

# Cell–Cell Interactions

*Methods and Protocols*

*Edited by*

**Sean P. Colgan**

## **Cell-Cell Interactions**

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Edited by

**Sean P. Colgan**

*Center for Experimental Therapeutics and Reperfusion Injury  
Brigham and Women's Hospital, Harvard Medical School  
Boston, MA*

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# Preface

Studies incorporating cell–cell interactions remain an exciting and ever-evolving discipline within the broader areas of biology. Although recent advances in genetics have provided much insight into gene- and disease-specific mechanisms, such models are often limited by tissue/organ complexity. The use of directed cell models to study the manner by which two or more cells interact has revolutionized our understanding of protein–protein interactions in a complex milieu and have provided an invaluable platform for the identification of new molecules.

The aim of *Cell–Cell Interactions: Methods and Protocols* is to provide a collection of diverse protocols, incorporating methods-based approaches both in vitro and in vivo. The authors of the various chapters are highly skilled experts in the development and utilization of cell–cell interaction assays. Their extensive expertise provides a detailed, step-by-step map of a reproducible protocol. Importantly, these protocols are readily adaptable for nearly any cell type and any organ system, and for this reason, I have tried to compile a diverse set of chapters incorporating numerous individual types of cells. It is my hope that this book will be both insightful to the beginner and inspiring to the experienced.

***Sean P. Colgan***



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## Contributors

- MICHAEL ASCHNER • *Department of Pediatrics Pharmacology and The Kennedy Center, Vanderbilt University School of Medicine, Nashville, TN*
- TANUSREE BHATTACHARYYA • *Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom*
- JOSEPH P. BRESSLER • *Department of Environmental Health Sciences, The Kennedy Krieger Institute, John Hopkins University Bloomberg School of Public Health, Baltimore, MD*
- MATTHIAS BRÜWER • *Department of General Surgery, University of Münster, Münster, Germany*
- GERALDINE CANNY • *Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- TRIANAFYLLOS CHAVAKIS • *Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD*
- NUNO CERCA • *The Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- NAN CHIANG • *Department of Anesthesiology, Perioperative, and Pain Medicine, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- ALEX C. CHIN • *Division of Gastrointestinal Pathology, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA*
- SEAN P. COLGAN • *Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- DIONNE DANIELS • *Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- HEIDI E. DE LUCA • *Gastrointestinal Cell Biology, Children's Hospital Boston, Harvard Medical School, Boston, MA*
- BRADLEY M. DENKER • *Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- ANA PAULA MARREILHA DOS SANTOS • *Faculty of Pharmacy, University of Lisbon, Lisbon, Portugal*
- TOBIAS ECKLE • *Department of Anesthesiology and Intensive Care Medicine, Tübingen University Clinic, Tübingen, Germany*
- HOLGER K. ELTZSCHIG • *Department of Anesthesiology and Intensive Care Medicine, Tübingen University Clinic, Tübingen, Germany*
- JANICE P. EVANS • *Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, John Hopkins University Bloomberg School of Public Health, Baltimore, MD*
- VANESSA A. FITSANAKIS • *Department of Pediatrics, Vanderbilt University Medical Center, Nashville, TN*

- ROBERT FUHLBRIGGE • *Harvard Skin Disease Research Center and Department of Dermatology, Harvard Medical School, Boston, MA*
- GLENN T. FURUTA • *Division of Pediatric Gastroenterology and Center for Experimental Therapeutics and Reperfusion, Brigham and Women's Hospital, Children's Hospital Boston, Harvard Medical School, Boston, MA*
- ALLISON J. GARDNER • *Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD*
- JUAN C. IBLA • *Department of Anesthesiology, Perioperative, and Pain Medicine, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Children's Hospital Boston, Harvard Medical School, Boston, MA*
- KIMBERLY K. JEFFERSON • *Department of Microbiology and Immunology, Virginia Commonwealth University, Richmond, VA*
- JOSEPH KHOURY • *Department of Anesthesiology, Perioperative, and Pain Medicine, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Children's Hospital Boston, Harvard Medical School, Boston, MA*
- AIMEE LANDRY • *Department of Molecular Biology, Massachusetts General Hospital, Boston, MA*
- WINSTON Y. LEE • *Division of Gastrointestinal Pathology, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA*
- WAYNE I. LENCER • *Gastrointestinal Cell Biology, Children's Hospital Boston, Harvard Medical School, Boston, MA*
- YAW-CHYN LIM • *Department of Pathology and Physiology, Immunology Program, National University of Singapore, Singapore*
- NANCY A. LOUIS • *Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- FRANCIS W. LUSCINSKAS • *Department of Pathology, Center for Excellence in Vascular Biology, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- ALICE MAGER • *Department of Anesthesiology and Intensive Care Medicine, Tübingen University Clinic, Tübingen, Germany*
- MICHELLE L. MATTER • *John A. Burns Medical School, University of Hawaii at Manoa, Honolulu, HI*
- BETH A. MCCORMICK • *Department of Pediatric Gastroenterology and Nutrition, Massachusetts General Hospital and Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA*
- ANITA J. MERRITT • *Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom*
- PETER NGO • *Fellow in Pediatric Gastroenterology, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*

- ASMA NUSRAT • *Department of Pathology and Laboratory Medicine, Epithelial Pathobiology Unit, Emory University School of Medicine, Atlanta, GA*
- NOBUKO OBARA • *Department of Oral Anatomy, School of Dentistry, Health Sciences University of Hokkaido, Hokkaido, Japan*
- LUISA OLIVI • *The Kennedy Krieger Institute, John Hopkins University Bloomberg School of Public Health, Baltimore, MD*
- VALERIA ORLOVA • *Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD; Department of Internal Medicine, University of Heidelberg, Heidelberg, Germany*
- CHARLES A. PARKOS • *Division of Gastrointestinal Pathology, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA*
- JONATHAN A. PHILLIPS • *Department of Gravitational Biology, Ames Research Center, Moffett Field, CA*
- PUNITHA RAMALINGAM • *Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- JOE W. RAMOS • *Cancer Research Center of Hawaii, University of Hawaii at Manoa, Honolulu, HI*
- ERNESTO SABATH • *Renal Division, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- ROBERT SACKSTEIN • *Harvard Skin Disease Research Center and Departments of Dermatology and Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- ANTHEA SCOTHERN • *Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom*
- CHARLES N. SERHAN • *Department of Anesthesiology, Perioperative, and Pain Medicine, Center for Experimental Therapeutics and Reperfusion Injury, Brigham and Women's Hospital, Harvard Medical School, Boston, MA*
- ALEXANDER SWIDSINSKI • *Innere Klinik, Gastroenterologie, Charit, Berlin, Germany*
- MARKUS UTECH • *Department of Pathology and Laboratory Medicine, Epithelial Pathobiology Unit, Emory University School of Medicine, Atlanta, GA; USA Department of General Surgery, University of Münster, Münster, Germany*
- SUSAN VOSS • *Division of Gastrointestinal Pathology, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA*
- THOMAS WEISSMÜLLER • *Department of Anesthesiology and Intensive Care Medicine, Tübingen University Clinic, Tübingen, Germany*
- GENEVIEVE B. WORTZMAN • *Division of Reproductive Biology, Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD*
- RAMNIK XAVIER • *Department of Medicine and Molecular Biology, Massachusetts General Hospital, Harvard Medical School, Boston, MA*



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## Blood–Brain Barrier and Cell–Cell Interactions

### *Methods for Establishing In Vitro Models of the Blood–Brain Barrier and Transport Measurements*

**Michael Aschner, Vanessa A. Fitsanakis, Ana Paula Marreilha dos Santos, Luisa Olivi, and Joseph P. Bressler**

#### **Summary**

This chapter describes *in vitro* methods for studying the blood–brain barrier. These methods include a cell line and isolated brain microvessels. The rat brain endothelial cell line 4 (RBE4) express many properties that are expressed by brain endothelial cells *in vivo*. Tissue culture methods allow the investigator to design experiments for studying transporters and permeability that would be much more difficult *in vivo*. A method for making preparations of isolated brain microvessels also is described. These preparations are highly enriched and also can be used for studying transport *in vitro*, but their short life span is a limitation. Two methods are discussed for measuring transport in cell culture. In one method, permeability is measured across a cell monolayer. This method is useful for measuring luminal and abluminal transport. The second method is especially designed for measuring the families of efflux transporters. These *in vitro* methods will complement many of the *in vivo* techniques, and they may be used as screening for more timely and expensive experiments, and also reducing the need for experimental animals.

**Key Words:** Blood–brain barrier; RBE4 cells; brain microvessels; transporters; permeability; multidrug resistance.

#### **1. Introduction**

The blood–brain barrier (BBB) is a specialized capillary bed separating the blood from the central nervous system parenchyma. It regulates amino acid, peptide, and protein transport, allowing for tight control of the composition of the brain's interstitial fluid composition. It also assures that changes in blood metabolite and ion composition (e.g., nutrients) are not reflected within the brain's milieu by preventing charged and large molecules from entering the brain.

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Two main factors contribute to the BBB's restrictive nature, and they include a physical barrier comprised of tight junctions between the endothelial cells (1,2) and a plethora of proteins on the endothelium, which play a key role in regulating influx and efflux of nutrients, ions, and other materials between the two (blood and brain) compartments (3,4). Brain capillary endothelial cells are polarized into luminal (blood-facing) and abluminal (brain-facing) plasma membrane domains. Nutrients are transported from the luminal side to the abluminal side by transporters. Additionally, the BBB expresses efflux pumps belonging to the superfamily of adenosine-5'-triphosphate (ATP)-binding cassette (ABC) proteins that prevent a broad range of amphiphatic chemicals from entering the brain. Efflux pumps that have been discovered thus far in the BBB include multidrug resistance glycoprotein, also referred to as P-glycoprotein (and most recently referred to as ABCB1), and breast cancer resistance protein (and most recently referred to as ABCG2 [5]). ABCB1 and ABCG2 are expressed on the luminal surface of brain endothelial cells in the BBB. The expression of members of the family of multidrug resistance-associated proteins (ABCC family) has been reported in cultures of bovine brain endothelial cells (6) but not in isolated human (7) or rat microvessels (8). ABCB1 and ABCG2 transport lipophilic and cationic compounds with overlap (9,10), whereas multidrug resistance proteins transport organic anions, glutathione, or glucuronide-conjugated compounds, as well as various nucleosides (11).

In vitro and in vivo methods have been described for studying permeability of the BBB. Obviously, the advantage of in vivo methods is that the integrity of the BBB is maintained, but these methods have distinct disadvantages when compared with in vitro methods, including cost and sensitivity. Also, the experimental design is more difficult when the experiments involve inhibitors and inducers of transporters. In an in vitro method, such as cell culture, it is relatively easy to modify the activity of transporters. Nonetheless, a distinct disadvantage of cultures of brain endothelial cells is that they do not fully mimic the properties of the BBB *in situ*. The most notable property that is not fully mimicked when using in vitro models of the BBB is the expression of a tight barrier (commonly measured as the electrical resistance [12]). Accordingly, a combination of both in vivo and in vitro methods is needed.

This chapter discusses two in vitro models for studying permeability and transport; these include cultures of the rat brain endothelial cell line (RBE4) and isolated brain microvessels. We also describe methods for examining transport permeability. In addition to RBE4 cell line, other cell culture models are available. Primary cultures of brain endothelial cells, for example, commonly are used. Monolayer cultures of porcine and bovine brain endothelial cells have

been reported to display higher electrical resistance than most cell lines (13–15). Nonetheless, a wide variation in electrical resistance has been observed in different preparations of primary cultures of endothelial cells from bovine and porcine brain from the same laboratory (13,14). Also, a practical issue in preparing primary cultures is the laboratory's proximity to the slaughterhouse. Poor cell yield will be obtained when brains are kept on ice for longer than 1 h. We refer the reader to manuscripts describing methods for preparing primary cultures of brain endothelial cells (14,15).

## 2. Materials

1. Minimal Essential Media (MEM), with Earle's salts (Gibco-BRL, Grand Island, NY; cat. no. 11095-080). MEM was modified to contain no  $\text{Ca}^{2+}$ , which can produce cell clumping as the result of interactions of extracellular matrix proteins.
2. Heat-inactivated horse serum (Gibco-BRL; cat. no. 26050-088). Repeated freeze–thaw cycles of serum should be avoided. To thaw frozen serum, place the bottle in a refrigerator for approx 24–48 h. If rapid thawing is needed, place in a water bath no warmer than 40°C. Once thawed, serum can be stored at 4°C for approx 1 wk; however, we suggest aliquoting into 50-mL tubes and storing at –20°C. These 50-mL tubes can then be thawed as needed at room temperature over a few hours.
3. Penicillin G (10,000 U/mL)–streptomycin sulfate (10,000 µg/mL; Gibco-BRL; cat. no. 15140-122). Storing penicillin/streptomycin in 10-mL aliquots at –20°C is suggested to avoid repeated freeze–thaw cycles and because it provides the correct amount for 1 L of media.
4. Fungizone® (Amphotericin B, 250 µg/mL; Gibco-BRL; cat. no. 15290-018): its use is optional to prevent fungal or yeast contaminations.
5. Trypan blue 0.4%. Trypan blue is used to determine cell viability. Intact cells are able to exclude trypan blue, whereas dead or damaged cells retain the dye. This is a useful method when determining plating density needed for the experiments.

### 2.1. Cell Culture

1. RBE4 cells: Neurotech SA (Evry, France) graciously provided the RBE4 cells.
2. MEM, alpha medium, F-10 nutrient mixture, glutamine, penicillin, streptomycin, heat-inactivated fetal bovine serum, and geneticin (G418).
3. T-75 flasks, which must be collagen coated and are obtained from BD Bioscience (Bedford, MA). Experiments are routinely conducted after microscopic and visual confirmation of confluence and are performed in HEPES buffer of the following composition: 25 mM HEPES/NaOH, 122 mM NaCl, 3.3 mM KCl, 0.4 mM  $\text{MgSO}_4$ , 1.3  $\text{CaCl}_2$ , 1.2 mM  $\text{KH}_2\text{PO}_4$ , 10 mM glucose, adjusted to pH 7.4, and at 25°C unless otherwise desired (for example, as in studies examining the effect of temperature-dependence on transport kinetics).

## 2.2. Efflux Transporters

1. Hank's Balanced Salt Solutions (HBSS; with Ca, Mg, and glucose) without phenol red. Verapamil is kept at 1 mM in HBSS in small aliquots at  $-20^{\circ}\text{C}$ . Working dilution is 20  $\mu\text{M}$  in HBSS, and the final concentration is 10  $\mu\text{M}$ .
2. Calcein AM is made at 1 mM in dimethyl sulfoxide and kept in small aliquots in the freezer at  $-20^{\circ}\text{C}$ . It is light sensitive. The final concentration is 2  $\mu\text{M}$  in HBSS.
3. MicroFluor 96-well black plates.
4. Phosphate-buffered saline with 1% Triton X-100 or an equivalent nonionic detergent. Store the buffer at room temperature.

## 2.3. Isolated Microvessels

1. Media 199.
2. Nytex nylon mesh ([www.sefar.com](http://www.sefar.com)).
3. Dextran.
4. Dounce homogenizer with a Teflon pestle (0.25-mm clearance). The homogenizer is purchased from a supply company and shaved by a machine shop.
5. Motorized homogenizer.
6. Dissecting tools, such as scissors and forceps.
7. Kim wipes.
8. 250-mL flat-bottom centrifuge tubes with lid.
9. Buchner funnel and fitting flask.
10. 3-mL syringe.
11. 0.25-mm glass beads.

## 3. Methods

### 3.1. Cell Culture

Our focus is on RBE4 cells in describing a cell culture method for studying the BBB. These cells were derived from rat brain microvascular endothelial cells immortalized with the plasmid pE1A-neo containing the E1A region of adenovirus 2 and a neomycin resistance gene (16–18). The RBE4 cell line has been used in our laboratory for a number of years with great success, and these cells have proven to be a useful tool for probing the characteristics of the BBB in vitro. RBE4 cells have been used extensively by others as well, representing a wide range of transporter studies, including multidrug transporters (19) serotonin (5-HT [20]; L-3,4-dihydroxyphenylalanine or L-DOPA [21]), organic cation transporter/carnitine transporter OCTN2 (22), and glucose transporter 1 (23) RBE4 cells also express the specific brain endothelial enzymes  $\gamma$ -glutamyltranspeptidase and alkaline phosphatase (16,24).

The RBE4 cell cultures are maintained in T-75 flasks coated with rat tail collagen I and subcultured into transparent six-well plates with porous nitrocellulose and collagen I-coated inserts (both from BD Biosciences, Bedford, MA)

for transport studies. The flasks and/or plates are routinely monitored by inverse microscopy before their doubling rate, approximately every 20 to 24 h, and the media are replaced after each doubling. In reality, the media is replaced approximately every 48 h to prevent the unnecessary disruption of the RBE4 cell monolayers (*see Note 1*). RBE4 monolayers in the flasks are subcultured every 3 to 4 d just as each flask's monolayer becomes confluent at approx  $1.5 \times 10^5$  cell/cm<sup>2</sup>. During this fractional splitting, these newly confluent monolayers are washed with 3 mL of 0.125% trypsin, detached with 1 mL 0.25% trypsin, centrifuged at very low speed (~1000g for 1 min), and fractionated 1:3 or 1:4, depending on the quality of the monolayer and the time frame in which they are needed. This procedure is routinely conducted for each flask and for each type of media (either astrocyte-conditioned media [ACM] or normal alpha medium) at approx  $10^5$  cell/cm<sup>2</sup>.

This method is a gentler way of manipulating the eukaryotic RBE4 cells and their collagen adherent proteins. Trituration, after the addition of trypsin, is accomplished more easily and with less shearing. The result is a more uniform and disbursed initial cell layer over the hydrated nitrocellulose collagen filters and a faster growing, uniformly confluent monolayer in the model chambers (*see Notes 2–4*).

The media specifications are largely dependent on the experimental design and the parameters that are to be tested. We have traditionally used two different types of medium for RBE4 growth. These include the traditional alpha-type media (**16,18**) and ACM, which is known to decrease transendothelial permeability by physically tightening the barrier and reducing transluminal diffusion (**25**) and thus representing a more amenable model system for probing issues related to transport properties of the BBB (*see Note 5*).

ACM is made just before use. It consists of equal parts (1:1, v/v) of fresh astrocyte growth media (MEM with Earl's salts containing 10% heat-inactivated horse serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL Fungizone) and used astrocyte growth media siphoned from stock primary astrocyte colonies maintained in the laboratory.

### 3.2. Transcellular Transport

The RBE4 cells are grown to confluent monolayers on porous, collagen I-coated, nitrocellulose filters and transferred to six-well plates. Each well has the porous filters attached to the base acrylic-walled inserts. Each insert sits securely suspended in its respective well, creating a top well above the cell monolayer and a bottom layer below each filter. Permeability and flux derivations are based on a one-dimensional permeability mathematical model derived from Fick's law:

$$J_F(x,t) = -D (\delta C(x,t)/\delta x)$$

where  $D$  is the gross coefficient of diffusion, and  $C$  is the chemical expression of concentration of distributed mass. The minus sign insures a stable, thermodynamically sound system with a downhill concentration gradient.

Unidirectional flux ( $J$ ) is calculated using the following equation:

$$J = \Delta Q / \Delta t * A$$

where  $Q$  is the fraction of the original radioisotope on the basolateral side of the culture model to the original activity applied to the apical side of the system. This value,  $Q$  (nanomoles), is divided by time of the experiment ( $t$ ; minutes), and area ( $A$ ;  $\text{cm}^2$ ) of the filter.

Concentration apparent permeability ( $\text{conc}P_{\text{app}}$ ) is calculated by dividing flux ( $J$ ) by the initial concentration ( $C_a$ ) used on the apical side of the model BBB system (26,27):

$$\text{conc}P_{\text{app}} = J / C_a.$$

Results are presented as both  $\text{conc}P_{\text{app}}$  relative to percent control and as equivalent nanomoles over the time course of the assay.

The permeability or flux of  $^{54}\text{Mn}^{2+}$  across confluent monolayers of RBE4 cells is quantified by liquid scintillation counting. The asymmetric timepoints chosen for measurement were 1, 5, 10, 30, 60, and 120 min. They proved to be spaced well enough apart for practical considerations and long enough in breadth between and in duration for Michaelis–Menton saturation kinetic calculations based on flux rate and concentration to be practicable as well.

The results are reported as mean  $\pm$  SEM. All experiments are analyzed routinely per well, with  $n \geq 9$  (one should use power analysis to calculate the number of wells and replicates needed to perform sound statistical analyses). In our laboratory, we routinely use InStat (GraphPad Software, El Camino Real, San Diego, CA) to analyze the data by one-way analysis of variance with a post-hoc test such as Tukey, when the number of groups is greater than two (to avoid multiple Student's  $t$ -tests). Results are considered to be statistically significantly from controls if the  $p < 0.05$ .

### 3.3. Efflux Pumps

The most direct method of assaying efflux pumps is a functional assay measuring intracellular accumulation of a radioactive or fluorescent substrate (probe) in the presence of a competing substrate. The competition will result in greater uptake of the probe. Alternatively, ATPase assays (28,29) have been described but are difficult to interpret because P-glycoprotein-associated ATPase activity is affected differently by different substrates. We prefer fluorescent probes in the functional assay because they are less expensive yet display the same level of sensitivity as radioactive probes. Instruments that have been used for measuring

fluorescence include fluorescence-activated cell sorters, image analysis systems, spectrofluorometers, and multiwell plate fluorescent readers. The choice depends on the experimental design. In studying heterogeneous populations of cells, fluorescence-activated cell sorters and image analysis systems have the best potential of determining the cell type expressing the efflux pump. A spectrofluorometer (or image analysis system) would enable the investigator to examine the same population of cells over time. A multiwell plate fluorescent reader allows high-input screening and might be more readily available to the investigator than the other instruments. Because of availability, we use the multiwell plate fluorescent reader. Also, multiplate fluorescent readers can be used because many of the commonly used fluorescent substrates are strong emitters that can be easily detected.

Several probes have been reported for measuring *ABCB1*, *ABCG2*, and *ABCC* family. In assaying *ABCG2*, mitoxantrone, BODIPY-prazosin (30), and pheophorbide have been used (31). An issue with the first two is that both are also substrates for *ABCB1* (32,33), whereas pheophorbide is specific for *ABCG2* (31). Rhodamine 123 often is used as a specific substrate for P-glycoprotein, but at least one study found an interaction between rhodamine 123 and *ABCC1* (34). Also, rhodamine 123 is not transported by wild-type BCRP but transported by a mutated form of BCRP (Arg 482 to Thr) The mutation is commonly found in different cell lines (35). Carboxyfluorescein diacetate A often is used for studying many of the members of the *ABCC* family of efflux pumps.

Inhibitors of *ABCB1* and *ABCG2* are verapamil (36) and fumitremorgin C (37), respectively. The latter compound is not available currently for commercial use but can be obtained from the Natural Products Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, and the National Cancer Institute. These drugs are often used as positive controls.

As we discussed previously, the underlying principal of the functional assay is that the putative substrate competitively inhibits efflux of the fluorescent probe, resulting in higher levels of probe inside the cells that is indicated by increased fluorescence. Most probes are lipophilic and may nonspecifically leak out of the cell, resulting in a lower intracellular fluorescence. To circumvent this problem, we have used calcein-acetoxymethylester (calcein-AM) to study *ABCB2* and members of the *ABCC* family. Upon entering the cell, the ester is cleaved by nonspecific esterases, and the calcein is impermeable and will not leak from the cell. We also would like to recommend caution when determining whether chemicals are substrates for efflux pumps. Many potential substrates are lipophilic and have a limited solubility in water.

The assay described here has been used for several cell lines. RBE4 cells, for example, are plated in 24-well dishes (collagen-coated wells) so that a confluent monolayer is achieved within 2 to 3 d. The investigator might be interested in coculturing endothelial cells with astrocytes to achieve higher levels of efflux

**Table 1**  
**Excitation and Emission Wavelengths of Commonly Used Probes**

Probe	Excitation	Emission	Final concentration
Rhodamine 123	507	529	2.5 $\mu\text{M}$
Calcein	493	515	1 $\mu\text{M}$
BODIPY-prazosin	488	530	250 $\mu\text{M}$
Pheophorbide	488	650	10 $\mu\text{M}$

pumps (*see Subheading 3.1.* for culturing endothelial cells with astrocytes or in the presence of AC [38]). Co-cultures can be achieved either by plating astrocytes and endothelial cells on opposite sides of polycarbonate filters or by placing inserts containing cultures of astrocyte monolayers into endothelial cell monolayers in 24-well dishes for a few days. The efflux assay is performed immediately on the endothelial cells immediately after removing the insert.

Cultures are washed three times with HBSS and incubated at 37°C with 500  $\mu\text{L}$  of verapamil and 500  $\mu\text{L}$  of calcein-AM to achieve a final concentration of 10  $\mu\text{M}$  and 1  $\mu\text{M}$  of verapamil and calcein, respectively. In examining potential substrates for the efflux pumps, we routinely preincubate the cells with the substrate for 10 min at 37°C before adding calcein-AM. After 60 min with calcein-AM, the cells are again given three washes, and the intracellular calcein is extracted by incubating cells with 200  $\mu\text{L}$  of phosphate-buffered saline/Triton X-100 for 30 min at 4°C. Because calcein-AM is light-sensitive, we shield the plates with aluminum foil. The extract is transferred to the microtiter plate, and the fluorescence is measured at an excitation of and emission as shown in **Table 1**. The data are expressed as relative fluorescence or computed as percent control (*see Note 6*).

### 3.4. Isolation of Microvessels From Bovine Brain

Isolated microvessels have been used for characterizing transporters and other brain capillary specific proteins and for studying signaling pathways. We use the term microvessel instead of capillary because the procedure isolates vessels that are 5 to 25  $\mu\text{m}$  in diameter, which will include venules and arterioles, as well as capillaries. The isolated microvessels consist of endothelial cells, abluminally located pericytes, and their surrounding extracellular basement but are void of perivascular astrocytes that in vivo ensheath the microvessel (39). Whereas primary cultures of endothelial cells lose the expression of some of the genes expressed in vivo and gain the expression of genes needed for sustenance and growth in culture, isolated microvessels are the best preparation of enriched endothelial cell protein. Indeed, typical fold enrichments of endothelial cell proteins (e.g.,  $\gamma$ -glutamyl peptidase and alkaline phosphatase) are approx 20 but

might vary depending on the brain region (40). The reader should be aware of two problems in using isolated microvessels. First, isolated microvessels have a brief viability because they cannot maintain adequate levels of ATP (41) (*see Note 7*). The second problem is associated with transport measurements. In vivo, transport from blood to brain across the BBB is unidirectional. Most substrates start their journey from the luminal surface, cross to the abluminal surface, and enter the parenchyma. In isolated microvessels, transport is assayed by measuring uptake into the microvasculature and transport is bidirectional because both the luminal and abluminal surfaces are exposed to the substrate undergoing transport. Furthermore, the luminal surface might not be accessible to the substrate because the microvessels could have been damaged during the preparation. Nonetheless, amino acid and glucose transporters, and efflux pumps have been characterized in isolated microvessels (42). More recently, a method for studying transport has been described using fluorescent substrates with confocal microscopy (43). This approach has two advantages. First, undamaged capillaries can be selected and assayed. Second, a microscopic assay requires less material.

The procedure is used for isolating brain microvessels from rats (44) and cows (40) in our laboratory and likely has been applied to other mammals. When working with larger mammals, it is advised to work with only gray matter unless there is a specific reason for studying capillaries from white matter. It is important to keep brains at 4°C throughout the procedure. The basis of the procedure for isolating brain microvessels is that the integrity of brain capillaries is not compromised when brain is homogenized in a Dounce homogenizer. The pestle of the homogenizer is shaved by a machine shop to achieve a clearance of 0.25 mm. Because the microvessels are intact, but the remaining cells in the brain are disrupted, microvessels can be isolated as described in the next paragraph (*see Note 8*).

The following protocol is tailored for isolating brain microvessels from adult rats. Dissect the brains and place them on cellophane wrap on ice. Wipe the surface with a Kim wipe to remove meninges containing superficial arteries and membranes. Cut away the cerebellum and brain-stem and open up the two halves of the cortex to remove the choroid plexus from the ventricles. With forceps, remove arteries along the surface. Place the brain in a 250-mL beaker containing 50 mL of ice-cold Media 199 that was pregassed with 95% O<sub>2</sub>/5% CO<sub>2</sub> containing 1% (bovine serum albumin [BSA]) solutions on ice. With four pairs of scissors, and two hands, mince the brain until it is the consistency of oatmeal. Divide the mince into four homogenization tubes and add 9 volumes of Media 199. Homogenize 20 strokes for 2 min slowly at 40 rpm with an electrical homogenizer so that there is no spillage.

Take the homogenate and dilute 1:10 with Media 199/BSA in flat-bottom centrifuge tubes that have closed lids. Shake the tubes for 1 min and centrifuge

for 10 min at 1000g. Aspirate the supernatant and add 15% (wt/vol) dextran in Media 199 (see **Note 9**). Several investigators have substituted 25% BSA. Shake vigorously for 1 min and centrifuge for 10 min at 4000g. The myelin floats and the cell pellet containing microvessels and nuclei. If not shaken properly, myelin will not separate from the cell pellet. Aspirate (do not pour) the supernatant and wipe fat clinging to the walls of the centrifuge tube with Kim wipes. Resuspend the pellet thoroughly in ice-cold Media 199/1% BSA using a broken capillary pipet.

The microvessels are separated from larger vessels by filtering a nylon screen. Secure a piece of 118- $\mu$ m mesh onto a 4-in. Buchner funnel with a rubber band. Wet the mesh and make a depression so liquid will flow to the center and not the edges. Add the suspension of microvessels to the filter and collect the microvessels that flow through. Fat and larger vessels will be retained. Keep washing the mesh with Media 199 while pushing aside fat and larger vessels with a spatula. Rinse the centrifuge tube making sure all of the microvessels have been removed. Approximately 10 mL of suspension will have resulted in at least 100 mL of flow-through. Centrifuge the flow-through at 4000g for 10 min and aspirate the supernatant. Resuspend the pellet with 4 mL of Media 199.

Microvessels are separated from nuclei through a column of glass beads. Plastic columns are prepared by cutting both ends of 3-mL syringe and securing one end with a small piece of 53- $\mu$ m mesh that is secured with a rubber band. Fill the columns 1.5 cm with glass beads (0.25 mm) and wash column with 10 mL of Media 199. Add 1 mL of suspension to each column and allow the capillaries to be trapped by the glass beads. Squirt 2 mL of Media 199 into each column, let it flow until the media is collected, and repeat five times. Remove the column, add the glass beads and nylon mesh to 10 mL of Media 199 in a 50-mL tube, gently swirl, and allow the beads to settle and mesh, and transfer the fluid to a new centrifuge tube. Place a drop on a microscope slide and view under phase contrast. Small thin capillaries should be observed without contaminating nuclei. Wash the beads again and combine the washings in one tube. Centrifuge for 5 min at 500g. In addition to visually monitoring purity, we recommend comparing the specific activity of alkaline phosphatase or gamma-glutamyl transpeptidase between brain homogenate and microvessel homogenate. Alkaline phosphatase activity should be approx seven-fold greater and  $\gamma$ -glutamyl transpeptidase activity should be 20-fold greater in microvessels.

#### 4. Notes

This section identifies major sources of problems associated with RBE4 cell culturing and notes how these problems can be identified and rectified. Specific

attention is directed to the subculturing and culturing techniques along with a detailed section on troubleshooting.

1. A new subculturing technique has been used in our laboratory to negate the occurrence of aberrant confluence in some BBB model culture dish wells. The new method requires a wash step to clear any serum from the T-75 flask monolayer before trypsinization.
2. Important variables to consider when performing studies with cultures are the effects of confluency and time in culture. Unlike tumor cells, these cell cultures stop growing and dividing once confluent because of cell–cell interactions. It is strongly recommended that the effects of confluency be determined by treating cells at various states of confluency. In a related issue, the time the cells are maintained in culture can have profound effects on the genotype and phenotype of the cells. Thus, preliminary studies should examine temporal changes in expression of the product of interest, and subsequent studies must contain appropriate controls to account for temporal changes in expression during treatments.
3. Microbial contamination of cell culture is probably the most common problem encountered once the initial cell isolation is successful. Contamination may be the result of invasion by bacteria, yeast, fungi, molds, mycoplasma, viruses, or any combination of these. Although isolated contamination of an individual culture plate or two is not uncommon, widespread or continual contaminations (in more than two separate cultures) require investigation into the source of the microbes. Once contamination is suspected, the rapid and accurate identification of the offending class of microbe can aid in determining the source of contamination. We highly recommend if contamination of cells is suspected that a sample be sent to a certified microbiological laboratory for identification of the class of microbe. Any infected culture plates must be disposed of immediately. All surfaces, including microscopes, pipettors, incubator and refrigerator door handles, also should be cleaned with 70% ethanol.
4. Because the growth media contains antibiotics (penicillin and streptomycin), bacterial contamination is uncommon. The addition of gentamycin sulfate may be beneficial in controlling bacterial and mycoplasma contamination. When adding new antibiotics, we suggest beginning with the lowest recommended concentrations (available in Gibco-BRL catalog) and performing a dose-toxicity curve in a few noninfected plates to assess effects on growth rate and cytotoxicity. In general, the highest concentration of antibiotic that does not adversely inhibit cell growth should be used. In our experience, the most common sources of contamination are yeast and fungi. We have found the addition of 0.25  $\mu\text{g}/\text{mL}$  Fungizone (Amphotericin B) effectively halts both yeast and fungal infections. We do not routinely add Fungizone to the growth media but use it only as necessary to control sporadic infections that seem to occur mainly during spring and summer. Cultures with yeast contamination can usually be readily identified by the examination of the media. The growth media appears cloudy and usually turns yellow as the result of acidification. The rapidly growing yeast consume the available glucose and convert

to anaerobic metabolism, which produces large quantities of lactic acid; it is this lactic acids which alters the pH (and thus the color) of the growth media. Microscopic examination of yeast-contaminated cultures reveals small round spores floating throughout the media. The source of yeast often is hard to identify and, thus, the list of possible suspects is long. Any item to be placed in the laminar flow hood should be cleaned with 70% ethanol, but special care must be taken when laboratory water baths are used to thaw or warm reagents for cell culture. The use of disposable plastic pipets can greatly reduce the possibility of contamination. Pipets should only be placed in the growth media once and used to feed one “stack” of culture plates. This prevents transfer of contaminants from a single plate to the growth media, which can then infect the entire culture. The Pasteur pipets used for removal of media should similarly be changed frequently. Fungus often is found growing directly on the cell layers, or even the walls of culture plates. It grows rapidly to form green to yellow sponge-like circles, and rapidly absorbs much of the growth media. When a fungal infection is discovered careful examination of the incubator is required, as this is often a reservoir of fungal spores. A thorough disinfection of the incubator with 70% ethanol is suggested. Mycoplasma contamination cannot be easily detected by visual inspection of the cultures. If cultures die before reaching confluency or are growing slowly, mycoplasma contamination may be suspected. Once again, we recommend sending samples to a certified microbiological laboratory for testing of mycoplasma. Alternatively, kits designed for mycoplasma detection are commercially available (Mycotest, Gibco-BRL, 15672-017 or Mycoplasma Detection Kit, Roche, 1296744).

5. The total time for the preparation of cultures is dependent upon the desired number of plates. Routinely splitting cells into 12 to 18 filters for the study of transport can take as long as 2 to 4 h. The changing of media (“feeding”) and subculturing usually requires approx 1 to 2 h but will of course vary with the number of plates/T-75 flasks.
6. Because potential chemical substrates of efflux pumps are lipophilic and have limited solubility in water, care should be exercised in solubilizing the chemical. The functional assay could be conducted in 96-well plates for high-input screening. **Table 1** lists dyes that have been described for studying different transporters. The concentrations are those that have worked for us using the protocol described.
7. Isolation of Microvessels. Because microvessels become energy depleted, brains should not be kept on ice for longer than 60 min.
8. The most common cause for poor recovery is associated with homogenizing tubes that do not provide a 0.25-mm clearance. Homogenizing tubes should be discarded when poor recovery is noticed.
9. Dextran is made at 33% in water (w/v) with constant heating and stirring. A 2X (30%) working solution is made by diluting it 1:10 with 10X Media 199.

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## Interactions of Intestinal Epithelial Cells With Bacteria and Immune Cells

*Methods to Characterize Microflora  
and Functional Consequences*

Geraldine Canny, Alexander Swidsinski, and Beth A. McCormick

### Summary

Epithelial cells at all mucosal surfaces are potentially apposed to bacteria, particularly in the intestine. It is established that intestinal epithelial cells (IECs) represent an important barrier between lamina propria cells and the potentially harmful luminal contents. In addition, IECs are important immunoeffector cells with the capacity to release cytokines, chemokines, and other molecules involved in antigen presentation and immune defense. The interaction of IECs with intestinal bacteria can result in a decrease in barrier function and the development of inflammation, which is known to be an important factor in the development of intestinal pathology. The potential role of such crosstalk between bacteria and other intestinal cell types in normal physiology and/or pathophysiology is therefore a topic of intense investigation. In this chapter, we provide protocols for the identification of bacteria that are associated with the epithelium and mucosa in addition to functional assays examining the interactions of neutrophils with epithelial cells and epithelial cell-mediated killing of bacteria.

**Key Words:** Structural organization of intestinal microbiota; fluorescence *in situ* hybridization (FISH); bacterial–epithelial interactions; neutrophil transepithelial migration; bacterial killing by epithelial cells.

### 1. Introduction

Intestinal epithelial cells (IECs) represent the first line of defense against potentially harmful bacteria present in the lumen. The human intestine is colonized by a huge number of bacteria, with approx  $10^{12}$  bacteria contained in a gram of colonic content, for example (*1*).

In addition, IECs are important immunoeffector cells with the capacity to release cytokines, chemokines, and other molecules involved in antigen presentation and immune defense (2,3). Although bacteria are clearly necessary for the development of normal immune function in the intestine, they also are involved in the pathogenesis of inflammatory bowel disease (IBD). Under normal circumstances, bacterial–epithelial interactions may result in the generation of tolerogenic signals, and no obvious inflammation ensues (2). The underlying mechanisms involved in normal individuals that are abrogated in IBD remain largely unknown. However, a significant number of both clinical and laboratory findings have provided evidence for the contribution of luminal bacteria to the pathogenesis of IBD (4), with considerable data from practically all animal models implicating bacteria in this process (5). The potential role of such crosstalk between bacteria and other intestinal cell types in normal physiology and/or pathophysiology is therefore a topic of intense investigation. In this chapter, we provide protocols for the identification of bacteria that are associated with the epithelium and mucosa in addition to functional assays determining the interactions of neutrophils with epithelial cells and the killing of bacteria by enterocytes. A large influx of neutrophils into the mucosa from the underlying vasculature occurs during intestinal bacterial infections (6,7). A marked infiltration of activated lymphocytes, macrophages, and granulocytes into the gut mucosa is also a salient feature in the pathology of IBD (8,9). This chronic influx and activation of immunocytes result in the sustained overproduction of reactive metabolites of oxygen and nitrogen, and it is thought that some of the intestinal injury and dysfunction observed in IBD results from the elaboration of these reactive species (10). Neutrophils traversing the intestinal epithelium can lead to the formation of crypt abscesses. Moreover, large-scale transepithelial polymorphonuclear (PMN) leukocyte migration causes decreased barrier function (11). Bacteria can use secretion systems to insert proteins into enterocytes and thus activate inflammatory signaling pathways. For example, SipA, a protein secreted by *Salmonella typhimurium*, is necessary and sufficient to drive PMN transmigration across model intestinal epithelia (12). The transepithelial migration protocol described in this chapter is of great use in understanding the dynamics and functional consequences of neutrophil interaction with enterocytes, the mediators produced by both cell types, how this process contributes to pathology, and how it might be prevented therapeutically (13).

The gastrointestinal expression of antimicrobial peptides is evolutionarily conserved (14) but it is a relatively recent discovery that epithelial cells have the capacity to actively kill bacteria. Indeed, antimicrobial peptides and proteins are thought to constitute an important facet of innate immunity in the intestine. Most antimicrobial peptides and proteins expressed by mammalian epithelial cells are

members of protein families that mediate nonoxidative microbial cell killing by phagocytes (15). Indeed enterocytes express members of the defensin family (3) and other antimicrobial proteins, such as BPI (16), which are thought to be involved in the maintenance of homeostasis toward intestinal microflora and their products such as lipopolysaccharide (LPS). It is noteworthy that a deficiency in the production of certain defensins may play a role in the aetiopathogenesis of Crohn's disease (17). The killing assay provided here can be used to determine the contribution of IEC to mucosal defense and whether this is subject to regulation.

Formation of sessile communities and their inherent resistance to antibiotics and host immune attack are increasingly identified as a source of many recalcitrant bacterial infections. These include periodontal disease, endocarditis, chronic obstructive lung disease, and foreign body-related infections (18,19). With the exception of *Helicobacter pylori*, the impact of biofilms on the pathogenesis of intestinal diseases is unknown. We describe an easy practicable fluorescence *in situ* hybridization (FISH) methodology achieving high-quality pictures by accurate resolution of spatial structure and the composition of the microbiota associated with intestinal tissues. Probes are inexpensive and can be purchased from many oligonucleotide manufacturers (such as MWG Biotech, Ebersberg, Germany) using different fluorochromes. Cy3, Cy5, or fluorescein isothiocyanate (FITC) fluorochromes do not bleach quickly, have little auto fluorescence background, and allow high-quality micrographs (Fig. 1). Cy3 provides better resolution than Cy5, and Cy5 is better than FITC. The results with other fluorochromes are less encouraging. Presently, the sequences of more than 200 FISH probes targeting the bacterial rRNA at domain group and species levels are described in the literature. Some of the probes frequently used for evaluation of fecal communities are listed in Table 1 (20–23).

Contrary to expectations, none of the FISH probes that we have tested are absolutely specific. Depending on the microbial community investigated (e.g., human or animal intestine, pancreatic duct, gallstones, biliary stents) all FISH probes demonstrated some kind of cross-hybridization at the conditions of optimal stringency. Many probes delivering specific results in human intestine were widely cross hybridizing in murine material. Cross-hybridization of probes observed when characterizing biofilms within biliary stents and gallstones did not occur while investigating colonic microbiota even within the same patient. The choice of FISH probes must therefore be adjusted to the specific requirements of the investigation. FISH is an excellent tool for the assessment of spatial structure. However, care must be taken when interpreting the results, and the presence of specific bacterial groups must be confirmed using alternative methods such as culturing, polymerase chain reaction with subsequent cloning, and sequencing. Taken together, the methods



Fig. 1. Bacterial flora attached to the ileal mucosa in a patient with Crohn's disease (at a magnification of  $\times 1000$ ). The triangle of lumen is at the bottom right. Bacteria are tightly attached to the intact mucosal surface. *Bacteroides* is detected using an orange-labeled Cy3 probe, Erec (*Eubacterium rectale* *Clostridium coccoides* group), is stained red (Cy5). No bacteria are found in normal controls (data not shown). (Please see color insert following p. 50 for a color version of this figure.)

**Table 1**  
**FISH Probes**

Name	Target	Reference
Eub338	Virtually all bacteria, Kingdom ( <i>Eu</i> ) <i>Bacteria</i>	20
Ebac	<i>Enterobacteriaceae</i>	21
Erec482	<i>Clostridium coccoides</i> - <i>Eubacterium rectale</i> group	22
Chis150	<i>Clostridium histolyticum</i> group	22
Clit135	<i>Clostridium lituseburense</i> group (incl. <i>Clostridium difficile</i> )	22
Strc493	<i>Streptococcus</i> group	22
Ecyl	<i>Eubacterium cylindroides</i> and other	23
Phasco	<i>Phascolarctobacterium faecium</i> group	23
Veil	<i>Veillonella</i> group	23
Rbro, Rfla	<i>Ruminococcus bromii</i> , <i>Ruminococcus flavefaciens</i> and other	23

**Table 2A**  
**Hybridization Buffer Composition**

Formamide (%)	Formamide ( $\mu\text{L}$ ) (=x)	Distilled $\text{H}_2\text{O}$ ( $\mu\text{L}$ )
0	0	1600
5	100	1500
10	200	1400
15	300	1300
20	400	1200
25	500	1100
30	600	1000
35	700	900
40	800	800
45	900	700
50	1000	600
55	1100	500
60	1200	400
65	1300	300

**Table 2B**  
**Posthybridization Wash Buffer Composition**

Formamide (%)	NaCl mM ( $\mu\text{L}$ )	5 M NaCl ( $\mu\text{L}$ ) (=y)	0.5 M EDTA ( $\mu\text{L}$ )
0	900	9000	0
5	636	6300	0
10	450	4500	0
15	318	3180	0
20	225	2150	500
25	159	1490	500
30	112	1020	500
35	80	700	500
40	56	460	500
45	40	300	500
50	28	180	500
55	20	100	500

$\text{H}_2\text{O}$ , 10 mL of 1 M HEPES, 9.5 g of HBSS– powder [Sigma], 0.05 mL 10 N NaOH; pH 7.4). All chemicals are obtained from Sigma.

- Polystyrene T162 Tissue Culture Flasks with Phenolic Cap supplied by Fisher Scientific (Costar).

provided here are of considerable use in characterizing intestinal bacterial flora and examining their interactions with other cell types in this environment to elucidate functional implications in health and disease.

## 2. Materials

### 2.1. Fluorescence In Situ Hybridization

1. Carnoy-solution (ethanol 6:glacial acetic acid 3:chloroform 1 from Sigma, St. Louis, MO) stored in the refrigerator.
2. Clean, filtered paraffin wax held at 2–4°C above its melting point (Kendall, Mansfield, MA).
3. A cold plate to rapidly cool the wax.
4. A supply of molds in which to embed the tissues.
5. SuperFrost® glass slides (Fisher Scientific, Pittsburgh, PA).
6. PAP-PEN (Birmingham, UK).
7. Forceps.
8. Xylene (Sigma).
9. Absolute alcohol.
10. FISH Probes. For commonly used probes, *see* [Table 1](#).
11. Hybridization buffer for (2 mL): 360 µL of 5 M NaCl; 40 µL of 1 M Tris-HCl, pH 7.4, x mL (*see* [Table 2A](#)) formamide, deionized, 10 µL of 10% sodium dodecyl sulfate (the amount will vary depending on the probe). With the exception of formamide, which must be deionized, all stock solutions can be stored at room temperature (RT) for 1 mo.
12. Posthybridization wash buffer (for 50 mL): 1 mL of 1 M Tris-HCl pH 7.4, y mL of 5 M NaCl (depending on the formamide concentration in the hybridization buffer, *see* [Table 2B](#)), 500 µL of 0.5 M EDTA (if formamide is used), 50 µL of 10% sodium dodecyl sulfate. This buffer should be made up fresh. Buffers can be made according to [Table 2B](#).
13. DAPI (Sigma), store at –20°C. Dilute to a working solution of 0.5 or 1 µg/mL.
14. For mounting SlowFade (Molecular Probes), ProLong anti-fade (Molecular Probes), or CitiFluor (CitiFluor, Ltd.) can be used.

#### 2.1.1. Cell Culture

1. Cell culture medium: T84 intestinal epithelial cells are grown as monolayers in a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 Nutrient mixture (Invitrogen) supplemented with 14 mM NaHCO<sub>3</sub>; 15 mM HEPES buffer, pH 7.5, 40 mg/L penicillin, 8 mg/L ampicillin, 90 mg/L streptomycin (all Sigma); and 5% newborn calf serum (Sigma).
2. Solution of trypsin (0.25%) and EDTA (1 mM) from Invitrogen.
3. Hank's Balanced Salts Solution (HBSS) buffers. HBSS with Ca<sup>2+</sup> and Mg<sup>2+</sup> is termed HBSS<sup>+</sup> (for 1 liter: 990 mL of deionized-distilled H<sub>2</sub>O, 10 mL of 1 M HEPES, 9.75 g of HBSS<sup>+</sup> powder [Sigma], 0.05 mL 10N NaOH; pH 7.4). HBSS without Ca<sup>2+</sup> and Mg<sup>2+</sup> is termed HBSS<sup>–</sup> (for 1 liter: 990 mL of deionized-distilled

5. 0.33-cm<sup>2</sup> (5 μm pore size) collagen-coated polycarbonate filters (Costar Corp., Cambridge, MA).
6. Triton X-100 (10% solution; Sigma).
7. *N*-formyl-Met-Leu-Phe (1 μM stock solution; Sigma).

#### *2.1.2. Solution of Rat Tail Collagen*

1. Rat tails.
2. 1% acetic acid (Sigma).
3. Spectropor no. 1 dialysis tubing (Fisher Scientific).
4. 70% ethanol.

#### *2.1.3. Bacterial Growth Medium*

1. Luria-Bertani broth (LB), per liter: 10 g tryptone/5 g yeast extract/10 g NaCl (Difco Laboratories Inc.).
2. Luria agar is LB containing 12 g of Bacto agar (Difco Laboratories Inc.) per liter.
3. MacConkey agar (Difco Laboratories) is prepared according to package instructions (50 g powder per liter of deionized-distilled H<sub>2</sub>O).
4. Gentamicin (Sigma): 50 μg/mL prepared in deionized-distilled H<sub>2</sub>O.

#### *2.1.4. Neutrophil Isolation*

1. 2% Gelatin (Sigma) prepared in HBSS. Make up 2% gelatin fresh per each use by preparing 3 g gelatin in 150 mL of HBSS— as follows: Preheat HBSS— to no more than 45°C (gelatin has collagen in it that could degrade). Then, add gelatin while stirring continually. Visually confirm that all of the gelatin is dissolved and let the solution cool to 37°C before using (do not over cool). Prepare 50 mL of 2% gelatin per 60 mL of blood drawn.
2. 13% Acid-citrate-dextrose per liter: 13.7 g of citric acid, 25 g of sodium citrate, 20 g of dextrose (all chemicals from Sigma).
3. Lysis buffer (keep at 4°C), per liter: 8.29 g of NH<sub>4</sub>Cl, 1 g of NaHCO<sub>3</sub>, 0.038 g of EDTA (all chemicals Sigma).
4. Hemocytometer.
5. Siliconized Pasteur pipets (Sigmacote; Sigma).
6. 2% Acetic acid (Sigma).

#### *2.1.5. Myeloperoxidase Assay*

1. 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) substrate solution. ABTS is made fresh before every use: 28 mg ABTS, 45 mL H<sub>2</sub>O, 5 mL citrate buffer.
2. Citrate buffer (store at 4°C), for 500 mL: 400 mL of deionized-distilled H<sub>2</sub>O, 73.5 g of sodium citrate, 52.5 g of citric acid, pH 4.2.
3. 30% H<sub>2</sub>O<sub>2</sub>. All chemicals obtained from Sigma.
4. 96-well plates.

## 2.2. Epithelial Cell-Killing Assay

1. Cell culture: Caco2 cells are cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, and 100 µg/mL Streptomycin sulfate. All reagents were obtained from Sigma. Other necessary cell culture materials are listed in **Subheading 2.1.1**.
2. Sterile HBSS– (Sigma). Store at 4°C. Killing assays are conducted in the presence of HBSS– as calcium and magnesium inhibit BPI-mediated activity ([24](#)); see **Note 1**.
3. Sterile water.
4. Sterilized Eppendorf tubes (Fisher Scientific).
5. Cell scrapers (Fisher Scientific).
6. Luria Bertani broth and agar plates, prepared as detailed in **Subheading 2.1.3**.

## 3. Methods

### 3.1. Fluorescence In Situ Hybridization

For Probe fixation, see **Note 2**.

1. Place the biopsy in an Eppendorf tube with 1.5 mL of cold Carnoy solution. Leave large probes longer in Carnoy (on average 1 h for each millimeter of tissue thickness).
2. Transfer the biopsy from Carnoy to the chilled absolute ethanol twice and from ethanol to xylene twice. Incubate the biopsy for 15 min in each. Embed the biopsy in liquid paraffin.
3. Paraffin embedding (see **Note 3**). Select the mold; there should be sufficient room for the tissue allowing for at least a 2-mm surrounding margin of wax.
4. Fill the mold with paraffin wax.
5. Using warm forceps, select the tissue, taking care that it does not cool.
6. Chill the mold on the cold plate, orienting the tissue and pressing it into the wax with warmed forceps. This step ensures that the correct orientation is maintained and the tissue surface to be sectioned is kept flat.
7. Insert the identifying label or place the labeled embedding ring or cassette base onto the mold.
8. Cool the block on the cold plate, or carefully submerge it under water when a thin skin has formed over the wax surface.
9. Remove the block from the mold.
10. Cross check block, label, and worksheet.
11. Cut the paraffin blocks to 2- to 10-µm thick slices and put them on super frost glass slides (two slices for each appointed FISH probe). It is important to have a properly fixed and embedded block and sharp knife or many biases can be introduced in the sectioning. Common biases include tearing, ripping, “venetian blinds,” holes, and folding.
12. Once sections are cut, they are floated on a warm water bath, which helps remove wrinkles. They are then picked up on a glass microscopic slide.

13. Place the glass slides in a warm oven at 50°C for approx 60 min to help the section adhere to the slide.
14. Deparaffinize the slides by running them through xylene to alcohol shortly before hybridization: 4X 3-min incubations in xylene and 4X 3-min incubations in absolute ethanol; all steps can be carried out at RT. Change the ethanol and xylene solutions every 40 slices containing intestinal contents to prevent fecal bacteria floating in the fluid and being found on the slices.
15. Heat the slices once more in a warm oven at 50°C for approx 25 min.
16. Demark the area of the hybridization with a PAP-PEN and air-dry.
17. Prepare the hybridization chamber using a Tupperware container that seals well. Darken the chamber to avoid bleaching of the fluorescence. A platform can be created for the slides, that must be absolutely horizontal over the hybridization time. Soak several paper towels with hybridization buffer to keep the chamber humidified so the hybridizations do not dry out. Individual slides can also be hybridized in humidified, 50-mL plastic screw cap tubes.
18. Mix 16 µL of hybridization buffer with 2 µL of the FISH probes (~10 ng of each probe) and distribute it over the biopsy surface. Be careful not to scratch the probe while distributing it. Do not use cover slips; they can mechanically disturb the mucus layer and spatial structures.
19. Place slides in box and incubate for 60 to 90 min in a moisture chamber at probe-specific temperature (*see Note 4*).
20. Posthybridization washes; remove the slides from the hybridization chamber. They should not be dry! Rinse the slides with MQ H<sub>2</sub>O and transfer them immediately into a 50-mL tube with washing buffer prewarmed to the required hybridization temperature (two slides in each tube). Wash for 5 min in a water bath at the required hybridization temperature.
21. After 5 min of washing, rinse the probes with MQ H<sub>2</sub>O, air-dry.
22. DAPI counter staining; apply 20 µL of DAPI to each section, stain for 5 min at RT, wash with MQ H<sub>2</sub>O, and air-dry.
23. Mounting; apply anti-fading mountant to the section. Add cover slip and view under an epifluorescent microscope and take pictures as desired (*see Note 5*). Because the slides fade quickly, they should be viewed within 2 d and stored in the dark.

### **3.2. Enteric Pathogen Interactions With Model IEC-Polarized Monolayers**

#### *3.2.1. Preparation of Rat Tail Collagen*

The isolation of rat tail collagen is performed by the method of Cereijido et al. (25).

1. Strip the skin from the rat tail; the tendons are almost pure collagen.
2. Soak 0.75 g of collagen in 70% ethanol for 20 min to dehydrate. Next, solubilize the tendons in 100 mL of 1 % acetic acid (v/v) precooled to 4°C. Stir the mixture overnight at 4°C.

At this point, the solution should be viscous and some undissolved material will remain.

3. Spin the collagen solution at 25,000g for 30 min at 4°C. Discard only the pellet and put the collagen preparation into dialysis tubing (Spectropor no. 1), which has been presoaked in distilled-deionized H<sub>2</sub>O for 1 h.
4. Place the tubing in a 2 L beaker, add cold distilled-deionized H<sub>2</sub>O, and dialyze for 24 h at 4°C against two to three changes of H<sub>2</sub>O.
5. Remove the collagen from the dialysis tubing and dilute the solution if necessary. The collagen solution should be a little thinner than the consistency of honey.
6. Store at 4°C for 2 mo.

### 3.2.2. Preparation of T84 Cells Grown on Collagen-Coated Polycarbonate Filters

1. Conventional inserts are prepared for the use in bacterial invasion assays (26). A 50- $\mu$ L aliquot of the rat tail collagen mixture is placed in each well. Care should be taken to make sure collagen is evenly distributed across the filter and that the collagen stock solution is maintained at 4°C.
2. Allow plates to completely dry (3–24 h).
3. Next, the T84 cells, which are grown on 162-cm<sup>2</sup> flasks are trypsinized, and  $7.5 \times 10^5$  cells are then suspended in 75  $\mu$ L of media and are placed in each transwell™ (Costar). A total 900  $\mu$ L of cell culture media is placed in the outside chamber. Polarized monolayers prepared in this fashion can be used 6 to 14 d after plating. A steady-state resistance is reached 4 to 6 d after plating, with variability largely related to the cell passage number.
4. Inverted monolayers are prepared for use in neutrophil transmigration assays (26). The transwells (Costar) are removed from their respective wells and are placed upside down in a large gridded Petri dish; one invert to each petri dish grid square (each Petri dish holds 24 inverts).
5. A 50- $\mu$ L aliquot of rail tail collagen is placed on the filter. When adding the collagen to the filter it is important to keep the solution at 4°C. Let the collagen dry on the filter overnight in the covered Petri dish.
6. Carefully add 75  $\mu$ L of a  $7.5 \times 10^5$  T84 cell suspension to each invert and incubate at 37°C overnight. The next day, use forceps to aseptically flip the inverts into a 24-well plate. Add 1 mL of cell culture medium to the outside well and 200  $\mu$ L to the inside of the invert. Polarized monolayers prepared in this fashion can be used 6 to 14 d after plating. A steady-state resistance is reached 4 to 6 d after plating, with variability largely related to the cell passage number.

### 3.2.3. Growth of Bacteria

Nonagitated microaerophilic cultures of *S. typhimurium* are prepared by inoculating 10 mL of LB broth with 0.01 mL of a stationary-phase culture, followed by overnight incubation (18 h) at 37°C. Bacteria from such cultures

are in late logarithmic phase of growth and represent approx  $5-7 \times 10^8$  colony-forming units (CFUs)/mL. CFUs are determined by serially diluting the bacteria and plating onto MacConkey agar medium.

#### 3.2.4. Neutrophil Isolation

The neutrophil isolation procedure is based on the method of Henson and Oades (27).

1. From a healthy human volunteer, draw 60 mL of blood directly into a 50-mL syringe that contains 6 mL of 13% acid-citrate-dextrose (pH 4.0).
2. Spin for the buffy coat (400g, 20 min at RT).
3. Using prepared, siliconized pasteur pipets, remove top yellow serum layer down through the white interface by gentle aspiration. The interface contains lymphocytes, platelets, and monocytes, while the neutrophils are in the red layer, and just below the white interface.
4. Once the buffy-coat is removed, dispense 20 mL of the blood per 50 mL Falcon™ centrifuge tube and then add the 2% gelatin bringing the final volume to 50 mL (i.e., should have three tubes/60-mL blood draw). Let stand for 25 min at 37°C, during which time the red blood cells will settle to the bottom of the tube.
5. Using a 10-mL pipet, combine as much of the clear supernatant as possible in new tubes and do not contaminate with the bottom red blood cell layer.
6. Spin the clear supernatant at RT in a tabletop centrifuge for 10 min at 400g.
7. Remove the supernatant by aspiration and gently resuspend the white pellet in 3 mL cold (4°C) lysis buffer per 50-mL tube.
8. Next, bring the volume of cold lysis buffer to 50 mL, and centrifuge at 4°C for 10 min at 1200g. Aspirate off the supernatant and discard.
9. Gently resuspend the pellet in 5 mL cold HBSS– and wash with 50 mL of cold HBSS–. Resuspend the pellet in 3 mL cold HBSS– and carefully break up the pellet by gentle pipetting.
10. To count the neutrophils, remove 5  $\mu$ L of the suspension and place this in 500  $\mu$ L of 2% acetic acid. Acetic acid prevents swelling of the neutrophils and lyses the red blood cells, permitting one to view the morphology of cells. Add 12  $\mu$ L of the neutrophil suspension to each side of the hemocytometer and count the number of cells in each of the chambers and average the counts.
11. Resuspend the neutrophils at a final concentration of  $5 \times 10^7$ /mL in HBSS–. PMN are kept on ice in HBSS– and are used within 1 hr of being isolated. It is also important to keep the PMN in HBSS– buffer since the addition of divalent cations will cause them to become activated.

#### 3.2.5. Invasion of *S. typhimurium* Into T84 Intestinal Epithelial Monolayers

Infection of T84 monolayers is performed by the method of Lee and Falkow (28) with slight modification (26).

1. Inserts with attached monolayers are lifted from the wells, drained of media by inverting, and gently washed by immersion in a beaker containing HBSS+, warmed to 37°C. Cells are incubated in HBSS+ buffer because the absence of divalent cations will cause the opening of intestinal epithelial tight junctions.
2. Next, the monolayers are placed in a new 24-well tissue culture plate bathed with 1.0 mL HBSS+ in the lower (outer) well and 0.05 mL HBSS+ added to the upper (inner well) and are then equilibrated for 30 min at 37°C.
3. 7  $\mu$ L of each HBSS+ washed, bacterial sample (representing an inoculation ratio of 10:20 bacteria:epithelial cell) is added per monolayer for 1 h. If necessary, the bacterial invasion can be followed throughout a time-course of 1 h. Two populations of bacteria generally are assessed: cell associated and internalized. Cell-associated bacteria represent the population of bacteria adherent to and/or internalized into the T84 monolayers and are released by incubation with 0.1 mL of 1% Triton X-100 for 20 min at 4°C. Internalized bacteria are those obtained from the lysis of epithelial cells with 1% Triton X-100, 90 min after the addition of gentamicin (50  $\mu$ g/mL). Gentamicin, an aminoglycoside antibiotic, does not permeate eukaryotic plasma membranes and is therefore cytolytic only to extracellular populations of bacteria while intracellular bacterial populations remain viable (29).
4. For both cell associated and internalized bacteria, 0.9 mL of LB is then added and each sample is vigorously mixed and quantified by plating for CFUs on MacConkey agar. To determine the number of adherent organisms the internalized population is subtracted from the cell-associated population (i.e., cell associated – internalized = adherent).

### 3.2.6. PMN Transepithelial Migration

Evaluation of neutrophil migration across monolayers of polarized intestinal epithelia stimulated by *S. typhimurium* is performed by the method of McCormick et al. (26) and is illustrated in Fig. 2.

1. T84 inverted monolayers are extensively rinsed in HBSS+ to remove residual serum components.
2. *S. typhimurium* is prepared by washing twice in HBSS+ and then are suspended to a final concentration of  $5 \times 10^9$ /mL. Then, 100- $\mu$ L aliquots of the bacterial suspension are microcentrifuged at 13,000g for 3 min and resuspended in HBSS+ a final volume of 25  $\mu$ L.
3. Inverted cell monolayers are removed from each well and placed in a moist chamber such that the epithelial apical membrane surface is oriented upward.
4. The bacterial suspension is gently distributed onto the apical surface and incubated for 45 min at 37°C.
5. Nonadherent bacteria are then removed by washing three times in HBSS+ buffer. The monolayers are then transferred, by inverting, back into the 24-well tissue culture tray containing 1.0 mL of HBSS+ buffer, in the lower (apical membrane now colonized with *Salmonella*) reservoir and 160  $\mu$ L in the upper (basolateral interface) reservoir.

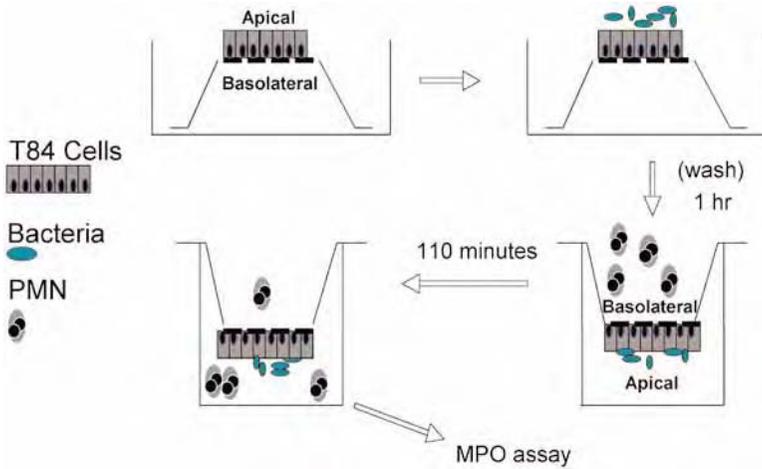


Fig. 2. Schematic for the protocol of *S. typhimurium*-induced neutrophil transepithelial migration. Inverted polarized, T84 cells are grown on the under-surface of, Transwell permeable supports. In this orientation, the apical surface of T84 cells was first colonized by *S. typhimurium* for 1 h. After the establishment of *S. typhimurium* infection, purified human peripheral blood neutrophils are then placed in the upper reservoir, interfacing with the epithelial basolateral membrane for 2 h. Such a maneuver is performed to achieve appropriate physiological orientation (i.e., neutrophils interacting basolaterally/*Salmonella* interacting apically).

- To the basolateral bath, 40 mL ( $10^6$ ) of isolated neutrophils are added to each monolayer and incubated for 110 min at 37°C. Positive control transmigration assays are performed by the addition of chemoattractant (1  $\mu$ M fMLP) to the opposing apical reservoir. All experiments are performed at 37°C.
- Transmigration is quantified by a standardized assay for the neutrophil azurophilic granule marker myeloperoxidase. After each transmigration assay, nonadherent PMN are washed from the surface of the monolayer and neutrophil cell equivalents, estimated from a standard curve, are assessed as the number of neutrophils that had completely transversed the monolayer (i.e., into the apical reservoir).

### 3.2.7. Myeloperoxidase Assay

This assay is used to determine neutrophil transmigration across epithelial monolayers (26).

- To assess the transmigrated neutrophil population, 50  $\mu$ L of 10% Triton X-100 stock solution is added to the bottom reservoir. Rotate the plate carefully (setting ~150 rpm) at 4°C for at least 20 min.
- During this time period, make up ABTS substrate solution and just before use add 1  $\mu$ L of 30%  $H_2O_2$  per mL of ABTS solution.

3. Prepare neutrophil standards by serially diluting  $1 \times 10^6$  neutrophils down to  $5 \times 10^3$ .
4. Add 50  $\mu\text{L}$  of citrate buffer to each well.
5. Remove 100  $\mu\text{L}$  of sample from each well and add it into a 96-well plate in duplicate.
6. Add 100  $\mu\text{L}$  of the ABTS solution (supplemented with  $\text{H}_2\text{O}_2$ ) to the prepared 96-well plates.
7. Place at  $37^\circ\text{C}$  and let develop for 15 to 20 min.
8. Read the plate in an ELISA plate reader at a wavelength of 405 nm.

### 3.3. Epithelial Cell-Killing Assay

1. Culture *S. typhimurium* in LB broth at  $37^\circ\text{C}$  until the culture reaches an absorbance of 0.8 when spaced at 600 nm. This corresponds to approx  $1 \times 10^9$  CFU/mL. If the bacteria have grown further, dilute the culture to this absorbance in HBSS<sup>-</sup>. These bacteria are in log phase.
2. Centrifuge the bacterial culture at 3000g for 10 min. Aspirate off the broth and resuspend the pellet in 5 mL of prewarmed HBSS<sup>-</sup>. Repeat this procedure once to wash the bacteria. Resuspend the bacteria in 5 mL HBSS<sup>-</sup>.
3. Fill and label Eppendorfs with 1 mL of HBSS<sup>-</sup>, these are used to dilute the samples. For purposes of accuracy it is important to pipet exact volumes.
4. Fill a 15-mL sterilin for each well of cells with 5 mL ice-cold sterile water and keep the sterilins on ice.
5. Take the plate of epithelial cells, aspirate off the medium and wash gently with 1 mL of warm HBSS<sup>-</sup>.
6. Reaspirate and add 1 mL of HBSS<sup>-</sup> to the cells.
7. Then incubate the cells with 100  $\mu\text{L}$  of the bacterial suspension. We generally use 6 well plates of 7- to 10-d-old Caco2 cells but this method can be modified for cells cultured on inserts by adding HBSS<sup>-</sup> apically and basolaterally and incubating the cells with apically applied bacteria, thereby mimicking the physiological scenario. Incubate HBSS<sup>-</sup> and bacteria in an additional plate to serve as a bacterial control. This bacterial standard allows for control of bacterial death and growth independent of epithelial cells and will be used to calculate percentage killing.
8. Incubate the cell culture plates at  $37^\circ\text{C}$  on a rotating platform set to 60 rpm.
9. At desired time-points, scrape the cells using a cell scraper along with the supernatants into 5 mL ice-cold water to hypotonically lyse the epithelial cells and prevent further killing or growth of bacteria.
10. Take samples from epithelial lysates and the bacterial standard and serially dilute them by transferring 10  $\mu\text{L}$  of lysate into 1 mL HBSS<sup>-</sup> and 100  $\mu\text{L}$  of this dilution into 1 mL HBSS<sup>-</sup>. To reduce variability this volume should be added without touching the liquid in the Eppendorf. Plate triplicate 50- $\mu\text{L}$  drops of 1/1000 and 1/10,000 dilutions on agar (this allows for replicates to be plated) and culture

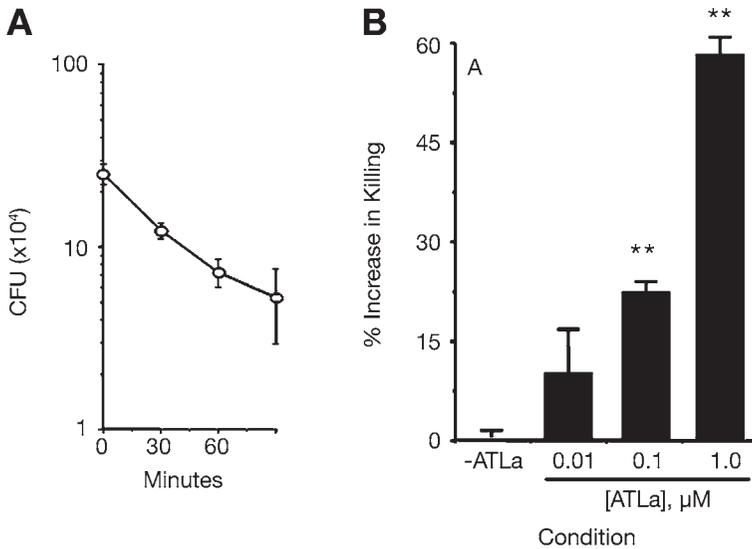


Fig. 3. (A) A time course depicting the killing of a smooth strain of *Salmonella typhimurium* by Caco2 cells. (B) Epithelial pre-exposure to the stable Lipoxin A<sub>4</sub> analog ATLa enhances this killing in a concentration-dependant fashion. Results are expressed as mean  $\pm$  SEM.

the plates at 37°C overnight. Determine the percentage bacterial killing using the following equation:

$$\text{Mean standard CFU} - \text{mean sample CFU} / \text{Mean standard CFU} \times 100 = \% \text{ killing}$$

where mean sample CFU was the mean of triplet drops for each dilution of the cellular lysate and standard was that of the bacteria alone. Results can also be expressed as CFU or CFU/mL. Alternatively, the plating may be performed using the “pour plate method” if desired. For Caco2 cells, significant killing has occurred by 15 min and, in our hands, it frequently is maximal by approx 60 min. For results of a typical experiment using a smooth strain of *Salmonella*, see Fig. 3. Care must be taken to ensure that the bacteria are not dying throughout the course of the experiment, thus negating the results. In this regard, *Salmonella* are viable for several hours in HBSS.

To prove that the killing is mediated by a specific peptide or protein, an inhibitory antibody or antisera may be used. In this case, prepare the antibody or antisera at the appropriate dilution and after washing the epithelial monolayer add 1 mL of this solution to the cells. Incubate for 30 min at 4°C. This is done to allow attachment of the antibody and prevent non-specific binding.

Thereafter, allow the plate to warm to 37°C, add the bacterial suspension and continue with the procedure above. Additional controls should be included to rule out the possibility of the antibody affecting bacterial growth.

#### 4. Notes

1. Initial growth curves should be conducted to relate absorbance to bacterial number. We have used this protocol to assess the contribution of BPI to bacterial killing mediated by intestinal epithelial cells but the assay can be adapted for other mucosal epithelial cells using a relevant strain of bacteria. Anionic sites in the Lipid A region of LPS are normally occupied by divalent cations ( $\text{Ca}^{2+}$  and/or  $\text{Mg}^{2+}$ ) which result in tight LPS packing. Bacterial/permeability increasing protein at antibacterial concentrations effectively competes for these sites in the LPS molecule and conversely, bound bacterial/permeability increasing protein can be displaced by these cations (24).
2. Before the spatial organization of intestinal microbiota can be investigated, it must be preserved. Shock frosting of the probes allows the preparation of native tissue slices with bacteria attached to the mucosa and intestinal contents. However, the fluorescent *in situ* hybridization takes place in an aqueous environment at temperatures of 46°C and higher. Hybridization occurs during a period of at least 90 min, which is necessary to achieve sufficient signals, but during this time bacteria are being progressively detached from the tissue and the spatial structure is lost. Formalin disintegrates spatial structures even before hybridization and tends to swell tissues rendering loose bacterial conglomerates within mucus more vulnerable to disintegration. The use of nonaqueous fixatives to preserve the mucus layer is crucial. We achieved excellent results preserving the material of human biopsies, surgically removed tissues and whole animal intestines with Carnoy solution. Once the structure of microbial communities is stabilized FISH proves to be a simple and reliable method.
3. The mechanical treatment of tissues while obtaining specimens leads to biases caused by dragging, crushing, and disrupting of normal anatomy. Fecal bacteria may be integrated into the distorted tissues and found at atypical anatomic sites and even within single cells. The optical integrity of the epithelial layer or of the tissue at the site of investigation is extremely important. Normally the autofluorescence background of human tissues allows good visualization and orientation within histological structures and the mucus layer. However, at least two additional stains (Alcian blue, hematoxylin and eosin, or other) for each biopsy or tissue specimens should be performed and are extremely helpful especially during optimization. No evaluation should be attempted when signs of tissue damage are obvious.
4. When optimizing probes for specificity, it is necessary to vary the hybridization temperature or formamide concentrations (increased temperature or formamide concentration means increased specificity). If possible do not use temperatures higher than 50°C or formamide concentrations higher than 55%. High temperatures result in mucus detachment and formamide introduces biases in eucaryotic tissues leading to bizarre fluorescent signals. Whenever possible, short hybridiza-

tion times of 90 min are preferable, and the hybridization should not exceed 6 h. In most cases, 90 min provides excellent resolution when Cy3, Cy5, or FITC fluorochromes are used.

5. The quantification of bacteria within spatial structures cannot be directly verified by stepwise dilution of bacteria. We use the approximation based on the following model. A 10- $\mu\text{L}$  suspension of bacteria of  $10^7$  cells per mL applied to a glass surface in a circle of 1 cm results in 40 cells per average microscopic field of 200  $\mu\text{m}$  diameter at a magnification of  $\times 1000$ . One bacterium thus occupies an area of 785  $\mu\text{m}^2$ . One bacterium averages 0.8  $\mu\text{m}$  in size. Under these assumptions, a surface of 100  $\mu\text{m}^2$  containing one (or 100) bacterial cells will correspond to a concentration of  $1 \times 10^9$  (or  $1 \times 10^{11}$ ) cells per microliter in tissue slides that are 10- $\mu\text{m}$  thick. 250 bacterial cells per 100  $\mu\text{m}^2$  does not allow the visual distinction of single bacteria but spaces between bacteria can still be seen; cases such as this were therefore assigned a concentration of  $10^{11}$  cells per milliliter. A homogeneous carpet without any empty spaces between them occurs as bacteria increase to 2500 cells/100  $\mu\text{m}^2$ ; these cases were assigned a concentration of  $10^{12}$  cells per milliliter. In cases of extremely high bacterial concentrations sections of 4 or even 2  $\mu\text{m}$  in size can be performed. The spatial organization and the composition of the biofilm can be evaluated using a cumulative multi-step extension analysis which includes detection of bacteria using single FISH probes (Cy3) and DAPI DNA counter-stain. For each sample, probes that positively hybridize with more than 1% of the bacteria that are visualized by DAPI are further combined with each other in pairs and triplets and applied simultaneously using different fluorochromes in a single hybridization (Cy3/FITC/Cy5/ DAPI corresponds to orange/green/dark red/blue). This permits a three- or four-color analysis of the population structure within the same microscopic field. The hybridizations with different probe combinations are cumulatively extended until the position of all relevant bacterial groups to each other and their relative concentrations are clarified. When probes for unrelated bacterial groups hybridize with the same bacteria, it is necessary to adjust the hybridization stringency (*see Note 4*) until a clear differentiation of the bacterial groups is possible. Probes cross-hybridizing even under high stringency conditions should be excluded from the evaluation.

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## The Role of Junctional Adhesion Molecules in Interactions Between Vascular Cells

Triantafyllos Chavakis and Valeria Orlova

### Summary

Adhesive interactions between cells regulate tissue integrity as well as the process of inflammatory cell recruitment. Such intercellular interactions are regulated by adhesion receptors and can be homotypic, that is, between cells of the same type, for example, between adjacent endothelial cells in the vasculature, as well as heterotypic, that is, between different cells, such as the leukocyte endothelial interactions that take place during leukocyte extravasation. Emerging evidence points to the importance of the family of junctional adhesion molecules (JAMs), which are localized in interendothelial contacts and are implicated in the regulation of leukocyte extravasation. JAMs are members of the immunoglobulin superfamily and can undergo both homophilic and heterophilic interactions. This chapter deals with the role of JAMs in the regulation of adhesive interactions between vascular cells.

**Key Words:** Cell adhesion; transmigration; junction; JAM, inflammation; homophilic; heterophilic; co-immunoprecipitation; leukocyte; endothelial.

### 1. Introduction

Junctional adhesion molecules (JAMs) belong to the CD2 subgroup of the immunoglobulin superfamily and consist of two extracellular Ig-like domains, followed by a single transmembrane region and a short cytoplasmic tail. The genes for human JAM-A, JAM-B, and JAM-C are localized on the chromosomes 1, 21, and 11, respectively, and the encoded mature glycoproteins have molecular masses of 32, 40, and 43 kDa, respectively (1–3). JAM-A is found on different circulating blood cells, including granulocytes, monocytes, lymphocytes, platelets, and erythrocytes, as well as on endothelial and epithelial cells (4,5). JAM-B particularly localizes on vascular and lymphatic endothelium and especially at high endothelial venules (6). JAM-C is expressed on endothelial

cells, and platelets (7–9) and on epithelial cells (10). All three JAMs have a class-II PDZ domain-binding motif at their final carboxy-terminus (three C-terminal amino acids). This motif predisposes the molecules to interact with PDZ-domain-containing molecules, such as the ones found in tight junctions (TJs [2]). Such PDZ-domain molecules include zonula occludens (ZO)-1 (11), AF6/afadin (12), MUPP 1 (multi-PDZ-domain-protein 1 [13]), and PAR-3 (partitioning defective), which forms a complex with atypical protein kinase C and PAR-6 (14,15). The association of JAMs with TJ-marker proteins such as ZO-1 can be studied with coimmunoprecipitation experiments. The codistribution of these molecules can also be confirmed by immunofluorescence studies. Thus, JAMs may be involved in a multiprotein complex located at TJs and, because ZO-1 and AF-6 are linked directly to the actin cytoskeleton (16), JAMs may be involved in the dynamic regulation of junction assembly. Here, a particular role may be assigned to the homophilic interaction between JAMs on two adjacent cells.

As opposed to the homophilic interaction, JAMs also can undergo heterophilic interactions and several lines of evidence point to the fact that JAMs also are engaged as counter-receptors for leukocyte integrins: in particular, JAM-A binds the leukocyte integrin  $\alpha$ L $\beta$ 2 (LFA-1 [17]), JAM-B associates with  $\alpha$ 4 $\beta$ 1 (VLA-4 [18]), and JAM-C interacts with  $\alpha$ M $\beta$ 2 (Mac-1 [9]). The latter interaction also mediates leukocyte-platelet interactions. Moreover, the cellular localization of JAMs and these heterophilic binding capacities pointed to a potential role of JAMs in leukocyte extravasation. These heterophilic interactions of JAMs with leukocyte integrins and their potential applications in leukocyte transendothelial migration can be investigated by specific assays, including the binding of isolated proteins in vitro, the adhesion of leukocytes, as well as their transendothelial migration in vitro.

## 2. Materials

### 2.1. Cell Culture and Lysis for the Preparation of Samples

1. Endothelial cell growth medium (PromoCell, Heidelberg, Germany) supplemented with growth factors and 5% FCS (PromoCell).
2. Alpha-Minimal Essential Media growth medium (PAA, Linz, Austria) supplemented with 10% heat-inactivated fetal calf serum (FCS; Biowest, Nuaille, France; see Note 1), 1% penicillin/streptomycin/glutamine, 1% Na-Pyruvate (GIBCO/BRL, Bethesda, MD).
3. Zeocin (Invitrogen, Carlsbad, CA); Accutase (PAA); Trypsin solution (10X) (GIBCO/BRL).
4. To prepare 0.2% gelatin solution, dilute 2% gelatin stock-solution (Sigma-Aldrich, Munich, Germany) in Hank's Balanced Salt Solution.
5. NP-40 lysis buffer: 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.5% NP-40. Buffer should be stored at 4°C. Before use, add protease inhibitor cocktail tablets (Complete Mini, EDTA-free, Roche, Mannheim, Germany), phosphatase inhibitor

cocktail 1 (Sigma), 2.5 mM Na-ortovanadate (Stock 200 mM, Sigma; *see Note 2*), and 1 mM NaF (Sigma).

6. Cell scrapers (Greiner Bio-One, Frickenhausen, Germany).

## 2.2. Immunoprecipitation

1. Mouse monoclonal antibodies against human JAM-C (Gi11) were provided by Dr. S. Santoso (Giessen, Germany [9]).
2. UltraLink Immobilized Protein A/G (PIERCE, Rockford, IL).
3. Sample buffer (4X): 240 mM Tris-HCl, pH 6.8, 40% (w/v) glycerol, 8% (w/v) sodium dodecyl sulfate (SDS), 0.1% (w/v) bromophenol blue.

## 2.3. SDS-PAGE

1. Separating buffer (4X): 1.5 M Tris-HCl, pH 8.8, 0.4% SDS. Store at 4°C.
2. Stacking buffer (4X): 0.5 M Tris-HCl, pH 6.8, 0.4% SDS. Store at 4°C.
3. 10% (w/v) Ammonium persulfate solution (APS), dissolve in water, and store at room temperature (RT; up to 1 wk) or at 4°C.
4. Thirty percent acrylamide/*bis* solution (37.5:1) and TEMED (Sigma).
5. Running buffer (5X): 125 mM Tris-HCl, 1.25 M glycine, 0.5% (w/v) SGS. Store at RT.
6. Prestained protein molecular weight marker (Fermentas, Hanover, MD).

## 2.4. Western Blot

1. Transfer buffer: 20 mM Tris, 150 mM glycine, 20% (v/v) methanol.
2. Gel-blotting paper (Schleicher & Schuell, Dassel Germany) and Nybond-Enhanced Chemiluminescence (ECL) Nitrocellulose membrane (Amersham Biosciences, Freiburg, Germany).
3. PBS-T20: Prepare 10X phosphate-buffered saline (PBS) stock solution of 1.37 M NaCl, 27 mM KCl, 81 mM Na<sub>2</sub>HPO<sub>4</sub>·x2H<sub>2</sub>O, and 18 mM KH<sub>2</sub>PO<sub>4</sub>. Dilute PBS to 1X and add 0.05% (v/v) Tween-20.
4. Blocking buffer: 5% (w/v) nonfat dry milk in PBS-T20.
5. Primary antibody dilution buffer: 1% (w/v) BSA in PBS-T20.
6. Primary antibody: Rabbit anti-ZO-1 (Zymed, South San Francisco, CA).
7. Secondary antibody: Pork anti-rabbit IgG-HRP (Dako Cytomation, Hamburg, Germany).
8. ECL Western blotting detection reagent and Hyperfilm ECL (Amersham Bioscience).

## 2.5. Immunofluorescence

1. Eight-well chamber slides (Permanox; Nunc, Roskilde, Denmark).
2. Human umbilical vein endothelial cells (HUVECs) grown on eight-well chamber slides precoated with 0.2% gelatin for 36 to 48 h at 37°C.
3. The methanol used to fix cells should be ice cold.
4. Primary antibodies: MAb Gi11 against JAM-C (*see Subheading 2.2.*) and rabbit polyclonal antibody against ZO-1 (Zymed).

5. Antibody-dilution buffer: 5% goat serum + 1% bovine serum albumin (BSA) in PBS.
6. Secondary antibodies: rhodamine-coupled anti-mouse IgG (Dianova, Hamburg, Germany) and fluorescein-coupled anti-rabbit IgG.
7. Vectashield to mount slides (Vector Laboratories, Burlingame, CA).

## **2.6. Enzyme-Linked Immunosorbent Assay for Ligand–Receptor Interactions**

1. Coating buffer (HEPES buffer): 20 mM HEPES, 150 mM NaCl, 1 mM  $Mn^{2+}$ , pH 7.4.
2. Blocking buffer: HEPES buffer supplemented with 3% BSA, pH 7.4.
3. Washing buffer: HEPES buffer supplemented with 0.05% Tween-20.
4. Binding buffer: HEPES buffer supplemented with 0.05% Tween-20 and 0.5% BSA (*see Note 3*).
5. Maxisorp plates (Greiner).
6. Purified Mac-1, LFA-1 were provided by Dr. C. Gahmberg (Helsinki, Finland). Soluble recombinant human JAM-C was produced as previously described (**19**). Purified recombinant intercellular adhesion molecule (ICAM)-1 was provided by Dr. S. Bodary (Genentech, CA; *see Note 4*).
7. Primary antibodies: mouse monoclonal antibody (MAb) to human JAM-C, Gi11 (**9**), and mouse MAb to human ICAM-1 (DAKO Cytomation). Secondary antibody: peroxidase-conjugated antibody to mouse immunoglobulins (DAKO Cytomation).
8. Substrate for quantification of bound secondary antibody: 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Boehringer Mannheim, Germany); ABTS-substrate solution: 0.022 g/mL in double-distilled (dd)  $H_2O$ ; ABTS-Buffer: 0.1 M Na-acetate, 0.05 M  $NaH_2PO_4$ , pH 5.5; Hydrogen peroxide solution: 30%  $H_2O_2$  (w/w). Mix ABTS-substrate solution/ABTS-Buffer/Hydrogen peroxide solution 4.76/95.17/0.07 (v/v/v), e.g., 0.75 mL of ABTS-substrate solution + 15 mL of ABTS-Buffer + 0.01 mL of hydrogen peroxide solution.
9. Microplate reader: BIO-TEK (Neufahrn, Germany).

## **2.7. THP-1 Cell Adhesion to Immobilized Soluble Human JAM-C**

1. THP-1 cells were cultivated in RPMI supplemented with 10% FCS (Gibco, Bethesda, MD).
2. Coating buffer: 15 mM  $Na_2CO_3$ , 35 mM  $NaHCO_3$ , pH 9.6.
3. Blocking buffer: PBS supplemented with 3% BSA.
4. Washing buffer: PBS.
5. Adhesion buffer: Serum-free RPMI (*see Note 5*).
6. Phorbol 12-myristate 13-acetate (PMA; Sigma) is dissolved at 1 mg/mL in dimethyl sulfoxide and stored in single use aliquots at  $-80^\circ C$ .
7. Antibodies that were used in the adhesion assay: MAb IB4 against  $\beta 2$  integrin subunit (CD18; Alexis, Gruenberg, Germany); MAb L15 against LFA-1 (kindly provided by Dr. C. Figdor, Nijmegen, The Netherlands); MAb LPM19c against

Mac-1 (kindly provided by Dr. A. May, Munich, Germany); MAb 6S6 against  $\beta$ 1 integrin subunit (CD29) (Chemicon, Temecula, CA).

8. Soluble recombinant human JAM-C (*see Subheading 2.6.*).
9. Ice-cold methanol-acetone 1:1 (v/v) for fixing cells.
10. Staining solution: 0.5% (w/v) Crystal violet (Sigma), 20% methanol, 80% dd H<sub>2</sub>O.
11. Destaining solution: acetic acid/methanol/dd H<sub>2</sub>O (10/30/60, v/v/v).
12. Tissue culture 96-well plates (Greiner).
13. Microplate reader (BIO-TEK, Neufahrn, Germany).

## 2.8. THP-1 Cell Transendothelial Migration

1. 6.5-mm Transwells with a 8- $\mu$ m pore size (Costar, Bodenheim, Germany).
2. HUVECs cultivated in endothelial cell growth medium (PromoCell) supplemented with growth factors and 5% FCS on 0.2% gelatin-precoated transwells.
3. Monocyte chemoattractant protein (MCP)-1 (R&D Systems, Wiesbaden, Germany) is dissolved at 10  $\mu$ g/mL in PBS containing 0.1% BSA.
4. Antibodies that were used in the adhesion assay: MAb LPM19c against Mac-1; MAb L15 against LFA-1; MAb Gi18 against platelet endothelial cell adhesion molecule-1 (PECAM-1) (kindly provided by Dr. S. Santoso, Giessen, Germany).
5. Soluble recombinant human JAM-C (*see Subheading 2.6.*).

## 3. Methods

### 3.1. Cell Culture and Lysis

1. Primary HUVECs were grown on gelatin-coated 100-mm dishes until confluence. When confluent, they were passaged with trypsin/EDTA to provide new cultures on 100-mm tissue dishes (for maintenance) or on eight-well chamber slides (for immunofluorescence), or on transwell filters (for transmigration). For maintenance, cells were split 1:4 to approach confluence after 72 h (*see Note 6*).
2. Chinese hamster ovary (CHO) cells transfected with JAM-C (**9**) were grown on 100-mm dishes in alpha-Minimal Essential Media media supplemented with zeocin 200 ng/mL. Cells were split 1:10 every 4 d with accutase treatment.
3. For each IP, approx  $2 \times 10^7$  JAM-C-transfected CHO cells were used. Cells were washed twice with ice-cold PBS and 500  $\mu$ L of NP-40 lysis buffer were added. After scraping, lysates were incubated for additional 30 min on ice and then pre-cleaned by centrifugation at 16,000g for 15 min at 4°C. The supernatant was transferred in to a fresh vial and then processed for immunoprecipitation. A 100- $\mu$ L aliquot was kept for further Western blot analysis and frozen at -20°C.

### 3.2. Immunoprecipitation

1. Extracts were pre-cleaned by rotating at 4°C with Protein A/G (50  $\mu$ L per 1 mL) for 30 min. After centrifugation, supernatants were transferred to a new tube.
2. MAb Gi11 against JAM-C or an irrelevant antibody (each at 4  $\mu$ g/mL) was added to the cell lysate. The lysate-antibody mixture was vortexed and incubated over night with gentle rotation at 4°C.

3. The next morning, 50  $\mu\text{L}$  of UltraLink Protein A/G are added to the cell extract and incubated rotating for additional 2 h at 4°C.
4. The beads are then washed three times with NP-40 lysis buffer, resuspended in 60  $\mu\text{L}$  of 2X sample buffer, and boiled for 5 min at 95°C. Thereafter, samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

### 3.3. SDS-PAGE

1. For SDS-PAGE, we used Mini Protean II Gel System (Bio-Rad, Munich, Germany). Glass plates were rinsed with detergent and dried with paper towel before the procedure.
2. A 1.5-mm thick 6% gel was prepared by mixing 3 mL of separation buffer (4X), 2.4 mL acrylamide solution, 6.5 mL of water, 120  $\mu\text{L}$  of APS solution, and 12 mL of TEMED. Pour 7.5 mL of the gel solution and overlay with 0.5 mL of water. After the gel is polymerized, remove water with Whatman paper. The stacking gel is prepared by mixing 1 mL of stacking buffer (4X), 0.4 mL of acrylamide, 2.56 mL of water, 100  $\mu\text{L}$  of APS, and 9 mL of TEMED. Pour the gel and insert the comb.
3. Prepare 1X running buffer by mixing four parts of water with one part of the 4X running buffer.
4. After the stacking gel is polymerized, remove the comb carefully and wash the wells with running buffer.
5. Add running buffer to the top and bottom chamber. Boil samples for 5 min at 95°C and load into the wells.
6. Run gel at 100 V for 2.5 h.

### 3.4. Western Blotting

1. Transfer is performed using Mini Trans-Blot System (Bio-Rad).
2. Prepare blotting buffer. Cut membrane and two pieces of blotting paper (they should be larger than the gel). Soak membrane with water and then equilibrate membrane and blotting paper in blotting buffer for 15 min.
3. Assemble blotting sandwich on transfer cassette as follows: sponge prewetted in blotting buffer, then blotting paper, membrane, and the gel. There should be no bubbles between blotting paper, membrane, and the gel. Cover gel with another sheet of blotting paper and sponge. Close transfer cassette and put it to the transfer tank filled with blotting buffer. Perform transfer for 3 h 100 mA at RT.
4. When transfer is finished, take out membrane and place it to the chamber with blocking buffer. Incubate membrane in blocking buffer for 1 h at RT with shaking and thereafter wash once with PBS-T20.
5. Add primary antibody against ZO-1, diluted 1:2500 in PBS-T20/1% BSA and incubate overnight at 4°C with shaking.
6. Discard antibody solution (*see Note 7*). Wash membrane three times for 5 min each in PBS-T20.
7. Add freshly prepared secondary antibody diluted 1:10,000 in PBS-T20/1% BSA and incubate with shaking for 45 min at RT.
8. Wash membrane in PBS-T20 three times for 5 min each.

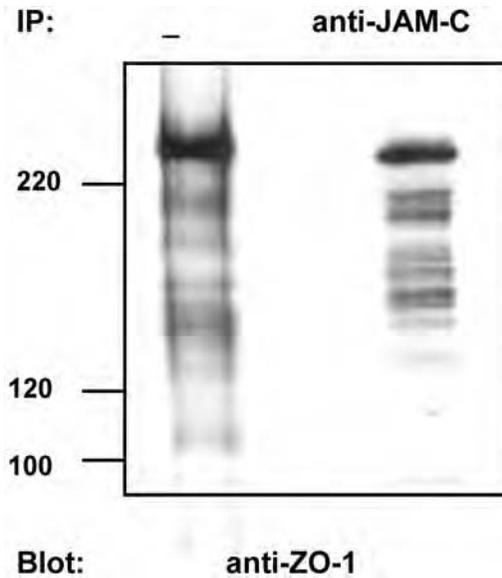


Fig. 1. Coimmunoprecipitation of JAM-C with ZO-1 in chinese hamster ovary (CHO) cells transfected with JAM-C. Detection of ZO-1 by Western blot in JAM-C transfected CHO cell lysates (— indicates no immunoprecipitation) or in CHO-JAM-C cell lysates that were subjected to immunoprecipitation with MAb against JAM-C, as indicated.

9. Discard secondary antibody and place membranes on saran wrap protein side up. Prepare ECL reagent by mixing equal parts of detection reagent 1 with detection reagent 2. Add ECL reagent to the membrane and incubate for 1 min. Discard ECL reagent and rinse membrane with 1X PBS. Then, cover membrane with new piece of saran wrap, put to the X-ray cassette and expose to film (**Fig. 1**).

### 3.5. Immunofluorescence

1. HUVECs were passaged and 30,000 cells/well plated onto eight-well chamber slides that were precoated with 0.2% gelatin (2 h at 37°C) and were allowed to adhere and grow to confluence for 36–48 h at 37°C.
2. After washing the cells three times with PBS, the cells were fixed with ice-cold methanol for 15 min.
3. Methanol is discarded, the cells are washed twice with PBS, and then blocked in antibody dilution buffer for 1 h at RT.
4. The blocking solution is removed and replaced with MAb Gi11 against JAM-C (10 µg/mL) and rabbit polyclonal antibody against ZO-1 (5 µg/mL) in antibody dilution buffer for 2 h at 22°C.
5. The primary antibody is removed and the samples washed three times for 2 min each with PBS. Then, rhodamine-coupled anti-mouse IgG or fluorescein-coupled anti-rabbit IgG is added (each at 1 : 100) for 1 h at 22°C.

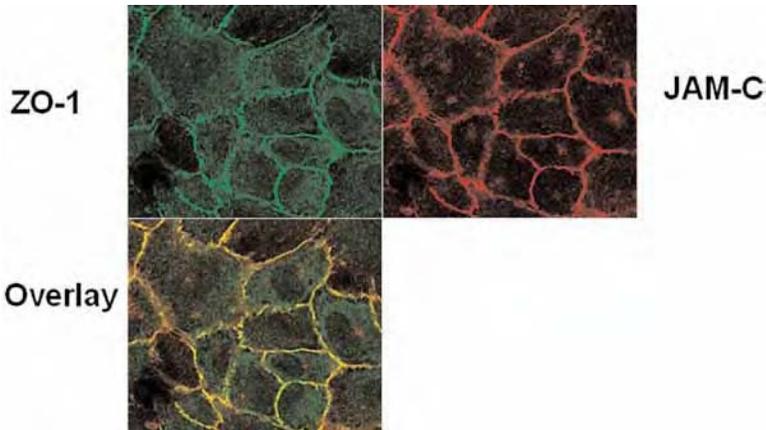


Fig. 2. JAM-C localization at interendothelial borders and colocalization with ZO-1. Representative immunofluorescence of HUVECs showing JAM-C distribution (incubation with MAb Gi11 followed by secondary rhodamine-coupled anti-mouse IgG) together with ZO-1 distribution (incubation with rabbit anti-ZO-1 followed by fluorescein isothiocyanate-coupled anti-rabbit IgG). Double stained images were merged to analyze colocalization of JAM-C with ZO-1. (Please *see* color insert following p. 50 for a color version of this figure.)

6. The secondary antibody is removed, the cells are washed three times with PBS, and DAPI is added for 10 min at RT to stain the DNA and identify the nuclei.
7. The samples are then washed five times in PBS. The gasket is then removed from the chamber slide, and then mounting medium (Vectashield) and a cover slip are added.
8. The slides are viewed and double-stained images are merged to analyze colocalization (**Fig. 2**).

### 3.6. Enzyme-Linked Immunosorbent Assay for Ligand–Receptor Interactions

1. The buffers used are described in **Subheading 2.6**.
2. Maxisorp plates are coated with Mac-1, LFA-1, or BSA (5  $\mu\text{g}/\text{mL}$ ) dissolved in coating buffer overnight at 4°C (*see Note 8*).
3. Discard the integrins and BSA and wash the wells twice in washing buffer. Wells are then blocked with blocking buffer for 1 h at RT.
4. The wells are then washed twice in washing buffer. Binding of soluble human JAM-C (0–20  $\mu\text{g}/\text{mL}$ ) or soluble human intercellular adhesion molecule-1 to the immobilized integrins or to BSA-coated wells is performed in a final volume of 50  $\mu\text{L}$  in binding buffer for 2 h at 22°C. In **Fig. 3**, binding of 10  $\mu\text{g}/\text{mL}$  JAM-C and 10  $\mu\text{g}/\text{mL}$  ICAM-1 is shown.
5. After the incubation period the ligands are discarded and wells are washed three times in washing buffer. To detect bound JAM-C or ICAM-1, the appropriate

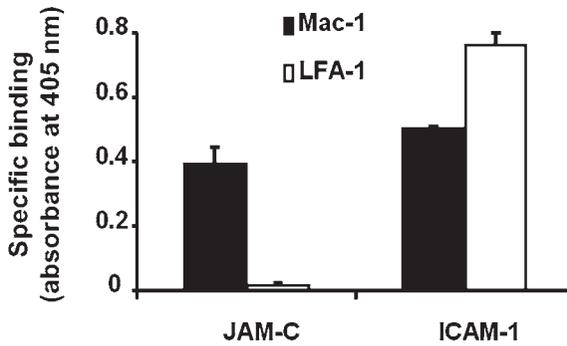


Fig. 3. Interaction between purified JAM-C and Mac-1 proteins. The binding of JAM-C or ICAM-1 to immobilized Mac-1 (**filled bars**) or to immobilized LFA-1 (**open bars**) is presented. Binding of ICAM-1 or JAM-C to BSA was subtracted to obtain specific binding to the integrins. Specific binding is expressed as absorbance at 405 nm. Data are mean + SD ( $n = 3$ ) of a typical experiment.

primary antibodies (*see Subheading 2.6.*) in binding buffer are added to the wells (50  $\mu\text{L}/\text{well}$ ) at a dilution of 1:1000 for 2 h at 22°C.

6. After the incubation period, the primary antibodies are discarded and the wells are washed three times in washing buffer. Then, the secondary antibody in binding buffer is added (50  $\mu\text{L}/\text{well}$ ) at a dilution of 1:2000 for 1 h at 22°C. The plate should be kept in a dark place after addition of the secondary antibody.
7. The secondary antibody is discarded and wells are washed four times with washing buffer.
8. Bound secondary antibody is quantified by using the substrate containing ABTS (*see Subheading 2.6.*; 150  $\mu\text{L}/\text{well}$ ). The plate should be kept in a dark place at RT and incubated for 5 to 120 min so that the change of color takes place.
9. The colorimetric reaction is quantified by measuring the absorbance at 405 nm in microplate reader. Nonspecific binding of JAM-C or ICAM-1 to BSA-coated wells is used as blank and is subtracted to calculate specific binding of these ligands to the integrins (**Fig. 3**).

### 3.7. THP-1 Cell Adhesion to Immobilized Soluble Human JAM-C

1. The buffers used are described in **Subheading 2.7.**
2. 96-well tissue culture plates are coated with JAM-C or BSA (each 10  $\mu\text{g}/\text{mL}$ ) dissolved in coating buffer overnight at 4°C.
3. Discard the JAM-C and BSA and wash the wells once with PBS. Wells are then blocked with blocking buffer for 1 h at RT.
4. During the 1-h incubation: THP-1 cells (cells grow in suspension) are collected from the tissue culture plate and washed in serum-free RPMI medium. For the washing, cells are centrifuged for 5 min at 1200 rpm, the pellet is resuspended in

10 mL serum-free RPMI (keep a small aliquot, e.g., 10 or 20  $\mu\text{L}$  to measure cell number), and then cells are centrifuged once more for 5 min at 200g. During this centrifugation step the cell number should be estimated (*see Note 9*). The cells should then be resuspended in serum-free RPMI at a density of  $2 \times 10^6/\text{mL}$ .

5. After the 1-h incubation, the wells are washed twice in washing buffer. 50  $\mu\text{L}$  of serum-free RPMI is added to each well without or with 50 ng/mL PMA (*see Notes 5 and 10*) and in the absence or presence of competitors (competitors are described in **Subheading 2.7.**; *see Note 11*). Immediately thereafter, 50  $\mu\text{L}$  of the THP-1 cell solution should be added per well (i.e., 100,000 cells/well). Incubate cells at 37°C for 40 to 60 min.
6. After the incubation period, the wells are washed twice with PBS, to remove non-adherent cells. Adhesion buffer or PBS should be aspirated at this washing step.
7. Adherent cells are then fixed with methanol acetone (1:1; 100  $\mu\text{L}$ /well) at 4°C for 30 min.
8. Discard methanol-acetone and stain adherent cells with staining solution (50  $\mu\text{L}$ /well) for 30 min at RT.
9. Remove the staining solution and wash the plates in a bucket with tap water for several times. Let the plate dry. Add the destaining solution (100  $\mu\text{L}$ /well) to the stained adherent cells. Wait for 5 min and then measure absorbance at 595 nm in a microplate reader. Nonspecific adhesion of THP-1 cells to BSA-coated wells is used as blank and is subtracted to calculate specific cell adhesion to JAM-C (*see Note 12; Fig. 4A*).

### 3.8. THP-1 Cell Transendothelial Migration

1. HUVECs are passaged and are plated on 0.2% gelatin-precoated 6.5-mm transwells with a 8- $\mu\text{m}$  pore size in endothelial cell growth medium supplemented with growth factors and 5% FCS at a density of 30,000 cells per well. At this density the wells are confluent after 36–48 h incubation in a humidified atmosphere (37°C, 5%  $\text{CO}_2$ ; *see Note 13*).
2. After this incubation period, chambers are examined microscopically for integrity and uniformity of endothelial monolayers. Monolayers are washed once with PBS and medium is switched to serum-free conditions (endothelial cell growth medium without FCS). If needed, stimuli are added (e.g., tumor necrosis factor- $\alpha$ , 10 ng/mL for 6 h at 37°C) to the endothelial cells, which will upregulate the expression of adhesion molecules on HUVECs.
3. At the beginning of the transmigration assay, THP-1 cells are collected from the tissue culture plate and washed in serum-free RPMI medium. For the washing, cells are centrifuged for 5 min at 1200 rpm, the pellet is resuspended in 10 mL serum-free RPMI (keep a small aliquot, for example, 10 or 20  $\mu\text{L}$  to measure cell number), and then cells are centrifuged once more for 5 min at 1200 rpm. During this centrifugation step, the cell number should be estimated. The cells should then be resuspended in serum-free RPMI at a density of  $4 \times 10^6/\text{mL}$ .
4. The transwell filters (inserts) are removed from their wells and 600  $\mu\text{L}$  migration assay medium (serum-free RPMI in the absence or presence of 50 ng/mL MCP-1)

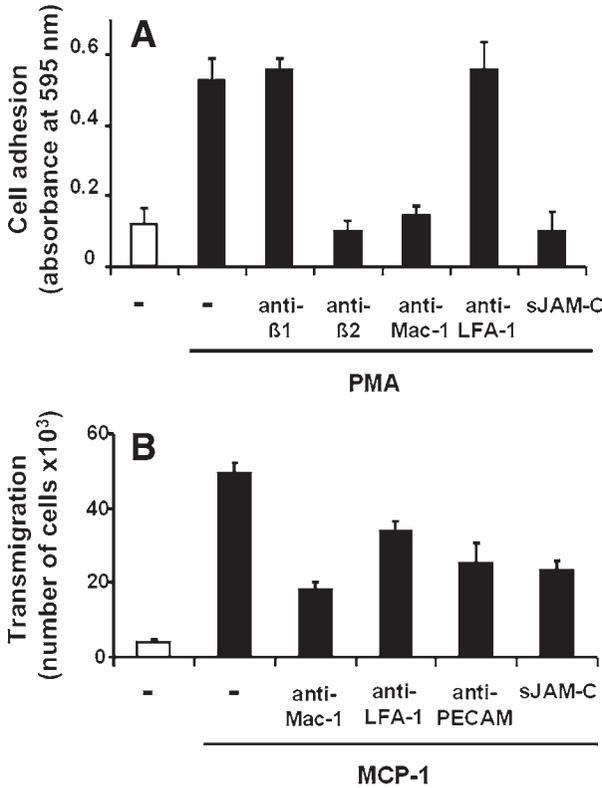


Fig. 4. Interaction between leukocyte Mac-1 and endothelial JAM-C. **(A)** Adhesion of THP-1 cells to immobilized human JAM-C is shown without (**open bar**) or with PMA (**filled bars**) and in the absence (-) or presence of blocking MAb to β1-integrin, MAb to β2-integrin, MAb to Mac-1, MAb to LFA-1 (each at 20 μg/mL), or soluble human JAM-C (20 μg/mL). Cell adhesion is represented as absorbance at 590 nm. Adhesion of THP-1 cells to BSA was subtracted to obtain specific adhesion. Data are mean ± SD (*n* = 3) of a typical experiment. **(B)** The *trans*-endothelial migration of THP-1 cells toward serum-free medium without (**open bar**) or with 50 ng/mL MCP-1 (**filled bars**) is shown in the absence (-) or presence of MAb to Mac-1, MAb to LFA-1, MAb to PECAM-1 (each at 20 μg/mL), or soluble human JAM-C (20 μg/mL). Data are mean ± SD (*n* = 3) of a typical experiment.

is added to the lower compartment (lower well). HUVECs are then washed twice with serum-free medium.

5. The transwell filters are brought back to their original wells, which now contain 600 μL of migration assay medium without or with MCP-1 in the lower compartment. The medium is then aspirated from the transwells and an aliquot of 100 μL of THP-1 cells ( $4 \times 10^5$ ) is then added to the upper compartment on top of the

endothelial monolayer. The competitors (different MAb or soluble proteins, as described under 2.8.) are immediately added (*see Note 11*) to the transwell filters.

6. After incubation for 4 h at 37°C the transwell filters (inserts) are removed from their wells to stop the experiment. The inserts are washed twice in PBS and fixed with methanol/acetone (1:1), stained with crystal violet and mounted on glass slides in order to confirm the confluency of the endothelial monolayer of the filters after the assay (for details on staining procedure please *see Subheading 3.7.*).
7. The number of transmigrated cells in the lower compartment is estimated by measuring cell number of an aliquot of the lower well with a cell counter (CASY-Counter, Schärfe-System, Reutlingen, Germany; *see Note 12; Fig. 4B*).

#### 4. Notes

1. Heat inactivation is performed by incubating FCS for 30 min at 56°C and thereafter serum is stored in single-use aliquots at -20°C.
2. Activated Na-ortovanadate solution is prepared by dissolving an appropriated amount of reagent into ddH<sub>2</sub>O, adjusting pH to 10.0 and boiling for approx 10 min (until it turns colorless). This procedure is repeated one more time and Na-ortovanadate is stored in 1-mL aliquots at -20°C.
3. To keep the immobilized integrins in an active conformation, the supplementation of 1 mM Mn<sup>2+</sup> to the coating, washing, blocking and binding buffers is extremely important. Without Mn<sup>2+</sup> binding of integrins to their ligands will be very weak.
4. Recombinant ICAM-1 or ICAM-1-Fc chimera can also be commercially purchased (e.g., by R&D Systems).
5. In cell adhesion experiments instead of using serum-free RPMI, a buffer containing 20 mM HEPES, pH 7.5; 140 mM NaCl; and 2 mg/mL glucose may be used as the adhesion buffer. In this case, 1 mM MnCl should be used as the stimulus for cell adhesion instead of PMA.
6. We recommend that early-passage HUVECs (up to fourth passage) are used.
7. It is possible to reuse primary antibody for several times. For storage 0.02% sodium azide should be added and solution might be kept at 4°C.
8. When the interaction between  $\beta$ 2-integrins and their ligands is investigated, it is strongly recommended that the integrin is immobilized and the ligand is in solution.
9. 10 or 20  $\mu$ L of the cells should be diluted in 10 mL of NaCl and the number of cells should be estimated in a cell counter. We use CASY-Counter (Schaefer-System). Alternatively, the number of the cells can also be measured in a Neubauer chamber.
10. Besides PMA and MnCl, another way to specifically stimulate  $\beta$ 2-integrin-dependent adhesion is by using stimulating mAb, such as Kim127 or Kim185 (use at 20  $\mu$ g/mL; kindly provided by Dr. M. Robinson, Slough Berkshire, UK).
11. In some cases an additional preincubation of the competitors with the THP-1 cells is recommended rather than the single coincubation of the competitors during the adhesion or transmigration assay in order to obtain higher degrees of inhibition. This can be performed by preincubating the antibodies or soluble proteins with the THP-1 cells for 30 min on ice. In this way the inhibitory antibodies such as the blocking MAb against Mac-1 (*see Subheading 2.7.*) or soluble JAM-C can already

bind to and block Mac-1. When cell–cell adhesion, e.g., THP-1 cell adhesion to HUVECs or THP-1 transendothelial migration is assessed, such a preincubation can be performed on both the THP-1 cells or the HUVECs as appropriate.

12. We have used the same protocols for adhesion and transmigration to test the HUVEC adhesion and transendothelial migration of freshly isolated neutrophils from peripheral blood (19,20). The pattern observed was comparable to results with THP-1 cells.
13. It is very important that no medium is added to the lower compartment before the beginning of the transmigration assay. In particular, serum-containing medium in the lower compartment might prevent HUVECs from growing to confluence on the transwell filter.

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## Cell–Cell Interactions on Solid Matrices

Nancy A. Louis, Dionne Daniels, and Sean P. Colgan

### Summary

Models to study molecular, biochemical, and functional responses in vitro generally incorporate an individual cell type or group of cells organized in a random fashion. Normal physiological responses in vivo require that individual cell types be oriented in an organized fashion with three-dimensional architecture and appropriately positioned cellular interfaces. Much recent progress has been made in the development and implementation of models to study cell–cell contact using substrate grown cells. Here, we summarize the use of membrane permeable supports to study functional responses in appropriately positioned cell types. These models incorporate two or more different cells cultured in physiologically positioned locales on solid substrates. Models incorporating nonadherent cells (e.g., leukocytes) in co-culture with such models also are discussed. Such models have been used extensively to discovery both cell-bound as well as soluble mediators of physiological and pathophysiological processes.

**Key Words:** Cell–cell interactions; substrate; endothelia; epithelia; leukocyte; co-culture.

### 1. Introduction

In intact tissues, matrix-bound cells must communicate not only with outside environment but also with other cells in the local microenvironment. For example, mucosal epithelial cells provide a barrier that separates luminal and vascular compartments. This monolayer of cells provides barrier function and serves as a conduit for vectorial ion movement, the transport event responsible for mucosal hydration (1). By secreting solutes and transporting fluid across the epithelium, epithelia are able to coordinate compositional changes of the luminal compartment. A number of stimuli, including hormones, neurotransmitters, and cytokines, have been shown to directly regulate both epithelial barrier function and ion transport (2).

In intact mucosal tissues, including the lung and intestine, epithelial cells are anatomically positioned in close proximity to a number of subepithelial cell

types, including lymphocytes, fibroblasts, smooth muscle cells, and endothelia. These subepithelial cell populations contribute to epithelial function through paracrine pathways (3). Under these circumstances, for example, the vascular endothelium functions as more than a passive conduit for blood components and synthesizes many compounds that precisely regulate blood vessel tone, vascular composition, and leukocyte movement (4,5). Under such conditions, endothelial cells themselves respond to a variety of proinflammatory stimuli that, in turn, can release inflammatory mediators such as cytokines and bioactive lipids (4). The vital role of the endothelium in coordinating inflammation and the proximity of the vasculature to the epithelium provides a potential paracrine crosstalk pathway between these two cell types.

Here, we discuss the design and implementation of models to study interactions between two or more cell types on solid matrices. These culture models provide ideal opportunities to identify paracrine mediators of physiological and pathophysiological importance and allow flexibility in the use of multiple functional responses. For the purposes of this chapter, we summarize models implementing mucosal epithelial cells as at least one component, but it should be noted that nearly any matrix-bound cell can be integrated into these models.

## 2. Materials

### 2.1. Epithelial and Endothelial Cell Culture

1. T84 cells: 1:1 ratio of Dulbecco's Modified Eagle's Medium (Gibco/BRL, Bethesda, MD) and Hams F-112 Medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 15 mM HEPES buffer (pH 7.5; Sigma, St. Louis, MO), 14 mM Na HCO<sub>3</sub> (Sigma), 40 mg/L penicillin (Sigma), 8 mg/L ampicillin (Sigma), and 9 mg/L streptomycin (Sigma).
2. Human microvascular endothelial cells (HMECs): Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum; 15 mM HEPES buffer, pH 7.5; 14 mM Na HCO<sub>3</sub>; 40 mg/L penicillin; 8 mg/L ampicillin; 9 mg/L streptomycin; 10 ng/mL epidermal growth factor (Collaborative Biomedical Products, Bedford, MA); and 1 μg/mL hydrocortisone (Sigma).
3. Membrane permeable supports in various formats and pore sizes are available from Corning-Costar (Cambridge, MA).
4. Endotoxin (List Biologicals, Campbell, CA).
5. Forskolin (Sigma): 10 mM stock solution made in DMSO (Sigma) and stored at -20°C.
6. Mineral oil (Sigma).
7. Hank's Buffered Salt Solution (HBSS) (Sigma) supplemented with 10 mM HEPES.
8. Modified HBSS (without Ca<sup>2+</sup> or Mg<sup>2+</sup>; Sigma) supplemented with 10 mM HEPES.
9. Voltage clamp (Iowa Dual Voltage Clamps, Bioengineering, University of Iowa) interfaced with an equilibrated pair of calomel electrodes and a pair of Ag-AgCl electrodes.
10. Evohm (World Precision Instruments Inc.).

## 2.2. Neutrophil Transmigration

1. Histopaque 1077 (Sigma).
2. Gelatin (100 bloom, Fisher Scientific).
3. Ammonium chloride lysis buffer: mix 8.29 g of  $\text{NH}_4\text{Cl}$  (Sigma), 1 g of  $\text{NaCO}_3$  (Sigma), and 0.038 g of EDTA (disodium salt, Sigma) in 1 L of distilled and deionized water.
4. Acid citrate dextrose is prepared by mixing 13.7 g of citric acid (Sigma), 25 g of sodium citrate (Sigma) and 20 g of dextrose (Sigma) in 1 L of distilled and deionized water.
5. Citrate buffer: titrate 1 M sodium citrate (Sigma) with 1 M citric acid (Sigma) to final pH of 4.2.
6. 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) reagent is made by adding 50 mg ABT to 100 mM citrate buffer and a final concentration of 0.03%  $\text{H}_2\text{O}_2$  (Sigma).
7. N-formyl-methionyl-leucyl-phenylalanine (fMLP; Sigma) prepared as a 10 mM stock in dimethyl sulfoxide and stored at  $-20^\circ\text{C}$ .

## 3. Methods

Cell culture models on solid matrices have proven important in the modeling of normal biochemical and molecular signaling in vitro, which is particularly important for cells that grow as polarized monolayers (e.g., epithelial cells). This principle has been defined to test and implement a number of cell–cell interaction models, including endothelial–epithelial interactions (3), fibroblast–epithelial interactions (6), neutrophil–epithelial interactions (7,8), mononuclear cell–epithelial interactions (9,10), and neutrophil interactions with residual matrix proteins (11). Examples of each of these cultures, as well as sample sets of data, are provided in this chapter.

### 3.1. Preparation of Epithelial–Endothelial Co-Cultures

1. T84 cells are trypsinized using standard methods of trypsin and EDTA solution (see Note 1).
2. T84 cells are washed in phosphate-buffered saline, diluted in media to a final concentration of  $\times 10^5/\text{mL}$ , and plated on the underside of polycarbonate inserts (termed *inverts*; see Notes 2 and 3), using no more than 82  $\mu\text{L}$  of cell suspension. For these purposes, cells are carefully beaded onto the underside of the insert. Alternatively, cloning rings (6.5 mm diameter) can be glued to the underside of supports to provide a reservoir for plating cells.
3. After plating, inverts are carefully transferred to a tissue culture incubator, and incubated at  $37^\circ\text{C}$  for 12 to 18 h. Monolayers are re-oriented (Fig. 1A) into T84 cell media.
4. HMECs are trypsinized using standard methods of trypsin and EDTA solution.

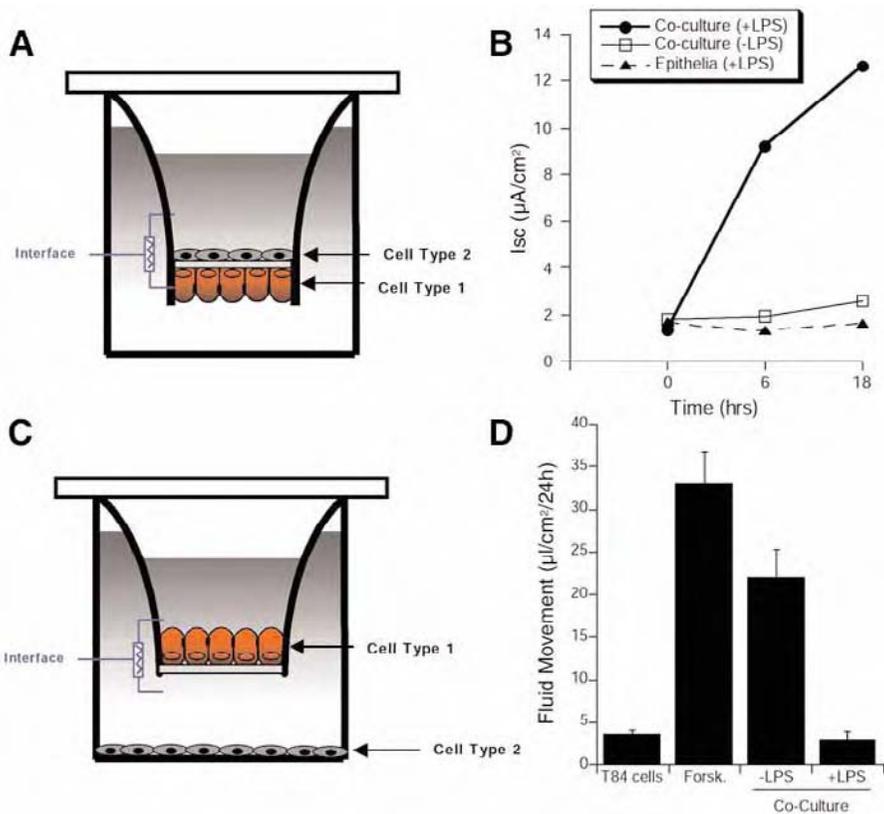


Fig. 1. Models of noncontact substrate grown co-cultures. **(A)** Cell type 1 (e.g., epithelia) and cell type 2 (e.g., endothelia) are plated on opposing surfaces of membrane permeable supports (0.4- $\mu\text{m}$  pores). Cells are allowed to grow to confluence, and functionally assessed. **(B)** Example of results derived from such a co-culture model. In this example, cultures of T84 intestinal epithelial cells (cell type 1) in co-culture with human microvascular endothelia (cell type 2) were incubated in the absence ( $\square$ ) and presence of lipopolysaccharide (LPS) (100 ng/mL,  $\bullet$ ) for indicated times and examined for generation of epithelial electrogenic  $\text{Cl}^-$  secretion (measured as a short circuit current, Isc). T84 monolayers alone ( $\blacktriangle$ ) were incubated with LPS as a control. Results represent data pooled from four monolayers in each condition. **(C)** Cell type 1 (e.g., epithelia) were plated on membrane permeable supports, whereas cell type 2 (e.g., endothelia) were plated in the adjacent well to the permeable support. **(D)** Provides an example of results derived from such a co-culture model. In this example, co-cultures were incubated in the presence and absence of LPS (100 ng/mL) for 24 h and examined for vectorial basolateral-to-apical fluid transport. T84 cells alone served as a negative control and T84 cells exposed to forskolin (10  $\mu\text{M}$ ) in the presence of 3-isobutyl-1-methylxanthine (5 mM) served as the positive control for induction of fluid transport. Data are pooled from three monolayers in each condition, and results are expressed as the mean  $\pm$  standard error of the mean (SEM) fluid movement over the course of 24 h. (Please see color insert following p. 50 for a color version of this figure.)

5. HMECs are washed in phosphate-buffered saline, diluted in media to a final concentration of  $3.5 \times 10^5/\text{mL}$ , and plated on the inner well of polycarbonate inserts using 100  $\mu\text{L}$  of cell suspension. HMECs are then fed 100  $\mu\text{L}$  of media on the apical surface and 1 mL of media on the basolateral surface. Plates are then incubated at 37°C for 3 to 5 d.
6. Typical experimental results assessing functional epithelial endpoints of endothelial activation is shown in **Fig. 1B**. For example, the addition of lipopolysaccharide (100 ng/mL) to endothelial–epithelial co-cultures (model shown in **Fig. 1B**) resulted in a time-dependent increase in epithelial chloride secretion (measured as a change in short circuit current, or  $\Delta\text{Isc}$ ; see **Note 4**).
7. Alternatively, endothelial cells can be plated opposing epithelial cells in the adjacent well of a 24-well plate (**Fig. 1C**). For these purposes, epithelial cells are plated in the inner well of polycarbonate supports.
8. Such co-cultures can be used to assess fluid transport in vitro (**3**). The apical solution of confluent T84 cell monolayers grown on 0.33-cm<sup>2</sup> permeable supports was replaced with 30  $\mu\text{L}$  of media and layered with 60  $\mu\text{L}$  of warm, sterile mineral oil to minimize evaporation. In some monolayers, the cyclic adenosine monophosphate agonist forskolin (50  $\mu\text{M}$ ) was added to the basolateral solution to promote fluid movement and serves as a positive control. After 24 h, the apical solution was collected, centrifuged at 10,000g and quantified with a calibrated pipet and weighed on a balance.

### 3.2. Neutrophil Transmigration Assays

1. T84 cells plated as inverts on the underside of polycarbonate inserts (see **Subheading 3.1.**). For these purposes, it is necessary to plate the cells on substrates with 5- $\mu\text{m}$  pores to allow neutrophils to passage through the substrate. Such studies can also be performed on co-cultures (see **Fig. 2A** and **Subheading 3.1.**).
2. Neutrophils are purified from whole venous blood by density centrifugation. Anti-coagulated whole blood. Tubes are centrifuged at 400g for 20 min at 25°C. Plasma and mononuclear cells are removed by aspiration. Red blood cells were sedimented using 2% gelatin, and residual red blood cells were removed by lysis in ice-cold  $\text{NH}_4\text{Cl}$  buffer.
3. Neutrophils are resuspended to a final concentration of  $5 \times 10^7$  in modified HBSS.
4. Neutrophils ( $1 \times 10^6$ ) are added to the upper chambers of transwell inverts (**Fig. 2**). A chemotactic gradient is established by adding fMLP to the lower chambers (1  $\mu\text{M}$  final concentration in HBSS).
5. Transmigration is carried out at 37°C for 60 to 120 min.
6. Transmigrated polymorphonuclear leukocytes are quantified by assaying for the neutrophil azurophilic marker myeloperoxidase. Neutrophils are lysed by the addition of Triton X-100 to a final concentration 0.5%. Samples are incubated at 4°C for 25 min on a rotating platform at 80 rpm.
7. The samples are acidified with citrate buffer (final concentration 100 mM, pH 4.2).

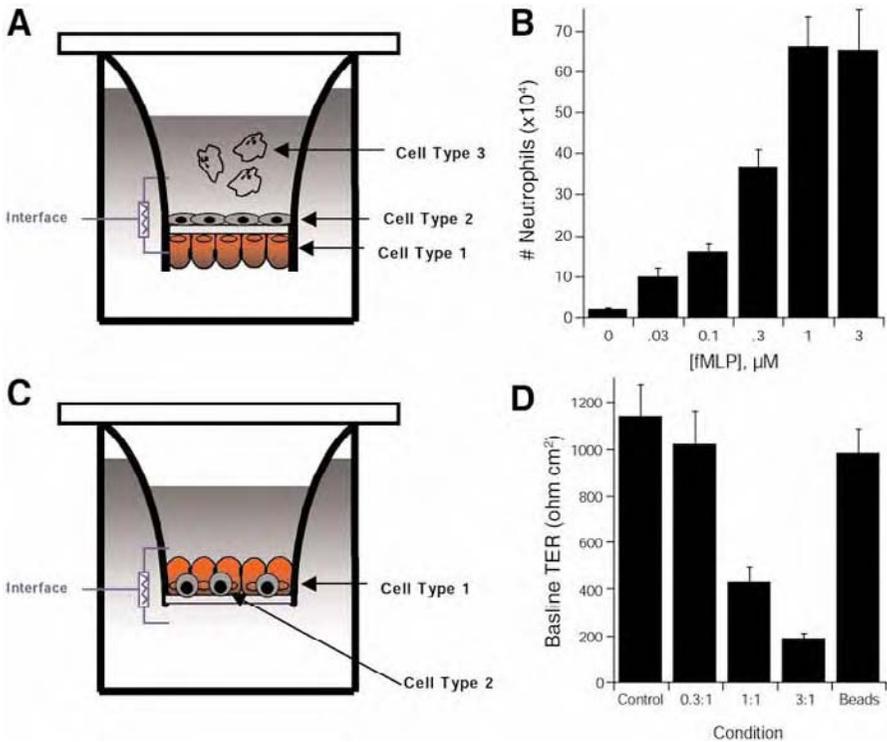


Fig. 2. Models of contact substrate grown co-cultures. **(A)** Cell type 1 (e.g., epithelia) and cell type 2 (e.g., endothelia) are plated on opposing surfaces of membrane permeable supports ( $5 \mu\text{m}$  pores). Cells are allowed to grow to confluence, and functionally was assessed as a substrate for migration of human neutrophils (cell type 3). **(B)** An example of results derived from such a model. In this example, cultures of T84 intestinal epithelial cells (cell type 1) in co-culture with human microvascular endothelia (cell type 2) were washed, and a gradient of indicated concentrations of the neutrophil chemoattractant fMLP was placed in the lower chamber. Purified human neutrophils ( $1 \times 10^6$ ) were placed added to the inner well and transmigration over the course of 90 min was determined by quantification of the detergent extractable neutrophil marker myeloperoxidase. Data are pooled from three monolayers in each condition, and results are expressed as the mean  $\pm$  SEM number of transmigrated neutrophils. **(C)** Cell type 1 (e.g., epithelia) were plated on membrane permeable supports ( $0.4 \mu\text{m}$ ) in direct co-culture with cell type 2 (e.g., mononuclear cells). **(D)** An example of results derived from such a co-culture model. In this example, co-cultures of T84 epithelial cells and human mononuclear cells were plated at indicated mononuclear cell:epithelial ratios (where control condition is epithelia alone and beads condition represents three latex beads per epithelial cell). Co-cultures were grown for 6 d and assessed for barrier function by measurement of transepithelial resistance. Data are pooled from three monolayers in each condition and results are expressed as the mean  $\pm$  standard error of the mean (SEM) transepithelial resistance ( $\text{ohm} \cdot \text{cm}^2$ ). (Please see color insert following p. 50 for a color version of this figure.)

8. An aliquot of sample (70  $\mu\text{L}$ ) is added to an equal volume of ABTS solution in a 96-well plate. The resulting absorbance is determined on a plate reader at 405 nm.
9. Typical results of a transmigration are shown in **Fig. 2B**.

### 3.3. Preparation of Epithelial–Mononuclear Cell Co-Cultures

1. Mononuclear cells are isolated from the interface of density gradients (*see Subheading 3.2.*) and quantified by counting on a hemocytometer.
2. T84 cells are trypsinized using standard methods of trypsin and EDTA solution.
3. After trypsinization, epithelial cells are mixed with mononuclear cells in various ratios; for example, 0.3 mononuclear cells per epithelial cell (0.3:1), 1 mononuclear cell per epithelial cell (1:1), and 3 mononuclear cells per epithelial cell (3:1; **Fig. 2C**). Latex beads ( $11.9 \pm 1.9 \mu\text{m}$  diameter, neutral charge, Seradyn Inc., Indianapolis, IN) were mixed with epithelial cells at the time of plating (three beads per epithelial cell).
4. Epithelial/mononuclear cell mixtures are plated on 0.33-cm<sup>2</sup> ring supported polycarbonate filters (0.4- $\mu\text{m}$  pore size).
5. Co-cultures are grown in T84 cell media to which human recombinant interleukin-2 is added at a final concentration of 2 nM. These epithelial–lymphocyte co-cultures are used 6 to 10 d after plating.
6. Typical experimental results assessing functional epithelial endpoints (e.g., barrier function) are shown in **Fig. 2D**.

### 3.4. Preparation of Residual Matrix Cultures

1. Endothelial or epithelial cultures are established on 0.33-cm<sup>2</sup> ring-supported polycarbonate filters (5- $\mu\text{m}$  pore size; *see Subheading 3.1.*).
2. Removal of cells from the polycarbonate filters is accomplished by incubation in 5 mM EDTA in modified HBSS at 37°C (*see Note 5*).
3. Filters are vigorously washed in HBSS and used to assess migration of neutrophils (*see Subheading 3.2.*).
4. Typical experimental results comparing neutrophil transendothelial and transmatrix migration are shown in **Fig. 3B**.

## 4. Notes

1. Trypsinizing T84 cells can take anywhere between 5 and 20 min. Trypsin–EDTA becomes less active with repeated freeze–thaw cycles. To reduce the time it takes to trypsinize T84 cells, use trypsin that has been aliquoted to avoid repeated freeze–thaw cycles.
2. Generally, cells grow better if the inserts are collagen coated the night before plating. The surfaces are coated with 50 to 150  $\mu\text{L}$  (depending on the diameter of the surface) of type IV collagen and allowed to dry for 12 to 18 h in the hood under ultraviolet light. The type IV collagen is dissolved in 0.2% acetic acid and then diluted in 60% EtOH. Dissolving the collagen can take up to 3 d. Allow the inserts to air dry, and plate cells directly over the collagen coating. No washing is necessary.

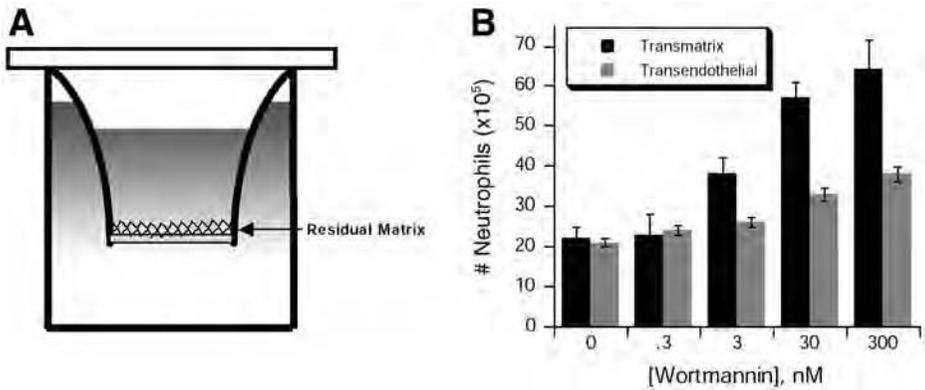


Fig. 3. Models of residual matrix co-cultures. (A) Cells (e.g., endothelia) are plated on membrane permeable supports (5- $\mu\text{m}$  pores) and allowed to grow to confluence. Cells are then removed by vigorous washing in EDTA (2 mM), resulting in residual matrix proteins attached to the membrane support. (B) An example of results derived from such a model. In this example, neutrophils were pre-exposed to indicated concentrations of the phosphoinositide-3-kinase inhibitor wortmannin and assessed for fMLP ( $10^{-8}\text{M}$ )-stimulated transmigration across confluent endothelia (■) or across matrix-coated substrates (■). Transmigrated neutrophils were quantified by determination of myeloperoxidase content after termination of the assay. Data are derived from four monolayers in each condition with results expressed as mean  $\pm$  standard error of the mean (SEM) number transmigrating neutrophils.

- Individual inserts are kept sterile and flipped upside down into a large Petri dish.
- To measure agonist-stimulated short circuit currents (Isc), transepithelial potentials and resistance, a commercially available voltage clamp (Iowa Dual Voltage Clamps, Bioengineering, University of Iowa) interfaced with an equilibrated pair of calomel electrodes and a pair of Ag-AgCl electrodes is utilized, as described in detail elsewhere (12). Using these values and Ohm's law, transepithelial resistance and transepithelial current were calculated. Fluid resistance within the system accounts for less than 5% of total transepithelial resistance. HBSS was used in both apical and basolateral baths during all experiments, unless otherwise noted.  $\text{Cl}^-$  secretory responses are expressed as a change in short circuit current ( $\Delta\text{Isc}$ ) necessary to maintain zero potential difference across the monolayer.
- An alternative method for generating a general matrix has also been used (11). Acellular, matrix-coated permeable support substrates can be generated as follows: bare polycarbonate permeable supports (Corning-Costar, Cambridge, MA, 5- $\mu\text{m}$  pore size) are precoated for 30 min with 50  $\mu\text{L}$  of 0.1% gelatin (derived from porcine skin, 175 Bloom, Attachment Factor, Cascade Biologics, Portland, OR) followed by the addition of media (containing 10% newborn calf serum, Gibco,

Grand Island, NY) for an additional 30 min. Inserts are rinsed in HBBS and polymorphonuclear leukocyte transmigration assessed using similar conditions as described in **Subheading 3.2**.

## Acknowledgments

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## Cell–Cell Interactions in the Kidney

### *Inducible Expression of Mutant G Protein $\alpha$ -Subunits in Madin–Darby Canine Kidney Cells for Studies of Epithelial Cell Junction Structure and Function*

**Ernesto Sabath and Bradley M. Denker**

#### Summary

Disrupted epithelial cell junctions are a hallmark of numerous disease processes. The signaling mechanisms regulating barrier function and re-establishment of intact junctions after injury and during development are complex and tightly regulated. We have shown that heterotrimeric G proteins regulate assembly and maintenance of epithelial cell barrier in cultured Madin–Darby canine kidney (MDCK) cells. The inducible expression of mutant signaling molecules (constitutively active or dominant negative) in polarized cells is a useful strategy for elucidating the role(s) for specific proteins. Using tetracycline-off inducible expression of wild-type and constitutively active  $G\alpha_{12}$ , we have demonstrated a fundamental role for  $G\alpha_{12}$  in regulating the junction of MDCK cells. Inducible expression permits the comparison of the identical cell line in the presence and absence of the protein of interest and minimizes variation arising from distinct clones. The methods described here are applicable to virtually any protein that may regulate maintenance or assembly of the epithelial cell junctional complex.

**Key Words:** G proteins; Madin–Darby canine kidney cells; tight junctions; signaling; epithelial cells; kidney; ZO-1.

#### 1. Introduction

G proteins (guanine nucleotide binding proteins) are molecular switches relaying information from upstream molecules (receptor or equivalent) to downstream targets (effectors). The fundamental “switch” in these proteins occurs after an upstream interaction (i.e., receptor) induces a change in conformation, resulting in the dissociation of bound guanosine diphosphate (GDP). Guanosine triphosphate (GTP) normally is present in cells at higher concentrations than GDP, and

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GTP binds to the G protein. The G protein remains activated and capable of downstream interactions until the intrinsic GTPase activity hydrolyzes bound GTP to GDP (reviewed in **refs. 1 and 2**). There are two broad families of G proteins: monomeric (single subunit) GTPases of the Ras Superfamily, and heterotrimeric (composed of  $G\alpha$  GTPase and  $G\beta\gamma$ ). Both families of G proteins regulate epithelial cell junctions (**3,4**). Regulating these signaling pathways holds promise for novel therapies to prevent or treat disorders resulting in disruption of junctional integrity. Madin–Darby canine kidney (MDCK) cells (derived from canine kidney, distal tubule, or collecting duct) are used widely for studies of epithelial cell polarity and cell junctions. MDCK cells have proven to be a useful model for studies of tight junction assembly through the “calcium switch” (**5**). When cultured in low calcium ( $\mu M$ ), cells lose polarity, junctional proteins become intracellular, and monolayer integrity is lost. With switching to normal calcium medium (“calcium switch”), there is coordinated assembly of the tight junctions thought to recapitulate fundamental processes involved in junctional biogenesis during development and after injury (**6**). Synchronized tight junction assembly without new protein synthesis allows the process to be studied biochemically. Functionally, the integrity of the barrier is monitored through the measurement of transepithelial resistance (TER) at specific time points during the switch process. Recently, this model has been modified to permit the effects of an induced protein on the development of the epithelial barrier to be studied. An example of an inducible system that will be described in detail in this chapter is the “doxycycline switch.” In the most common MDCK cell system (tetracycline-off [Tet-off]), the removal of doxycycline (dox) induces protein expression, permitting the effects of the transfected protein on the cell to be studied. We have used this methodology to elucidate the role of the  $G\alpha_{12}$ -subunit on the epithelial cell barrier (**7,8**).

## 2. Materials

### 2.1. Plasmids and DNA Preparation

1. pTRE (Clontech, Palo Alto, CA).
2. Complimentary DNA (cDNA) of interest and control.
3. Restriction enzymes.
4. Gel extraction kit (QIAquick; Qiagen, Valencia, CA).
5. Calf intestinal alkaline phosphatase (New England Biolabs, Beverly, MA).
6. T4 DNA Ligase 400,000 U/mL (New England Biolabs).
7. DH5 $\alpha$  competent cells (Invitrogen, Carlsbad, CA).
8. Luria-Bertani (LB) broth powder (Sigma, St. Louis, MO).
9. Bacteriological agar (Sigma).
10. Ampicillin sodium salt powder (Sigma).

11. Petri dishes 100 mm (BD Falcon).
12. Qiagen Plasmid Mini or Midi-Kit (Qiagen).

## 2.2. Cell Culture

1. MDCK type II cells stably expressing the tetracycline (Tc)-controlled transactivator for use in the Tet-off gene expression system (Clontech, Palo Alto, CA).
2. Dulbecco's Modified Eagle's Medium (500 mL) with 4.5 g/L glucose and L-glutamine (Cellgro, Herndon, VA).
3. Fetal bovine serum Tet System Approved (Clontech). Heat inactivate at 55°C for 30 min. Use at 5% (25 mL) final concentration.
4. Solution of 10,000 U/mL penicillin G sodium and 10,000 µg/mL streptomycin sulfate (Gibco-BRL, Carlsbad, CA). Add 5 mL of stock solution to medium.
5. G418 Powder, 1 g (Cellgro, Herndon, VA). Prepare stock solution at a concentration of 50 mg/mL. Add 1 mL to 500 mL of medium.
6. Hygromycin B from *Streptomyces hygroscopicus*. Stock solution (50 mg/mL; Roche Diagnostics, Mannheim, Germany.) Add 1 mL to medium.
7. Dox (Sigma) or Tc (*see Note 1*). Store in aliquots (concentration 1 mg/mL) at –20°C, store in the dark and protect from light with foil. Add 20 µL for a final concentration of 40 ng/mL.
8. Phosphate-buffered saline (PBS) tablets (Sigma). To prepare 1X solution, dissolve one tablet/200 mL H<sub>2</sub>O and sterilize.
9. Trypsin 0.25% and EDTA (Sigma).
10. Tissue culture microscope.

## 2.3. Stable Transfection and Selection of Antibiotic-Resistant Clones

1. Lipofectamine 2000 1 mL (Invitrogen).
2. OptiMEM I Reduced Serum Medium for transfections (Gibco, Carlsbad, CA).
3. pTRE Vector-Gene X.
4. pTK-Hyg (*see Note 2*; Clontech).
5. Cloning cylinders (Scienceware, Pequannock, NJ). An alternative is to cut 200-µL pipet tips approx 0.5 cm from wide end and autoclave.
6. High vacuum grease (Dow Corning, Fisher Scientific). Autoclave.
7. Tissue culture plates 24-, 12-, and 6-well plates, and 60-mm plates (BD Falcon).

## 2.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

1. Stacking buffer (4X): Tris-HCl 0.5 M, pH 6.8, 0.4% sodium dodecyl sulfate (SDS).
2. Separating buffer (4X): Tris-HCl 1.5 M, pH 8.8, 0.4% SDS.
3. 30% Acrylamide Solution (**Caution:** When acrylamide is unpolymerized, it is a neurotoxin that may be harmful if inhaled, swallowed, or exposed directly to skin).
4. TEMED (Sigma).
5. Ammonium persulfate (APS). Prepare 10% solution in water and store at –20°C (Sigma).
6. Isobutanol, water saturated.

7. Electrode (running) buffer (10X): 0.25 M Tris-HCl, 1.9 M glycine; add SDS 0.1% before use.
8. Sample buffer (3X): 187.5 mM Tris-HCl, pH 6.8, 6% SDS, 30% glycerol, 0.003% bromophenol blue. Add  $\beta$ -mercaptoethanol 5% before use.
9. Prestained molecular weight markers (Bio-Rad, Hercules, CA).

## 2.5. Cell Lysis

1. PBS (*see Subheading 2.2., item 8*).
2. Tissue Buffer: 10 mM Na-HEPES, pH 7.5, 1% NP-40, 0.25% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, protease inhibitor cocktail (Complete, Roche. One tablet in 1 mL of water makes a solution 50X);  $\text{Na}_3\text{VO}_4$  (tyrosine-phosphatase inhibitor) 1 mM; NaF (serine-phosphatase inhibitor) 25 mM; and 1-mL tuberculin syringe with 27-gage needle.
3. Tabletop centrifuge in a cold room at 4°C.
4. Liquid nitrogen (*see Note 3*).
5. Water bath at 37°C.

## 2.6. Western Blot

1. Electrophoretic transfer cell (Bio-Rad).
2. Nitrocellulose Pure Transfer Membranes (GE Osmonics Labstore, Minnetonka, MN).
3. Transfer buffer (glycine 39 mM, Tris-base 48 mM, SDS 0.04%, methanol 20%).
4. Filter paper, Whatmann 3MM (Fisherbrand).
5. GB003 thick 0.8 filter paper (Schleicher & Schuell BioScience, Keene, NH).
6. Ponceau-S Solution (Sigma).
7. Tris-buffer saline with Tween-20 (TBST): 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20.
8. Blocking-buffer: TBST containing 5% dry milk or 2% albumin powder.
9. Enhanced chemiluminescent reagents (Pierce, Rockford, IL).
10. Rabbit  $\text{G}\alpha_{12}$  antibody (Santa Cruz Biotechnologies, Santa Cruz, CA).
11. Goat anti-rabbit IgG, secondary antibody (Santa Cruz Biotechnologies).

## 2.7. TER Measurements

1. Polycarbonate filters (Transwell, Costar, Acton, MA): tissue culture-treated, 12-mm diameter, and 0.4- $\mu\text{m}$  pore size.
2. MDCK II cells (stably transfected).
3. Millipore ERS Electrical Resistance System (Millipore, Billerica, MA).
4. Dulbecco's Modified Eagle's Medium with additives.

## 3. Methods

The Tet systems permit gene expression to be tightly regulated in response to varying concentrations of Tc or dox. In the Tet-off system, gene expression is turned on when Tc or dox is removed from the culture medium. In contrast, expression is turned on in the Tet-on system by the addition of dox (*see Note 1*

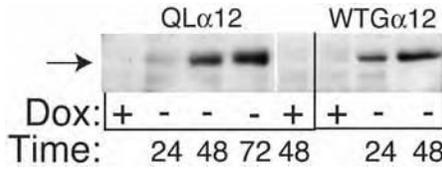


Fig. 1. Gα12 Western blot of inducible Gα12 expression in MDCK cells. Approximately 50 μg of cell lysates were analyzed by SDS-PAGE and Western blot as described. Monolayers were maintained in +dox for several days before switching to -dox for the indicated times. There is a small amount of endogenous Gα12 visible in the +dox lysates that is also seen in Tet-off MDCK cells (not shown). Both wild-type and activated (QL) α12 are rapidly induced with dox removal. The readdition of dox for 48 h (QLα12) demonstrates complete resuppression of protein expression.

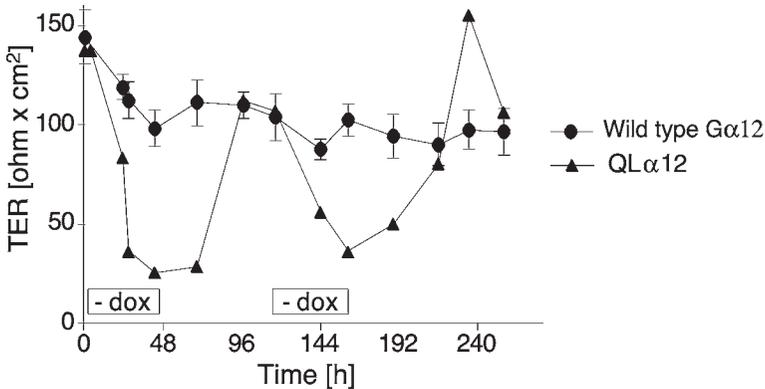


Fig. 2. QLα12 expression results in loss of barrier function. TER was monitored daily for 10 d in wild-type and QLα12-MDCK cells ± dox. The rectangles denote the time interval during which dox was removed. In wild-type Gα12-MDCK cells, there was little change in TER during the 10-d period regardless of Gα12 expression. With QLα12 expression, there was a significant decrease in TER correlating with QLα12 expression, and the effect on TER was completely reversible with readdition of dox.

and **Figs. 1** and **2**). Background or leaky expression of Protein X in the absence of induction is low with pTRE; however, it is important to add fresh dox every 2 d (*see Note 4*). A major advantage of this system is the comparison of the same clone in conditions ± dox (without or with *gene X* expression). In addition to ± *gene X* expression, it is also recommended to prepare a parallel transfection with a control gene. For example, in our analysis of Gα12, two different cell lines were established; one expressing the constitutively activated QLα12 gene and another expressing the wild-type protein (*see Figs. 1* and **2**).

### 3.1. Creating the Gene-Specific Expression Vector

1. Transform each of the plasmids into a DH5 $\alpha$  *Escherichia coli* (or equivalent) to ensure a renewable source of DNA.
2. Linearize pTRE Tet-vector using the appropriate restriction enzymes contained in the multicloning site. Cut your X-gene cDNA with the same restriction enzymes.
3. Run an agarose gel, check the digestion of the plasmids, and excise the bands corresponding at the Tet-vector and X-gene cDNA with a new razor blade; avoid long exposure to the ultraviolet light because it can crosslink the DNA (see **Note 5**). Take a picture from the remaining gel to be sure that no DNA remains.
4. Purify the DNA using a gel-extraction kit or a polymerase chain reaction-purification kit.
5. Add calf intestinal alkaline phosphatase to the pTRE vector for 1 h at 37°C to decrease the probability for self-ligation.
6. To perform ligation, use a ratio of pTRE:X gene cDNA of 1:3, add 10X ligase buffer (see **Note 6**), 1  $\mu$ L of T4 Ligase, and water (to complete 10–15 $\mu$ L/reaction). Incubate for 1 h at room temperature. For blunt-end ligation, incubate for 16 h at 16°C (see **Note 7**).
7. Perform transformation in DH5 $\alpha$  cells.
8. Purify DNA from bacteria with Miniprep kit according to the manufacturer's instructions.
9. Corroborate your ligation product by restriction analysis and/or sequencing.
10. Produce 50–100  $\mu$ g of DNA using the Midiprep kit according to manufacturer's instructions.

### 3.2. Cell Culture

1. Culture the MDCK II cells in Dulbecco's Modified Eagle's Medium with 4.5 g/L glucose and L-glutamine. Add fetal calf serum 5%, penicillin–streptomycin (5 mL), and G418 (1 mL) to the medium.
2. Grow the cells at 37°C in a humidified chamber with 5% CO<sub>2</sub>. Split (trypsinize) the cells when 90 to 100% confluent. Remove the Dulbecco's medium, wash twice with PBS (prewarmed to 37°C), add trypsin–EDTA (2 mL/10 cm plate). Incubate at 37°C for 5 to 15 min. Check under microscope for detachment of cells. Add Dulbecco's Medium (8 mL) to quench the trypsin and split into new plates at 1:5–10 (see **Note 8**).

### 3.3. Preparing Frozen Stocks

1. Trypsinize the desired number of plates or flasks.
2. Centrifuge cells at 100g for 3 to 5 min and aspirate the supernatant.
3. Resuspend the pellet in freezing medium. We resuspend cells from a confluent 10-cm plate in 1 mL and divide into 2  $\times$  0.5 mL aliquots for long-term storage. Freezing medium can be purchased from Sigma (cat. no. C6164) or cells can be frozen in 90% fetal bovine serum and 10% dimethyl sulfoxide.
4. Dispense 0.5-1-mL aliquots into sterile cryovials.

5. Freeze gradually. Start with  $-20^{\circ}\text{C}$  for 1 to 2 h. Transfer to  $-80^{\circ}\text{C}$  overnight and then store in liquid nitrogen.

### 3.4. Titrating Antibiotics (Kill Curve)

Before using hygromycin or puromycin to establish your stable cell lines, it is recommended to titrate the selection agent to determine the optimal concentration for selection.

1. Plate cells at normal dilutions (usually 1:10) in a six-well plate. Allow them to attach for 24 h, then add complete medium plus varying amounts (0, 50, 100, 200, 400, 800  $\mu\text{g}/\text{mL}$ ) of hygromycin.
2. Incubate the cells for 10 to 14 d, replacing the selective medium every 2 to 3 d. Examine the dishes for viable cells every 2 d.
3. For selecting stable transformants, use the lowest concentration that demonstrates significant cell death at approx 5 d and 100% mortality at 10 to 14 d. For Tet-off MDCK cells, we have found 200  $\mu\text{g}/\text{mL}$  hygromycin to be optimal but it may vary from lot to lot.

### 3.5. Stable Transfection and Colony Selection

The goal with the stable transfection is to generate a cell line that gives low background and high expression of Protein X. Both expression levels and induction of Protein X can be significantly affected by the site of plasmid integration. Insertion near an enhancer may result in high basal expression of Protein X, whereas other insertion sites may result in suboptimal induction. To find the clone with the highest induction and lowest background, we recommend that you grow and analyze as many clones as feasible. We usually aim for 30 to 50 clones initially; 20 to 50% will be lost on subsequent processing (to minimize the loss of clones, it is important to avoid long exposure times to trypsin).

1. Grow cells to 80% confluency in complete medium.
2. Transfect pTRE-*Gene X* (25  $\mu\text{g}/100\text{ cm}^2$  dish) and your Selection Marker (pTK-Hyg, or pPUR; 2.5–5  $\mu\text{g}/100\text{ cm}^2$  dish). The plasmids can be linearized before transfection and this may increase transfection efficiency.
3. To transfect MDCK II cells, use a lipid agent, such as Lipofectamine 2000.
4. We recommend adding dox (40 ng/mL) to the transfection medium within 10 to 12 h of the transfection. This step is particularly important if your protein product may be toxic to the cells.
5. Split the cells (1:10) 24 h after the transfection. Usually, a 10-cm plate will be split into ten 10-cm plates.
6. Allow cells to grow 24 h, then add hygromycin (or puromycin) at the optimal concentration (previously determined by killing curve). Sometimes we have added hygromycin immediately after the first split (**step 5**) and have found comparable results.

7. Replace medium containing hygromycin (or puromycin) every 2 d. Fresh dox must be added every 2 d for Tet-off cells (*see Note 4*).
8. After about 5 d, clear patches of plastic devoid of cells should begin to be apparent at low magnification under the tissue culture microscope. Ideally, cells at this stage should not be trypsinized and split (however, if your cells become confluent, you should trypsinize and split again).
9. After 2 to 4 wk, hyg- (or puro-) resistant colonies will begin to become visible to the naked eye and can be viewed by low power on the tissue culture scope. View and circle with a marker on the bottom of the plate to know where to place the cloning cylinder or pipet ring. Avoid waiting until your colonies get confluent, but ideally they should be greater than 50 cells.
10. Viewing the plate from the bottom, you may distinguish small white spots that are colonies. Mark them and confirm under the microscope. Expect 0 to more than 30 distinct colonies per 10-cm plate. It is important that you look carefully at plate periphery because sometimes the colonies prefer this location.
11. Wash entire plate twice with PBS (prewarmed to 37°C).
12. According to the size of the colony (usually 10–100 cells) choose a cloning cylinder, place vacuum grease on lower lip, and place over the margins of your colony. Add trypsin–EDTA 50 to 100  $\mu$ L per ring. Incubate at 37°C for 5 min and look for cell detachment under the microscope. To avoid excessive trypsinization, check cells every 2 min. After cells detach, add complete medium and transfer the cells to a 24 well-plate.
13. Grow the cells with complete medium plus hygromycin and dox. When confluent, transfer to a 12-well plate. When clones nearly confluent, repeat this process for a six-well plate.
14. When the cells are confluent in a six-well plate, we recommend splitting the entire well into a 10-cm plate. When this plate is confluent, we trypsinize and freeze 90% of the cells to insure a continued lineage. The remaining 10% of cells are placed in a six-well plate for analysis.
15. When a clone in the six-well plate is confluent, the media is changed to –dox for 72 h to induce protein expression. Make a cell lysate and determine protein expression by SDS/Western blot. Controls include one +dox cell lysate and one Tet-off cell lysate on the Western (*see Note 9*).

### 3.6. Cell Lysis

1. Wash monolayer with PBS three times.
2. Scrape with rubber policeman in 0.5 to 5 mL of tissue buffer plus protease inhibitors and place in a 1.5-mL Eppendorf tube.
3. Freeze in liquid nitrogen; thaw in 37°C water bath. Be careful not to let sample warm. Repeat two more times.
4. Triturate by passing 10 to 20 times through a 27-gage needle.
5. Centrifuge samples at 3000 rpm for 5 min to pellet nuclei and debris.
6. Determine protein concentration and store samples at –80°C.

### 3.7. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

$\alpha$ -subunits migrate at 39 to 43 kDa on SDS-polyacrylamide gel electrophoresis (PAGE). Therefore, we use 10 to 12% acrylamide gels.

1. The glass of your gel system should be cleaned with a rinsable detergent (Alconox, Alconox, NY). Rinse extensively with distilled water and do the last wash with ethanol 70%. Dry at room temperature.
2. Assemble your gel system. Make sure that your system is not leaky.
3. Prepare a 0.75-mm thick, 10% gel using 30% acrylamide, 1.25 mL of separating buffer, 25  $\mu$ L of 10% APS, and 5  $\mu$ L of TEMED. Pour the gel, leaving space for the stacking gel, and overlay with water-saturated isobutanol. The gel should polymerize in approx 25 to 30 min.
4. Pour off the isobutanol and rinse with distilled water until alcohol odor is gone.
5. Prepare the stacking gel. Pour the stacking gel solution on to the top of the separating gel, and insert the comb. Once your gel has polymerized, remove the comb and fill the spaces with running (electrode) buffer.
6. Add sample-buffer to your cell lysate (usually 10–60  $\mu$ g of protein); heat your samples for 4 to 5 min at 95°C.
7. With an electrophoresis tip, fill the wells of the stacking gel with your samples and add your molecular weight marker (Bio-Rad) to one or more lanes.

Connect your device to the power supply. The gel can be run overnight at 40–50 V (constant voltage) or at 15–20 mA/gel until the sample has exited the stacking gel and entered the running gel. Current can be increased to 30–40 mA/gel until the blue dye reaches the bottom (usually ~2–3 h). Gels run in this manner should be cooled.

### 3.8. Western Blot

1. After SDS-PAGE, transfer is done with the Bio-Rad transfer system.
2. Disconnect the gel unit from the power supply and disassemble. Discard the stacking gel.
3. To assemble your transfer system, you need two foam sheets, two thick filter paper sheets, two thin filter paper sheets, and one nitrocellulose membrane. We usually use squares of 14 cm<sup>2</sup>; try to get all pieces to be the same size. Pour transfer buffer in a big tray and assemble components immersed in transfer buffer. Assemble a transfer cassette (be sure to assemble in order): place two foam sheets, one thick filter paper sheet, one thin filter paper sheet, and then nitrocellulose. The gel is laid on the nitrocellulose and the sandwich completed by placing thin filter paper followed by thick filter paper and the foam sheet.
4. Place your cassette in the transfer tank; your nitrocellulose membrane should be oriented to the anode (red color) and the gel to the cathode (black color). To avoid overheating your system, place your transfer tank on ice and in a cold room. You can transfer your proteins overnight at 0.25 mA or in 2 h at 0.5 to 0.75 mA.

5. Once the transfer is complete, remove the cassette and disassemble. Mark the location of the prestained markers. To assess the quality of transfer, place membrane in Ponceau-S solution for 3 min, wash first with water and then with TBST and look for protein bands. Optional: take a photocopy for later comparison.
6. Incubate nitrocellulose membrane in blocking solution (TBST + 5% dry milk) for 1 h at room temperature or overnight at 4°C on a rocking platform.
7. Discard blocking solution and rinse membrane once with TBST.
8. Place membrane inside a plastic sealable bag, add G $\alpha$ 12 primary antibody (diluted 1:1000 in TBST), and seal. Incubate overnight at 4°C on a rocking platform.
9. Remove the primary antibody (*see Note 10*) and wash the membrane three times, 10 min each with TBST.
10. Add goat anti-rabbit IgG secondary antibody (diluted 1:10,000 in TBST) and incubate for 1 h at room temperature in a rocking platform.
11. Remove the secondary antibody and wash six times for 5 min each with TBST.
12. Prepare enhanced chemiluminescent solution mixing 2 mL of each component. Add this solution to membrane, taking care to cover all the surface of your membrane. Incubate for 5 min.
13. Remove membrane and place into film cassette with intensifying screens and expose to X-ray film for various times to optimize visualization of the protein.

### 3.9. TER Measurements

1. Split cells with trypsin–EDTA.
2. Plate the cells on polycarbonate filters (Transwell, Costar) at confluent density ( $3 \times 10^5$  cells/cm<sup>2</sup>). From a 10-cm plate in 10 mL of medium, place 0.5 mL of cell suspension in the top chamber. Add 1 mL of medium to the lower reservoir. Leave one filter blank with no cells.
3. Allow 24 to 48 h for attachment.
4. TER stabilizes over the course of 2 to 4 d after plating; once TER is stabilized you can switch your cells to –dox.
5. To measure TER, rinse the Millipore ERS electrodes in 70% ethanol, allow them to air-dry, and then rinse them with sterile PBS. When obtaining measurements, it is important to always place the electrode in the same orientation (*see Note 11*). One prong of the chopstick electrode (shorter) is placed inside the suspended filter and the other in the outer well. Press the measure button, wait approx 1 s for the value to stabilize and record. Subtract the value of your blank (this number should be around 100  $\Omega$ /cm<sup>2</sup>). (**Note:** we find our Tet-off cells with TER  $\sim$ 75–90  $\Omega$ /cm<sup>2</sup>.)

## 4. Notes

1. Dox has some advantages over Tc, including a lower concentration for inactivation and a longer half-life in culture.
2. There are other vectors with the Tet-responsive cassette. The pTRE2pur (Clontech, Palo Alto, CA) confers resistance to puromycin instead of hygromycin. There is no clear advantage for either of the antibiotics. The pTRE-Tight (Clontech) is reported to minimize the basal expression of most proteins. Other TRE vectors

permit placement of an epitope tag to aid in protein detection (i.e., pTRE-myc; Clontech).

3. There are other alternatives to extract proteins from the cell lysate, such as sonication on ice.
4. This is the most important procedure to avoid leaky protein expression. Store Dox in the dark at  $-20^{\circ}\text{C}$  for no more than 6 mo. Add fresh Dox to medium every 2–3 d, and replace the medium of cells every 2–3 d.
5. This step is important for success in ligation. Exposure of DNA to ultraviolet light can result in damage to the ends and lower ligation efficiency.
6. Use fresh ligase buffer and avoid many thaw–freeze cycles because this may inactivate the ATP.
7. For ligations, we have obtained the same results incubating the ligation mixture for 1 h at room temperature in comparison with 16 h at  $16^{\circ}\text{C}$ .
8. When trypsin is thawed at  $37^{\circ}\text{C}$ , it can lose activity. Aliquot your trypsin to avoid freeze–thaw cycles.
9. On occasion, well-characterized stable cell lines can lose their responsiveness to Tc or Dox. This can occur after changing lots serum and may be the result of contamination with antibiotics. If a sudden loss of responsiveness is observed, check serum by performing a dose–response curve. It is recommended to subclone and freeze stocks of your cells at various stages.
10. The primary antibody can be saved for subsequent experiments. Add 0.02% final concentration of sodium azide (prepare a 10% stock solution), and storage at  $4^{\circ}\text{C}$ .
11. TER measurements are inherently slightly variable. Measure TER with all the filters in the same orientation, angle and depth of electrodes, media volume and duration of “pulse.” Filters that have had TER recently measured (within last 15–20 min) may have TER values that are noticeably higher if remeasured within this time frame.

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## Nucleotide Metabolism and Cell–Cell Interactions

Holger K. Eltzschig, Thomas Weissmüller, Alice Mager,  
and Tobias Eckle

### Summary

Interactions between the vascular endothelium and polymorphonuclear leukocytes (PMNs) are central to PMN emigration into inflamed tissues, and to neutrophil–endothelial crosstalk pathways that modulate inflammatory responses and vascular barrier function. For example, during episodes of inflammation, the transendothelial migration (TEM) of PMNs potentially disturbs vascular barrier and gives rise to intravascular fluid extravasation and edema. However, because of the close special relationship between PMNs and the vascular endothelium, TEM creates an ideal situation for neutrophil–endothelial crosstalk. While investigating innate mechanisms to dampen intravascular fluid loss and edema occurring during TEM, we observed that PMNs release adenine nucleotides after activation (adenosine triphosphate [ATP] and adenosine monophosphate [AMP]). ATP and AMP are metabolized by endothelial cell-surface enzymes, the ecto-apyrase (CD39, metabolizes ATP to AMP) and the 5′-ecto-nucleotidase (CD73, metabolizes AMP to adenosine). Adenosine generated in this fashion can activate endothelial adenosine receptors, leading to increases in intracellular cyclic AMP and resealing of the endothelial junctions, thereby promoting vascular barrier function. This crosstalk pathway provides an endogenous mechanism to dampen vascular leak syndrome during neutrophil–endothelial interaction. In other words, during TEM, neutrophils close the door behind them.

**Key Words:** Adenosine; adenosine monophosphate; adenosine triphosphate, ectonucleotidase; endothelium; neutrophil; CD73; CD39; inflammation.

### 1. Introduction

Neutrophil-mediated tissue injury is a prominent component in organ damage during inflammation (**1,2**). Endothelial cells, which line the inner lumen of blood vessels and largely determine overall vascular permeability (**3**), are primary targets for polymorphonuclear leukocyte (PMN) during uncontrolled inflammatory responses (**4**). Endothelial injury may result in increased endothelial paracellular permeability and decreased endothelial barrier function. The resultant local tissue

edema and intravascular loss of fluid and solutes may further exacerbate tissue injury via interference with cellular function and limitation of regional blood flow (5).

To understand such interactions between PMNs and the vascular endothelium and potentially identify therapeutic targets for limiting vascular leak syndrome associated with inflammation and hypoxia, the first step is to identify biological mechanisms that control endothelial barrier function and regulate vascular leak. For example, macromolecule transit across blood vessels has evolved to be tightly controlled. Relatively low macromolecular permeability of blood vessels is essential for maintenance of a physiologically optimal equilibrium between intravascular and extravascular compartments (6,7). Endothelial cells comprise the inner cell layer of blood vessels and are the predominant cell type determining vascular permeability (3). Disturbance of endothelial barrier during disease states can lead to deleterious loss of fluids and plasma protein into the extravascular compartment. Such disturbances in endothelial barrier function are prominent in disorders such as shock and ischemia-reperfusion and contribute significantly to organ dysfunction (1,2,8). However, the direct relationship between PMN activation and increased endothelial permeability is not clear. Although PMN accumulation and increased vascular permeability often are coincidental, particularly at sites of inflammation/hypoxia, PMN activation can occur with limited or no net changes in endothelial permeability (9–12). Moreover, although PMN depletion has been demonstrated to decrease organ injury in some models of ischemia and reperfusion (13), PMNs have been demonstrated to exert a protective effect in other models (14–16). Therefore, further information regarding PMN–endothelial cell interactions and their influence on endothelial permeability may provide a better understanding of the regulation of endothelial permeability.

Approximately 70 million PMNs exit the vasculature per minute (17). These inflammatory cells move into underlying tissue by initially passing between endothelial cells that line the inner surface of blood vessels. This process, referred to as transendothelial migration (TEM), is particularly prevalent in inflamed tissues. Understanding the biochemical details of leukocyte–endothelial interactions is currently an area of concentrated investigation, and recent studies of genetically modified animals have suggested that specific molecules may establish “bottlenecks” to the control of the inflammatory response (18). For example, detailed studies have revealed that the process of leukocyte TEM entails a concerted series of events involving intimate interactions of a series of leukocyte and endothelial glycoproteins that include selectins, integrins, and members of the immunoglobulin supergene family (e.g., intercellular adhesion molecule-1 [19–21]). Moreover, histological studies of TEM reveal that PMNs initially adhere to endothelium, move to nearby interendothelial

junctions via diapedesis, and insert pseudopodia into the interendothelial paracellular space (22). Successful TEM is accomplished by temporary PMN self-deformation with localized widening of the interendothelial junction. After TEM, adjacent endothelial cells appear to “reseat,” leaving no residual interendothelial gaps (22). These histological studies are consistent with the observation that leukocyte TEM may result in little or no change in endothelial permeability to macromolecules (9–12,23). In the absence of this tight and dynamic control of endothelial morphology and permeability, interendothelial gap formation during leukocyte TEM could lead to marked increases in endothelial permeability. However, only limited information exists regarding the biochemical events which maintain and dynamically regulate endothelial permeability in the setting of either PMN activation or TEM (22,24).

Despite the severe course in which inflammatory diseases can proceed, most inflammation is self-limiting. One important factor may be increased production of endogenous adenosine, a naturally occurring anti-inflammatory agent (25–27). Several lines of evidence support this assertion. First, adenosine receptors are widely expressed on target cell types as diverse as leukocytes, vascular endothelia, and mucosal epithelia and have been studied for their capacity to modulate inflammation (27). Second, murine models of inflammation provide evidence for adenosine receptor signaling as a mechanism for regulating inflammatory responses in vivo in endogenous anti-inflammation. For example, mice deficient in  $A_{2A}$ -adenosine receptor ( $AdoRA_{2A}$ ) show increased inflammation-associated tissue damage (28). Third, hypoxia is a common feature of inflamed tissues (29) and is accompanied by significantly increased levels of adenosine (30–33). At present, the exact source of adenosine is not well-defined but likely results from a combination of increased intracellular metabolism and amplified extracellular phosphohydrolysis of adenine nucleotides via surface ecto-nucleotidases.

Previous studies have indicated that activated PMN release a number of soluble mediators that regulate vascular permeability during transmigration. For example, PMNs have been demonstrated to actively release adenine nucleotides (in the form of adenosine triphosphate [ATP] or adenosine monophosphate [AMP]). On the endothelial surface, ATP can be converted by the ecto-apyrase (CD39) to AMP, which in turn is converted by the 5'-ectonucleotidase (CD73) to adenosine (14,15,34,35). Adenosine generated in this fashion is then available to activate adenosine receptors either on the endothelial surface or on neutrophils to modulate inflammatory responses.

To pursue studies of neutrophil–endothelial interaction, we first describe an isolation technique of fresh PMNs from human blood and a high-performance liquid chromatography (HPLC)-based technique to measure nucleotide/nucleoside release from activated neutrophils. Then, we introduce an in vitro model of endothelial permeability that allows the in vitro measurement of endothelial

barrier function. Finally, we describe techniques to measure induction of CD73 or CD39 protein and function on the endothelial surface.

## 2. Materials

### 2.1. Isolation of Human Neutrophils

1. Citrate blood: 10 mL of Monovets containing Na-citrate, 1 mL (Sarstedt, Nümbrecht, Germany).
2. Percoll (Amersham Biosciences, Uppsala, Sweden).
3.  $\text{NH}_4\text{Cl}$  lysis buffer: 420 mg of  $\text{NH}_4\text{Cl}$ , 4 g of  $\text{NaHCO}_3$ , and 18.5 mg of EDTA; bring with  $\text{H}_2\text{O}$  to 500 mL.
4. Hank's Balanced Salts Solution (HBSS)- (Gibco/Invitrogen) containing no calcium and no magnesium, buffered with HEPES (Gibco/Invitrogen) 10 mM to pH 7.4 (10 mL of the 1 M stock solution is added to 1 L of HBSS).

### 2.2. Preparation of Activated PMN Supernatants and Measurement of Nucleotide Contents

1. *N*-formyl-Met-Leu-Phe (fMLP; Sigma-Aldrich, Munich, Germany).
2. HBSS- (Gibco/Invitrogen) containing no calcium and no magnesium, buffered with HEPES (Gibco/Invitrogen) 10 mM to pH 7.4 (10 mL of the 1 M stock solution is added to 1 L of HBSS).
3. HBSS+ (Gibco/Invitrogen) containing calcium and magnesium, buffered with HEPES (Gibco/Invitrogen) 10 mM to pH 7.4 (10 mL of the 1 M stock solution is added to 1 L of HBSS).
4. CHRONO-LUME reagent (Crono-log Corp, Haverton, PA).
5. For activation of murine PMN, use Leukotriene B4 (Calbiochem, San Diego, CA).

### 2.3. Cell Isolation and Cell Culture

1. HBSS- (Gibco/Invitrogen) containing 1% antibiotic-antimycotic solution (Sigma-Aldrich).
2. Lactated Ringer's Solution (Fresenius, Bad Homburg, Germany).
3. Collagenase A solution (Roche, Mannheim, Germany) in HBSS- to 1 mg/mL.
4. Modified Medium 199, Medium 199 (Cambrex, Verbier, Belgium) and fetal calf serum (FCS; PAA, Cölbe, Germany) + 1% Na Pyruvate (Sigma-Aldrich) + 1% antibiotic/antimycotic solution (Sigma-Aldrich).
5. Antibiotic solution: antibiotic-antimycotic solution (Sigma-Aldrich).
6. Endothelial cell basal medium (Promo Cell, Heidelberg, Germany) supplemented with endothelial cell growth media (Promo Cell).
7. Accutase (PAA).
8. Trypsin-EDTA (Cambrex).
9. Trypsin neutralizing solution (Cambrex).
10. Human microvascular endothelial cells (HMEC) media: modified MCDB 131 (Gibco/Invitrogen) with L-glutamin 5 mM (Gibco), 10% fetal calf serum (PAA),

1  $\mu\text{g}/\text{mL}$  hydrocortisone (Sigma), and epidermal growth factor (BD Biosciences, Bradford, MA).

#### **2.4. Endothelial Permeability Assay**

1. HBSS+ (Gibco/Invitrogen) containing calcium and magnesium, buffered with 10 mM HEPES (Gibco/Invitrogen) pH 7.4 (10 mL of the 1 M stock solution is added to 1 L of HBSS).
2. HBSS– (Gibco/Invitrogen) containing no calcium and no magnesium, buffered with 10 mM HEPES (Gibco/Invitrogen) pH 7.4 (10 mL of the 1 M stock solution is added to 1 L of HBSS).
3. 70-kDa fluorescein isothiocyanate (FITC)-labeled dextran (Molecular Probes, Eugene, OR).

#### **2.5. Immunoprecipitation**

1. Biotin (Sigma-Aldrich, 1 mM final concentration).
2.  $\text{NH}_4\text{Cl}$  buffer (150 mM): for 50 mL add 0.4 g of  $\text{NH}_4\text{Cl}$  to 50 mL of  $\text{H}_2\text{O}$ .
3. Radio-immunoprecipitation (RIPA) buffer: 250 mM NaCl, 10 mM Tris-HCl, pH 8.1, 1% Triton X-100 (Sigma-Aldrich) 1%; NP 40 (Igepal/CA630, Sigma-Aldrich); and 1 mM EDTA. Add protease/phosphatase inhibitors immediately before use: 1 mM phenylmethylsulfonyl fluoride, 1  $\mu\text{g}/\text{mL}$  leupeptin, and 100  $\mu\text{M}$  sodium orthovanadate.
4. Protein G–Sepharose (Amersham Bioscience).
5. Reducing sample buffer: 2.5% sodium dodecyl sulfate, 0.38 M Tris-HCl, pH 6.8, 20% glycerol, and 0.1% bromophenol blue.
6. Wash buffer: 30 g of NaCl, 2 mL of Tween-20 (Sigma-Aldrich), and 10 mL of 1 M HEPES to 1000 mL with  $\text{H}_2\text{O}$ .
7. Blocking buffer: use wash buffer with 3% bovine serum albumin.
8. Streptavidin-peroxidase (Sigma-Aldrich).
9. Enhanced chemiluminescence (Amersham Pharmacia Biotechnology).

#### **2.6. Measurement of Endothelial Surface Enzyme Activity of the Ecto-Apyrase (CD39) and the 5'-Ectonucleotidase (CD73)**

1. Etheno-ATP (E-ATP; Molecular Probes).
2. Etheno-AMP (E-AMP; Sigma-Aldrich).
3. Etheno-adenosine (Sigma-Aldrich).

Avoid frequent thawing of the etheno-compounds because they will lose their activity. Thaw once, and aliquot them as needed for each experiment.

### **3. Methods**

#### **3.1. Isolation of Human Neutrophils (PMNs)**

PMNs have to be freshly isolated from whole blood obtained by venipuncture from human volunteers and anticoagulated with acid citrate. We use 10-mL

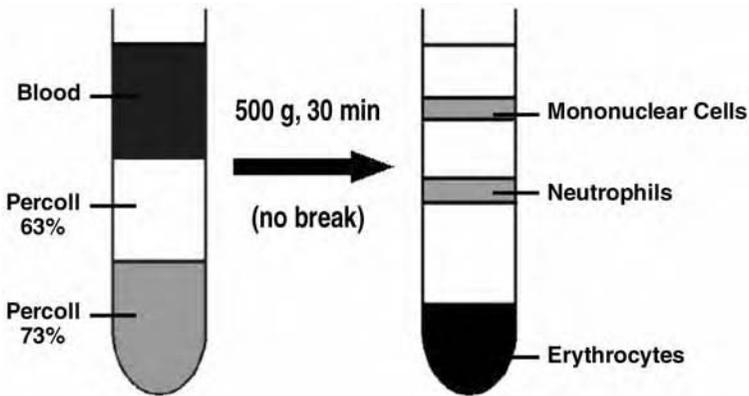


Fig. 1. Isolation of PMNs using double-density centrifugation of whole blood. Whole blood is obtained by venipuncture from human volunteers and anticoagulated with acid citrate. To remove platelets, plasma, mononuclear cells and erythrocytes, a double-density centrifugation technique is applied using the Percoll system. For this purpose 4 mL of Percoll, prepared to a density of 63% (*see* manufacturer's instructions), is placed into a clear 15-mL tube, and 4 mL of Percoll, density 73%, is carefully placed into the bottom. Then, 4 mL of whole blood is placed on top (**left**). The tubes are centrifuged at 500g for 30 min at room temperature, no break. After the centrifugation, three bands can be visualized in the tube: below are the erythrocytes, the second band consists of the PMNs, and the third band contains the mononuclear cells (**right**).

monovets containing Na-citrate (Sarstedt, Nümbrecht, Germany) to draw the blood. To remove platelets, plasma, mononuclear cells, and most of the erythrocytes, a double-density centrifugation technique is applied using the Percoll system (*see* **Note 1**).

1. In short, 4 mL of Percoll, prepared to a density of 63% (*see* manufacturer's instructions) is placed into a clear 15-mL tube, and 4 mL of Percoll, density 73%, is carefully placed below.
2. Then, 4 mL of the anticoagulated whole blood is placed carefully on top (**Fig. 1**).
3. The tubes are centrifuged at 500g for 30 min at room temperature, no break.
4. After the centrifugation, three bands can be visualized in the tube. At the bottom are the erythrocytes, the second band represents the PMNs, and the third band contains the mononuclear cells (**Fig. 1**). To isolate the neutrophils, the plasma and the band containing the mononuclear cells are removed to gain easier access to the PMN-containing band.
5. Next, the band with the neutrophils is transferred into a 50-mL tube. From this point forward, PMNs should be kept strictly at 4°C (*see* **Note 2**).

Residual erythrocytes are removed by lysis in cold  $\text{NH}_4\text{Cl}$  buffer. For this purpose, PMN are resuspended into 30 mL of ice-cold  $\text{NH}_4\text{Cl}$  buffer, incubated at

4°C for 10 min, and centrifuged at 170g (10 min, 4°C, break on). The supernatant is discarded, and the cells are washed with HBSS– twice (400g, 10 min, 4°C, break on). Remaining cells should consist of 97% or greater PMNs, as can be assessed by microscopic evaluation. PMNs should be studied within 2 h of their isolation. In general, approx 0.5 to  $1 \times 10^8$  PMNs can be isolated from 50 mL of fresh blood.

### **3.2. Preparation of Activated PMN Supernatants and Measurement of Nucleotide Contents**

To measure nucleotide release from activated PMN, freshly isolated human neutrophils are activated with fMLP, and samples from the supernatant are taken at different time-points after activation and analyzed by HPLC.

1. In short, freshly isolated human PMNs ( $10^7$  cells in 1 mL of HBSS+ with  $10^{-6}$  M fMLP) are incubated end-over-end at 37°C.
2. At 1, 5, 10, and 15 min, 200- $\mu$ L samples are transferred into an ice-cold Eppendorf cup and immediately pelleted (1000g for 20 s at 4°C).
3. The resultant cell-free supernatants are filtered (0.45- $\mu$ m Phenomenex, Torrance, CA) and kept strictly on ice until assessed by HPLC. Alternatively, supernatants can be stored at –80°C until HPLC measurements are performed. To determine nucleotide content, the cell-free supernatants are resolved by HPLC (Hewlett-Packard, Palo Alto, CA; model 1050) with an HP 1100 diode array detector by reverse-phase on an HPLC column (Luna 5- $\mu$ m C18, 150  $\times$  4.60 mm; Phenomenex, Torrance, California, CA) with 100% H<sub>2</sub>O mobile-phase. ATP and adenine nucleotides can thus be identified by their chromatographic behavior (retention time, ultraviolet absorption spectra, and co-elution with standards).

In experiments when only the ATP content of the supernatant is of interest, a luciferase-based technique can be applied. This technique has a higher resolution because ATP concentrations in the picco-molar range can easily be detected. To do this, PMN are activated as outlined previously, supernatants are collected, and the ATP content can be quantified using CHRONO-LUME reagent (Crono-log Corp, Haverton, PA). Luciferase activity can be assessed on a luminometer (Turner Designs Inc., Sunnyvale, CA) and compared with internal ATP standards (*see* **Notes 3** and **4**).

### **3.3. Endothelial Cell Isolation and Culture**

For assessment of endothelial barrier function *in vitro*, human umbilical vein endothelial cells (HUVECs) or immortalized HMEC-1 were used. HUVECs were isolated from a 10- to 15-cm long “fresh” piece of umbilical cord (minimum 10 cm). The umbilical cord was transported on ice from the labor floor to the laboratory in a sterile bottle without any solution. The residual blood within the umbilical cord provides the best media for the cells until isolation.

To isolate the HUVECs from the cord, the umbilical cord is first flushed with HBSS– (containing no calcium and no magnesium). The vein is identified by its larger size (compared with the arteries) and lack of a muscular tissue surrounding the lumen, and filled all the way up with collagenase A (1 mg/mL). Both ends of the umbilical cord are sealed with strings, and the collagenase A filled umbilical cord is incubated in 37°C in warm lactated Ringer’s solution for exactly 5 min. The collagenase A solution (containing the umbilical vein endothelial cells) is then flushed out of the vein using modified Medium 199 (*see Subheading 2.1.*) into a 50-mL Falcon tube. The cells are centrifuged at 110g for 8 min, no break at room temperature. The supernatant is discarded and to wash the cells, the pellet is carefully resuspended into 20 mL of modified Medium 199 and centrifuged again (110g, 8 min, room temperature, no break). After discarding the supernatant, the pellet is resuspended into 20 mL of endothelial cell basal medium and transferred into a collagen-coated cell culture bottle. Cells are cultured at 37°C containing 5% CO<sub>2</sub>. The media has to be changed the day after the primary cell preparation and thereafter every other day. The time until the first passage of the cells differs considerably; therefore, cells have to be observed carefully before the first passage. Full confluency usually is reached within 1 to 2 wk. At that time cells should be split using trypsin–EDTA and trypsin-neutralizing solution (*see Subheading 2.1.*) and cultured as described previously. HUVECs can be used up to four passages; then, the cells should be discarded and new cells should be freshly prepared from an umbilical cord (*see Note 5*).

HMEC-1 are harvested with Accutase and incubated at 37°C in 95% air/5% CO<sub>2</sub>. Modified MCDB 131 is used for cell culture. Endothelial cell purity can be assessed by phase microscopic “cobblestone” appearance and uptake of fluorescent acetylated low-density lipoprotein.

### **3.4. Endothelial Permeability Assay**

To study details of neutrophil endothelial interaction with regard to endothelial barrier function, we culture endothelial cells (HMEC-1 or HUVECs) on a permeable support system with a separate apical and basal portion (so-called “insert”). As soon as the cultured endothelium forms a confluent monolayers, the system can be used to investigate endothelial barrier function. For example, activated neutrophils can be added in different concentration to the apical portion of the insert. By using a tracer substance that is applied to the apical compartment, the flux of the tracer substance can be measured at different time points from the basal portion, thereby determining transcellular flux rates and measuring endothelial barrier function. We have previously used this technique to measure endothelial barrier function in the presence of different concentrations of activated neutrophils or the supernatant of activated neutrophils. Interestingly, activated PMNs or their supernatant decreased endothelial flux

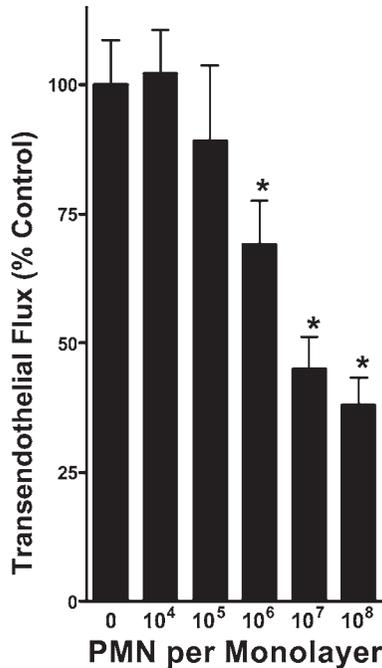


Fig. 2. Activated PMNs promote endothelial barrier function. PMNs activated with fMLP ( $10^{-6}$  M) and FITC-dextran 70 kDa were added to HMEC-1 monolayers. Transendothelial flux was calculated by linear regression (six samples over 60 min) and normalized as percent of control (HBSS+ only). Data are derived from six monolayers in each condition; results are expressed as mean of percent control flux  $\pm$  standard deviation. \* $p < 0.05$ .

of 70 kDa dextran, thereby promoting barrier function in the below described model (Fig. 2).

For preparation of experimental endothelial monolayers used to measure endothelial barrier function, confluent endothelial cells are seeded at approx  $1 \times 10^5$  cells/cm<sup>2</sup> onto either permeable polycarbonate inserts or 100-mm Petri dishes. In short, HMEC-1 are seeded on polycarbonate permeable inserts (0.4- $\mu$ m pore, 6.5-mm diameter; Costar Corp., Cambridge, MA). To do this, HMEC-1 from a cell culture bottle (area 75 cm<sup>2</sup>) of fully confluent HMEC-1 are detached with trypsin and suspended in 20 mL of HMEC media. As next, 1 mL of HMEC media (without cells) is placed on the basal portion of the insert and 80  $\mu$ L of the cell suspension is carefully placed on the apical portion of the insert. A few drops of media are then added to the apical portion. Endothelial barrier function can be studied when controls seeded on Petri dishes reach full confluency (usually 5–7 d after seeding; see Note 6). To study endothelial

barrier function, inserts are placed in HBSS+-containing wells (0.9 mL), and HBSS+ (alone or with PMN, PMN supernatant, or ATP) is added to the apical portion (100  $\mu$ L). At the start of the assay ( $t = 0$ ), FITC-labeled dextran 70 kDa (concentration 3.5  $\mu$ M) is added to fluid within the apical portion of the insert. The size of FITC-dextran, 70 kDa, approximates that of human albumin, both of which have been used in similar endothelial paracellular permeability models (36,37). Fluid from the opposing well (basal portion) are sampled (50  $\mu$ L) over the course of 60 min ( $t = 20, 40,$  and 60 min). Fluorescence intensity of each sample can then be measured (excitation, 485 nm; emission, 530 nm; Cytofluor 2300; Millipore Corp., Waters Chromatography, Bedford, MA) and FITC-dextran concentrations are determined from standard curves generated by serial dilution of FITC-dextran. Paracellular flux is calculated by linear regression of sample fluorescence (37). Consistent with observations of other investigators, control experiments demonstrate decreased paracellular permeability with forskolin or 8-bromo-cyclic AMP (38) and increased paracellular permeability with thrombin and hydrogen peroxide (39).

### 3.5. Immunoprecipitation

To show expression or induction of a specific cellular protein on the endothelial surface, surface proteins can be labeled with biotin. Using an avidin blot, these proteins can be quantified. We have previously used this technique to show expression and induction of the CD39 and CD73 by hypoxia (14,34).

To pursue this, HMEC-1 or HUVECs are grown to full confluency in 100-mm Petri dishes. The labeling has to be performed in the cold (4°C) to stabilize surface proteins (see Note 7). Therefore, Petri dishes with cells are taken to a cold room (4°C) and placed on a cold surface (4°C). Cells are washed twice with ice-cold HBSS+ and are labeled with biotin (final concentration 1 mM). For this purpose, cover cells completely with 1 mM Biotin in HBSS+, enough to cover all cells (minimum 3 mL), and place cells on a rotator (slow settings) for 20 min at 4°C. As next, cells are rinsed twice with HBSS+. Then, endothelia are incubated with 150 mM NH<sub>4</sub>CL buffer (10 mL/plate, 30 min at 4°C), rinsed twice with HBSS+, and lysed with RIPA buffer. For this purpose, 1.25 mL of RIPA buffer is given to each 100-mm Petri dish, cells are scraped with a cell-scraper or a pipet tip and transferred into an Eppendorf cup (avoid bubbles). The Eppendorf cups are rotated end-over-end for 30 min, and cell debris is removed by centrifugation (1000g at 4°C for 5 min). Immunoprecipitation is then performed with mono or polyclonal antibodies (e.g., for human CD39 [Research Diagnostics, Inc. Flanders, NJ; 5  $\mu$ g/mL], CD73 with MAAb 1E9 [5  $\mu$ g/mL, a kind gift from Dr. Linda Thompson, Oklahoma Medical Research Foundation]) followed by addition of 100  $\mu$ L of pre-equilibrated protein

G-Sepharose (use RIPA buffer for pre-equilibration) and overnight incubation (end over end at 4°C; *see Note 8*). Washed immunoprecipitates are boiled in reducing sample buffer, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose, and blocked overnight in blocking buffer. Biotinylated proteins are labeled with streptavidin–peroxidase and visualized by enhanced chemiluminescence (*see Note 9*).

### **3.6. Measurement of Endothelial Surface Enzyme Activity of the Ecto-Apyrase (CD39) and the 5'-Ectonucleotidase (CD73)**

To assess enzyme activity of CD39 and CD73 on the surface on endothelia, the etheno-compounds E-ATP (Molecular Probes) and E-AMP (Sigma-Aldrich) can be used, respectively (*see Note 10*). Because of their etheno-groups, these compounds exhibit different elution characteristics on the HPLC compared with the native compounds (ATP or AMP) and, therefore, endogenous nucleotide concentrations will not affect the measurements. To measure CD39 activity, HMEC-1 or HUVECs are grown to full confluency on a 60-mm Petri dish. HBSS+ (with addition of the CD73 inhibitor  $\alpha\beta$ -methylene-adenosine 5'-diphosphate [ADP; 50  $\mu\text{M}$ ] to prevent further metabolism of E-AMP to E-adenosine) is added to the endothelial monolayers. After 10 min, E-ATP (final concentration 5  $\mu\text{M}$ ) is added. Samples are taken at 0, 5, 10, and 30 min, removed, acidified to pH 3.5 with HCl, spun (10,000g for 20 s at 4°C), filtered (0.45  $\mu\text{m}$ ), and frozen (–80°C) until analysis via reverse-phase HPLC using a Reprosil-Pur C18-AQ 5  $\mu\text{M}$  column (Maisch, Ammerbuch, Germany). The ratio of E-ATP and E-AMP is measured with a mobile phase (1 mL/min over the course of 15 min) of 15% methanol/ $\text{NH}_4$ -acetate (0.1 molar) buffer adjusted to pH 5.0. Absorbance is measured with a fluorescence detector at 410 nm with excitation at 315 nm. Retention time for E-ATP is approx 3 min, for E-AMP approx 6 min. CD39 activity is expressed as percent conversion of E-ATP to E-AMP in this time frame (*see Note 11*).

To measure CD73 activity, cells are washed with HBSS+, and HBSS+ with or without  $\alpha\beta$ -methylene-ADP is added to the endothelial monolayers (grown on 60-mm Petri dishes). After 10 min, E-AMP (final concentration 5  $\mu\text{M}$ ) is added. Samples are taken at 0, 5, 10, and 30 min, removed, acidified to pH 3.5 with HCl, spun (10,000g for 20 s, 4°C), filtered (0.45  $\mu\text{m}$ ), and frozen (–80°C) until analysis via reverse-phase HPLC using a Reprosil-Pur C18-AQ 5  $\mu\text{M}$  column (Maisch, Ammerbuch, Germany). Ratio of E-AMP and E-adenosine are measured with a mobile phase (1 mL/min over the course of 8 min) of 25% methanol/ $\text{NH}_4$ -acetate (0.1 M) buffer adjusted to pH 5.0. Absorbance is measured with a fluorescence detector at 410 nm after excitation at 315 nm. Retention time for E-AMP is approx 3 min; for E-adenosine, it is approx 5 min.

Retention time for E-adenosine is confirmed using commercially available E-adenosine (Sigma-Aldrich). CD73 activity is expressed as percent conversion of  $\alpha\beta$ -methylene-ADP inhibited E-AMP conversion to E-adenosine in this time frame.

#### 4. Notes

1. For neutrophil isolation, it is critical that the freshly drawn blood and both Percoll solutions have the same temperature. Otherwise, the double-density centrifugation cannot be performed successfully, and the different bands cannot be distinguished clearly.
2. To be able to measure release of nucleotides upon PMN activation, it is critical to avoid activation of PMNs in the process of isolation and washing. Therefore, all solutions used during this process should not contain calcium (e.g., HBSS<sup>-</sup>). Also, after the initial isolation, PMNs should be kept on ice all the time, and centrifuges should be used at 4°C. Do not let keep PMNs in the pellet; instead, keep them in solution in HBSS<sup>-</sup>.
3. To activate PMNs, neutrophils in HBSS<sup>-</sup> are pelleted by centrifugation (400g for 10 min at 4°C) and transferred into warm HBSS<sup>+</sup> (37°C), containing fMLP (100 nM).
4. In case that murine PMNs are studied, the neutrophils cannot be activated by fMLP because murine PMNs do not express fMLP receptors. Instead of fMLP, murine PMN can be activated using leukotriene B<sub>4</sub> (i.e., leukotriene B<sub>4</sub> [40]).
5. Don't use PBS to wash or flush HUVECs.
6. When changing the media or the HBSS solution of inserts used to measure endothelial barrier function in vitro, one has to be extremely careful not to touch the cell layer with a pipet. If that happens, the barrier is lost, and the tracer substance will immediately equilibrate between apical and basal compartment.
7. For surface labeling of endothelial proteins with biotin, it is critical to perform the whole experiment at 4°C to stabilize the confirmation and position of surface proteins.
8. When performing end-over-end incubations for immunoprecipitation, the Eppendorf cups should be locked tightly and sealed with plastic foil to avoid leakage and loss of the lysates.
9. Do not perform an avidin blot with milk powder. Instead, use 3% bovine serum albumin resolved in wash buffer.
10. The etheno-compounds in the supernatant of HMEC-1 or HUVECs are unstable, unless acidified and cooled to 4°C immediately.
11. It is important to filter the samples thoroughly before injection into the HPLC. Otherwise, HPLC filter systems may be blocked or increased pressure readings will occur.

#### Acknowledgments

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## Analysis of Mammalian Sperm–Egg Membrane Interactions During In Vitro Fertilization

Genevieve B. Wortzman, Allison J. Gardner, and Janice P. Evans

### Summary

The interactions between egg and sperm are among the most fascinating in cell biology. These interactions include cell–cell adhesion and then membrane fusion between the two gametes. This chapter details the experimental methods used to for gamete culture and in vitro fertilization using mouse sperm and eggs.

**Key Words:** In vitro fertilization; cell adhesion; membrane fusion; cell–cell fusion.

### 1. Introduction

This chapter focuses on the cell–cell interactions between mouse sperm and egg, namely the interactions between sperm and egg plasma membranes, which include sperm–egg adhesion (or binding) and sperm–egg fusion. General aspects of the cell and molecular biology of fertilization have been addressed in recent review articles (1,2). It should be noted that normally the sperm interacts with the egg on three different levels. The first two interactions are with the egg's two extracellular coats, the cumulus layer and the *zona pellucida* (ZP). After penetrating these two layers, the sperm gains access to the perivitelline space, between the inner surface of the ZP and the egg plasma membrane; it is in this space that sperm–egg membrane interactions occur. For most experiments for the study of sperm–egg adhesion and fusion, the cumulus layers and the ZP are removed from the eggs, as addressed in the chapter, with relevant aspects of gamete cell biology discussed in the appropriate portions of the Notes section. Mammalian eggs have not yet completed meiosis (in contrast to the sperm, which complete meiosis during spermatogenesis in the testis). Instead, most mammalian eggs are arrested in metaphase of meiosis II, with

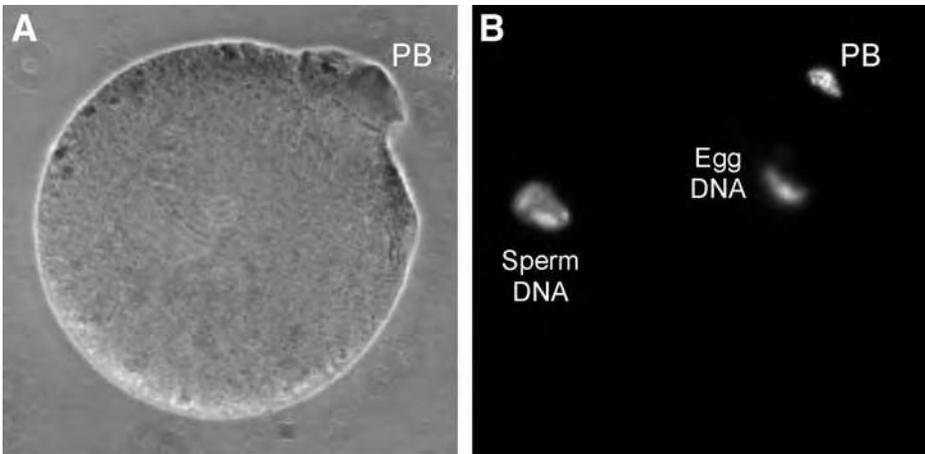


Fig. 1. DAPI-stained fertilized egg, approx 60 min after insemination. (A) Phase contrast image. (B) Fluorescent image of the DAPI-stained DNA. This egg has nearly completed meiosis, as indicated by the emission of the polar body (PB). The sperm nucleus is present in the egg cytoplasm; this will ultimately form a male pronucleus which will fuse with the female pronucleus to create the new diploid organism.

fertilization by the sperm providing the trigger to complete meiosis (3). This activation by the sperm results in cytokinesis that produces a small cell, called the polar body, containing half of the egg's DNA (Fig. 1). This leaves a haploid set of DNA in the egg that will ultimately fuse with the DNA delivered by the sperm, at approx 7 to 8 h after fertilization.

The processes of fertilization are highly conserved between mammalian species. For experimental studies, the mouse is most commonly used because gamete collection and in vitro fertilization (IVF) procedures have been very well refined (Fig. 2). IVF is more difficult in other species, for example, low fertilization rates are common with rat gametes, and polyspermy (fertilization by more than one sperm) is common with pig gametes. It should be noted that the main barrier for species specificity of fertilization is the ZP, whereas sperm–egg membrane fusion is much less species-specific than sperm–ZP interactions. Mouse sperm can fuse with the eggs of rat, guinea pig, and rabbit (4). ZP-free eggs from golden hamsters can be fertilized by sperm from a very wide variety of species (including human, bat, and dolphin [4]). Because of logistical and ethical problems associated with doing experiments with human sperm and eggs, it is common for studies of human sperm to use ZP-free hamster eggs. However, for general interests in cell–cell interactions, it is more appropriate to use sperm and eggs from the same species because potentially misleading results can come from studies of heterologous gametes (5). This chapter

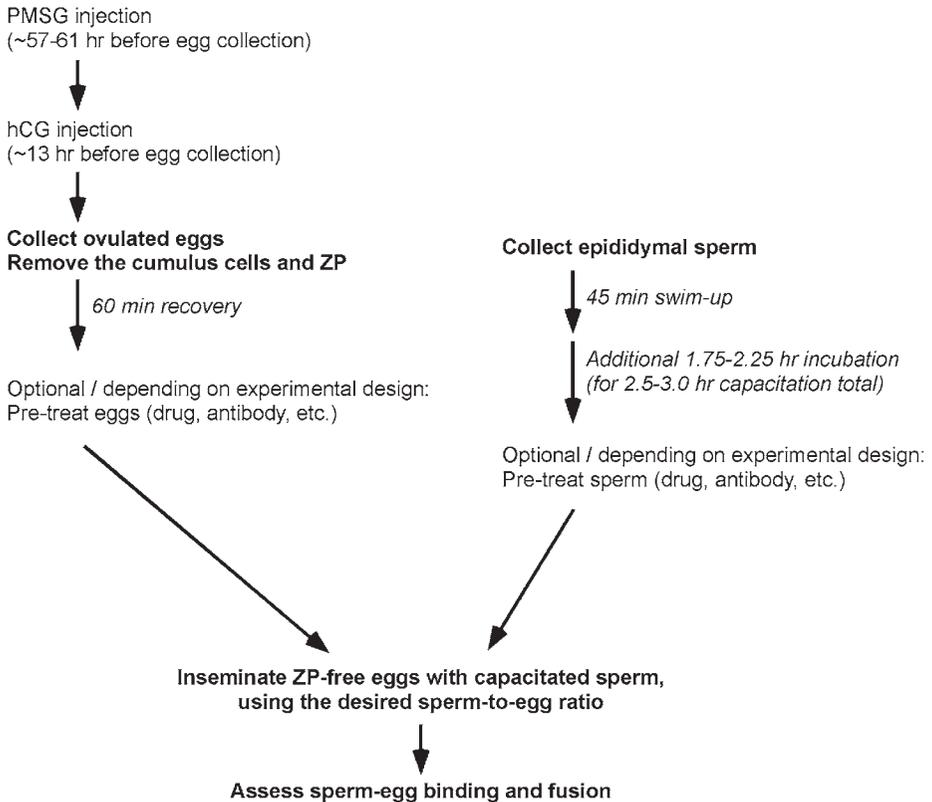


Fig. 2. Flow chart of the experimental design for an IVF study.

presents protocols for gamete collection and IVF using the mouse as an experimental model system. The reader also is advised that there are a number of other issues related to the experimental procedures used to assess sperm–egg interactions that are beyond the scope of this chapter (the interested reader is referred to Section 3 in **ref. 6**).

## 2. Materials

1. Mice: the females most commonly used for superovulation and egg collection are CF-1 (non-Swiss albino) or CD-1 (ICR) from Harlan or Charles River. An inexpensive choice for males is CD-1 retired breeders; sperm quality from these males generally is good, although there will be occasional mice with suboptimal sperm (e.g., low count, self-aggregating and clumpy). An alternative source of sperm are F1 hybrid males, such as from Jackson Laboratories, although these tend to be significantly more expensive. Insemination conditions (optimal sperm concentrations, insemination times) may vary for gametes from mice of different strains.

2. Gonadotropins are the hormones used to induce females to ovulate. Pregnant mare's serum gonadotropin (PMSG) has activity similar to the naturally occurring pituitary hormone follicle-stimulating hormone and is used to prime the females for ovulation. Human chorionic gonadotropin (hCG) has activity similar to the naturally occurring pituitary hormone luteinizing hormone, and is used to induce ovulation.  
PMSG (Calbiochem, San Diego, CA; cat. no. 367222, 5000 IU) is dissolved in 100 mL of phosphate-buffered saline (PBS) for a final concentration of 5 U/0.1 mL. hCG from human pregnancy urine (Sigma, St. Louis, MO; cat. no. CG-10, 10,000 U, or cat. no. C1063, 2500 IU) is dissolved in PBS for a final concentration of 5 U/0.1 mL. Store aliquots of 600  $\mu$ L (enough for three to six mice) at  $-20$  or  $-80^{\circ}\text{C}$ . (PMSG in particular may lose effectiveness when stored below  $-20^{\circ}\text{C}$ .)
3. Whitten's medium stock (7): begin with high-quality water (such as sterile surgical irrigation water; Abbott Laboratories, North Chicago, IL or water for embryo transfer; Sigma; cat. no. W1503). Add 109.5 mM NaCl, 4.7 mM KCl, 1.2 mM  $\text{KH}_2\text{PO}_4$ , 1.2 mM  $\text{MgSO}_4$ , 5.5 mM glucose, 0.23 mM pyruvic acid, 4.8 mM lactic acid, 0.01% gentamycin, and 0.0001% Phenol Red. For Whitten's-HEPES medium (W-H), add 7 mM  $\text{NaHCO}_3$  and 15 mM HEPES and adjust pH to 7.4. For Whitten's bicarbonate medium (W-B), add 22 mM  $\text{NaHCO}_3$ . Filter through a surfactant-free cellulose acetate 0.2- $\mu$ m filter, and store at  $4^{\circ}\text{C}$  (see **Note 1**).
4. W-B medium with 15 mg/mL bovine serum albumin (W-B/15). This medium is made the night before or the day of the IVF experiment by adding 15 mg/mL of bovine serum albumin (BSA; Albumax I, Gibco-BRL, Gaithersburg, MD) to the desired volume of W-B. Typically 5 to 10 mL is made, depending on the scale of the experiment. Invert gently to dissolve the BSA into solution (do not vortex). Add approx 5  $\mu$ L of 1 N NaOH per 1 mL of medium; the medium color will change from orange-ish to pinkish-purple. Gently filter through a 0.2- $\mu$ m surfactant-free cellulose acetate syringe filter, and then incubate at  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  in air (see **Note 2**).
5. Whitten's bicarbonate medium with 0.01–0.1% polyvinyl alcohol (W-B/PVA) (PVA, Sigma; cat. no. P-8136). We make this in batches of approx 50 mL, and our preferred concentration of PVA is 0.05% (see **Note 3**).
6. Pipets for handling eggs: Pasteur pipets made of borosilicate glass (Fisher; cat. no. 13-678-20D; do not use not soda lime glass) are heated in a flame and pulled thin. Using a Bunsen burner, hold both ends of the pipet so the flame touches one spot on the narrow region of the pipet. As the glass softens, in one motion quickly lift the pipet out of the flame and pull the ends of the pipet apart. Done correctly, the pipet is intact with a very narrow section in the middle. After the glass has cooled, slide the thinned section of glass between your index finger and thumb with light pressure until the pipet breaks. This will result in a pipet with a narrow tip and a clean edge (see **Note 4**).

Egg handling is managed by mouth pipetting. Homemade mouth pipets are made of mouthpieces (HPI Hospital Products, Altamonte Springs, FL) and conventional rubber tubing (Latex amber thick wall tubing, 1/8 in. inner diameter, 1/16 in. wall thickness, 1/4 in. outer diameter; Fisher; cat. no. 14-178-5B).

7. Culture dishes: sterile polystyrene, not tissue culture treated, 35 × 10 mm (Falcon; cat. no. 351008) and 60 × 15 mm (Falcon; cat. no. 351007).
8. Mineral oil: Sigma cat. no. M-3516, stored at 37°C, 5% CO<sub>2</sub>.
9. Hyaluronidase 0.025% in W-H with 3 mg/mL BSA (W-H/HY): measure 2.5 mg of hyaluronidase (type IV-S from bovine testis, Sigma cat. no. H3884) and 30 mg BSA (Gibco-BRL Albumax I) into a 15-mL polystyrene culture tube. Add 10 mL of W-H; invert or vortex to mix.
10. Acidic Minimal Essential Medium (MEM)-compatible (MEMCO) for ZP removal (**8**): in high-quality water (*see Subheading 2.3.*), dissolve 10 mM HEPES, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 5.4 mM KCl, and 116.4 mM NaCl. Adjust pH to 1.5. Store aliquots of 10 mL at –20°C, heat to thaw. Working stock can be kept at room temperature.
11. Chymotrypsin for ZP removal: stock of α-chymotrypsin (type II from bovine pancreas; Sigma) is dissolved at 10 mg/mL in PBS and stored at –20°C. Working solutions are prepared immediately before use by diluting to 10 μg/mL final concentration and/or 100 μg/mL final concentration in W-B with 3 mg/mL BSA added (W-B/3, as described for W-B/15 previously; *see also Note 5*).
12. MitoTracker for labeling of sperm: MitoTracker Green (Molecular Probes, Eugene, OR; cat. no. M-7514) is dissolved in dimethyl sulfoxide to a stock concentration of 1 mM and stored at –20°C protected from light. Working solutions are prepared by diluting to 100 nM in Whitten's medium containing 15 mg/mL BSA (*see Note 6*).
13. DAPI for labeling of live eggs: 1–10 μg/mL DAPI (Sigma), diluted in W-B/15.

### 3. Methods

#### 3.1. Superovulation of Mice

Three days before the experiment, female mice are given 100 to 200 μL of PMSG by intraperitoneal injection, approx 57 to 61 h before egg collection and 44 to 48 h before the hCG injection. PMSG will stimulate maturation ovarian follicles. The evening before the experiment, each mouse is given an injection 100 to 200 μL of hCG. This hCG injection is done 44 to 48 h after injection of PMSG and 12 to 13 h before the planned collection of eggs (*see Note 7*).

#### 3.2. Egg Collection

1. Prepare a 35-mm dish with five 35 μL drops of W-B/15 covered with warm mineral oil.
2. Sacrifice females with CO<sub>2</sub> followed by cervical dislocation.
3. Open the body cavity by making a U-shaped incision in the lower abdomen up to the liver; push aside the skin flap and intestines to the side to gain access to the reproductive tract.
4. Remove the oviducts (just below the ovary) and place in a 200 to 300 μL of W-H to rinse off blood and other fluid.

5. In a 60-mm culture dish, put a drop of W-H/HY for every two oviducts and two drops of W-B for washing the eggs.
6. Transfer the oviducts to the W-H/HY drops and puncture the swollen ampulla region (in which a “cloud” of eggs should be visible) with a 27-gage needle to release the cumulus-encased eggs. The eggs can be gently pipetted through a wide-bore pipet to help break up the complexes and give the hyaluronidase access to the cumulus cells’ extracellular matrix. As the cumulus cells disperse from the eggs in the W-H/HY, transfer the cumulus cell-free eggs to a wash drop of W-B (see **Note 8**).
7. Wash the cumulus-free eggs through the second W-B drop and then a fresh W-B/15 drop.
8. Transfer the eggs to the previously prepared 35-mm culture dish with five 35- $\mu$ L drops of W-B/15, covered with warm oil. Eggs should be cultured in W-B/15 at 37°C, 5% CO<sub>2</sub> until the next step.

### 3.3. Sperm Preparation (see Note 9)

1. Sacrifice one male mouse with CO<sub>2</sub> followed by cervical dislocation. Clip away the skin/muscle layer of the lower abdomen in an inverted “U” shape; pull flap up. Gently clip the cauda epididymis and upper part (~1 cm) of vas deferens free from surrounding tissue.
2. Add the two caudae epididymides to 250  $\mu$ L of W-B/15 covered in warm mineral oil in a 35-mm Petri dish. Clean scissors and gently mince the tissue. Incubate the drop at 37°C, 5% CO<sub>2</sub> for 10 min to allow sperm to swim out into the medium. Remove the tissue from the sperm suspension. Gently draw up the 250- $\mu$ L sperm suspension into a pipet and carefully transfer the sperm suspension to the bottom of a 1.5 mL of W-B/15 in a 12  $\times$  75-mm polystyrene culture tube. After 45 min, remove the top 440  $\mu$ L from the tube and place this sperm suspension in a fresh culture dish and cover with warm mineral oil (see **Note 10**).
3. To determine the concentration of sperm (sperm/milliliter), prepare a 1:10 dilution of swim-up sperm in water and transfer 10  $\mu$ L of this to a hemacytometer. Count the number of sperm in the 4  $\times$  4 double-line-bordered square. Repeat for two other squares and average these values. To determine the number of sperm/mL, multiply this average sperm count value by the dilution factor (which is 10, for the 1:10 dilution in water) and also by 10<sup>4</sup> (a correction factor for the hemacytometer). This will give you the number of sperm per mL in the swim-up stock suspension (see **Note 11**).

### 3.4. Two Methods of ZP Removal From Eggs (see Note 12)

#### 3.4.1. Acid Solubilization of the ZP

1. Set up a Petri dish with one drop of acidic MEMCO and several drops of W-B/15.
2. Work in batches of 5 to 20 eggs, depending on skill level. Transfer ZP-intact eggs to the drop of Acidic MEMCO and pipet the eggs up and down for approx 15 s, watching closely as the ZP dissolve.

3. As soon as the ZP have dissolved, wash the eggs by transferring them through adjacent drops of W-B/15. Transfer ZP-free eggs to fresh drops of W-B/15 covered in warm mineral oil; culture at 37°C, 5% CO<sub>2</sub> in air for 60 min to allow eggs to recover from ZP removal.

#### 3.4.2. Protease Treatment and Mechanical Removal of the ZP

1. Set up a Petri dish with one drop of 10 µg/mL chymotrypsin in W-B/3, one drop of 100 µg/mL chymotrypsin in W-B/3, three drops of W-B/3, and three drops of W-B/15, covered in warm mineral oil. (Each drop can be ~50–100 µL.)
2. Transfer eggs to the drop of 10 µg/mL chymotrypsin in W-B/3 at 37°C and check for ZP swelling after approx 2 min. If the ZP appears swollen, use a thin-bore pipet to shear off the ZP from the egg. If the ZP is not swollen, incubate an additional approx 2 min. If the ZP is not swollen after this second incubation, transfer to the drop containing 100 µg/mL chymotrypsin in W-B/3 for approx 1 min (*see Note 5*).
3. After shearing off the ZP, immediately wash eggs through the W-B/3 drops then the W-B/15 drops.

#### 3.5. Optional Additional Step: Labeling of Live Sperm With MitoTracker Green (*see Note 13*)

1. Collect epididymal sperm as described in **Subheading 3.3.** (sperm collection) and allow the sperm to capacitate for 2.5 to 3 h before use.
2. Approximately 15 min before the start of IVF, prepare the labeling solution for the sperm. Dilute the 1 mM MitoTracker Green stock to 200 nM with W-B/15 (i.e., double the final working concentration of MitoTracker Green).
3. Dilute the 200 nM MitoTracker Green solution to the working concentration of 100 nM by adding an equal volume of sperm to be labeled (*see Note 14*).
4. Incubate the sperm in the MitoTracker Green-containing medium for 10 min at 37°C in a dark, humidified atmosphere of 5% CO<sub>2</sub>.
5. Dilute the labeled sperm to the desired concentration for IVF with W-B/15 (*see Note 6*).

#### 3.6. Optional Supplement: Loading Live Eggs With DAPI (*see Note 15*)

1. Dilute DAPI in W-B/15 as noted in **Subheading 2.11.** (1–10 µg/mL final concentration). Prepare approx 25-µL drops of this medium and cover with warm mineral oil.
2. Approximately 25 min before the start of the insemination, transfer eggs to the W-B/15 containing DAPI and incubate for 15 min, then wash through 3 to 4 50- to 100-µL drops of W-B/15 before insemination (*see Note 16*).

#### 3.7. IVF (*see Notes 17 and 18*)

1. Prepare a suspension of sperm at the desired concentration in W-B/15, typically 25,000 to 100,000 sperm/mL (*see Note 17*). Make 10-µL drops of this sperm suspension and cover with warm mineral oil. (If you have 20 eggs per experimental group, make two 10-µL drops of sperm suspension.)

2. Add 10 eggs per 10- $\mu$ L drop of sperm suspension. Co-culture the eggs with sperm in these insemination drops at 37°C, 5% CO<sub>2</sub> in air for desired length of time (*see Note 17*).
3. To remove loosely attached sperm from the eggs, wash the eggs using a pipet approx 1.5X the diameter of the eggs. Transfer the eggs to the top of an approx 75- $\mu$ L drop of W-B/15, blowing gently (as if blowing a whistle quietly); allow the eggs to settle to the bottom. Repeat two more times (*see Notes 19 and 20*).
4. To prepare the eggs for viewing on a fluorescent microscope to assess sperm–egg interactions, fix the eggs in 3.7% to 4.0% paraformaldehyde in PBS for 15 to 60 min. Fixed eggs are then permeabilized in PBS containing 0.05% Triton X-100 (3–15 min), washed briefly in PBS two to three times, and then mounted in mounting medium (e.g., VectaShield, Vector Laboratories) containing 1.5  $\mu$ g/mL DAPI to stain the DNA in the eggs and sperm. Eggs also can be viewed live (particularly if DAPI-loaded eggs were used to assess sperm–egg fusion).

#### 4. Notes

1. The culture medium should be prepared using glassware designated only for media preparation. Do not use metal spatulas to weigh out the dry reagents. W-H and W-B can be prepared together. To prepare 500 mL of each, add all the ingredients up to the phenol red and bring up the volume to 900 mL; then divide into two batches of 450 mL and add the remaining reagents for either W-H or W-B.
2. We have had good success with Whitten's medium for IVF. There are also commercially available media for IVF (e.g., Human Tubal Fluid), although making your own Whitten's medium is more economical. W-H medium is used for collection and other work in regular atmosphere, whereas W-B medium, containing bicarbonate only (no HEPES), is used for culture in 5% CO<sub>2</sub>. Care should be taken to maintain appropriate pH of the W-B medium, as shifts in the pH may affect the health of eggs and sperm and fertilization success.
3. The addition of BSA or PVA keeps eggs from sticking to the culture dish. If making W-B with 0.1% PVA, it is likely that not quite all the PVA will go into solution; it is recommended that the medium be filtered before use.
4. Pipet pulling is a process of trial and error; you pull a couple dozen pipets, and then check them once you are at the dissecting microscope working with the eggs. The size of the pipet opening will vary, but a standard size for moving eggs will have a diameter slightly larger than the egg. It is advisable to have separate pipets for separate experimental groups (e.g., a control antibody and an experimental antibody), to avoid contamination between groups.
5. The times stated are approximate, but it is recommended that the eggs not be incubated for an extended period of time with chymotrypsin. As noted in **Note 9**, with the chymotrypsin treatment some eggs may never undergo ZP swelling; these eggs should be discarded.
6. Because the concentration of MitoTracker Green in the medium needs to be diluted before fertilization to reduce levels of background staining (i.e., dye uptake by the mitochondria of the eggs), label a sperm concentration that is about five fold higher

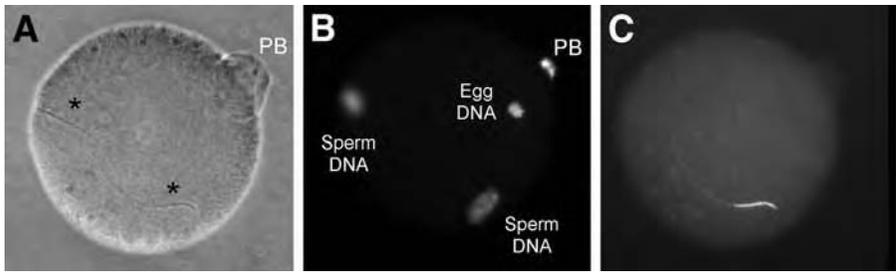


Fig. 3. Fluorescent images of an egg inseminated with unlabeled and MitoTracker Green-labeled sperm. (A) Phase contrast image with asterisks indicating the location of two sperm tails. (B) Fluorescent image showing DAPI staining of the egg DNA, polar body DNA (PB), and two sperm nuclei. (C) Image photographed with the FITC filter, to show MitoTracker Green staining. This image shows that one of the two sperm that fertilized the egg was labeled with MitoTracker Green.

than what will be used for insemination. Immediately before IVF, dilute the sperm to the desired concentration with W-B/15. See Fig. 3 for images of MitoTracker-labeled sperm fertilizing an egg.

7. The number of female mice used will depend on the planned scope of an experiment. It is preferable to have 20 to 30 eggs per experimental group for a given day's experiment (with a given experimental series being repeated at least three times). Average egg yield is approx 20 eggs per mouse but can vary widely. Healthy eggs are round with clear cytoplasm; the first polar body (the product of the first meiotic division) may or may not be present. Discard eggs that have a brown, granular cytoplasm, eggs that are fragmented, eggs that still have a germinal vesicle, or eggs that have extruded a second polar body. Eggs should be collected at approx 12 to 13 h after hCG injection for best quality and egg health (9).
8. Cumulus cells are somatic cells that are derived from the ovarian follicle. Cumulus cell detachment from the eggs happens somewhat asynchronously; some eggs are clean in approx 1 min and some require 5 min. Pipetting through a wide-bore pipet can facilitate cumulus removal and exposure of the hyaluronic acid (the extracellular matrix in which the cumulus cells are embedded) to the hyaluronidase. Transfer an egg to W-B as soon as it is cumulus cell-free. Try to minimize the time the eggs are in hyaluronidase (excessive exposure to hyaluronidase has been associated with spontaneous egg activation). It is not necessary to remove 100% of the cumulus cells, as a few residual cumulus cells will detach with ZP removal.
9. Sperm must undergo a maturational change known as capacitation in order to be able to fertilize eggs. Capacitation occurs *in vitro* in the appropriate culture medium (such as Whitten's medium) containing energy substrates, a cholesterol acceptor (e.g., BSA),  $\text{NaHCO}_3$ ,  $\text{Ca}^{2+}$ , and low  $\text{K}^+$  and physiological  $\text{Na}^+$  concentrations (10). During capacitation, a subset of sperm will undergo spontaneous acrosome exocytosis; this is crucial for fertilization of ZP-free eggs as only acrosome-reacted

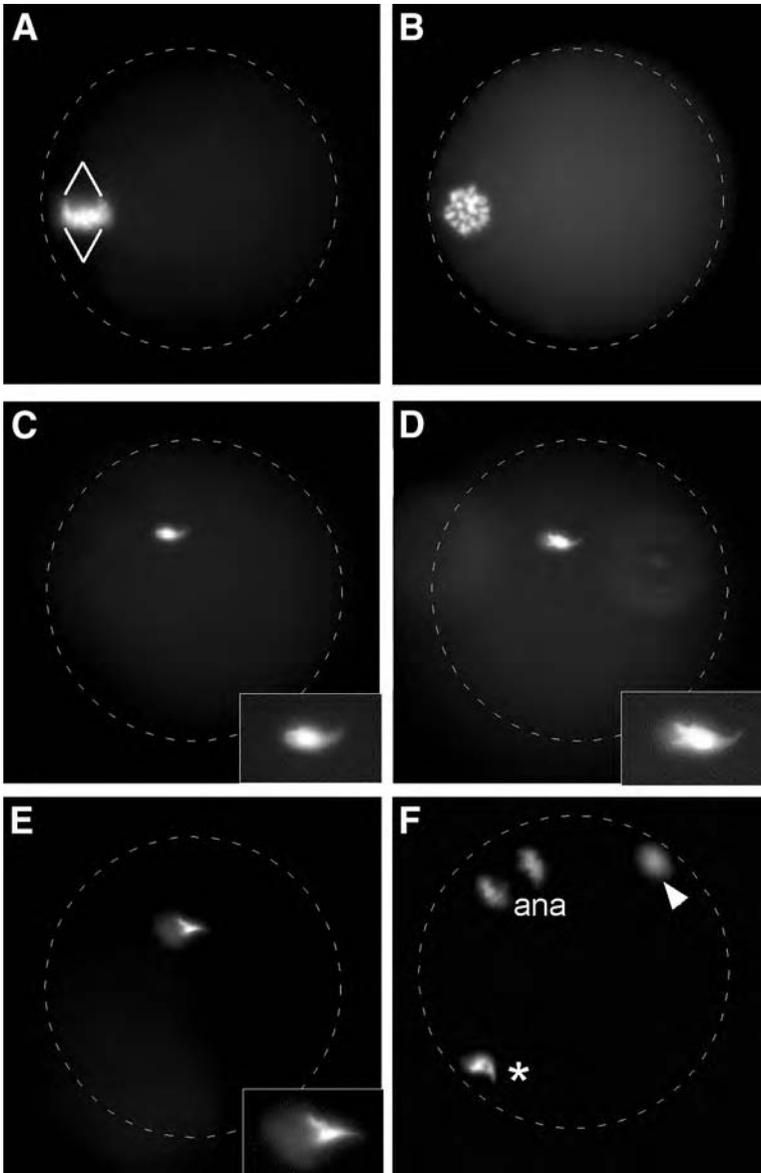


Fig. 4. DAPI-stained eggs, showing morphology of egg DNA and sperm DNA at different stages of fertilization. Dotted lines show the approximate outline of the egg; mouse eggs are approx 80  $\mu\text{m}$  in diameter. (A) Unfertilized metaphase II egg, with a side view of the chromosomes on the metaphase plate. White lines indicate the directions of the microtubules holding the chromosomes in place. (B) Unfertilized metaphase II egg, with an en face view of the chromosomes on the metaphase plate. Microtubules of the meiotic spindle are extending backward and forward from the

sperm are capable of binding and fusing with the egg plasma membrane (11). It is advisable to allow 2.5 to 3 h of capacitation time for these IVF experiments. Therefore, this is a key factor when planning the timing of sperm collection.

10. The “swim-up” preparation for sperm selects for the most motile sperm and reduces the risk of including any dead or immotile sperm in the preparation. A typical sperm concentration for the sperm swim-up preparation is  $1\text{--}5 \times 10^6$  sperm/mL.
11. For example, if you counted 20, 19, and 30 sperm in three separate squares on the hemacytometer, the calculation would be to determine the sperm concentration would be:

$$(20 + 19 + 30)/3 \times 10 \times 10^4 = 2.3 \times 10^6 \text{ sperm/mL}$$

Please note that it is important to prepare the sperm dilution in water (not medium) so that the sperm stop swimming to facilitate counting.

12. There are two commonly used methods for ZP removal: low pH to solubilize the ZP and chymotrypsin treatment to induce ZP swelling and thus facilitate mechanical removal. (It is possible to remove the ZP by mechanical manipulation alone, but this is extremely difficult and often results in low yields of ZP-free eggs because so many eggs lyse during the procedure.) We primarily use the acid solubilization method, although there are advantages and disadvantages to each method. The chymotrypsin-mechanical method has the disadvantage of resulting in lower yields of eggs than the acid solubilization method, as some ZP will never swell sufficiently for removal. The exposure of surface proteins to a protease like chymotrypsin can also be disadvantageous, although the exposure of surface molecules to low pH has the potential to alter them (we have observed the reversible modification of one monoclonal antibody epitope on eggs exposed to low pH [12]).
13. MitoTracker Green-labeling of sperm can be useful for a variety of experimental designs. We have used labeled sperm to inseminate early zygotes, that is, eggs that have been previously fertilized. Sperm labeling also can be used to distinguish between two different experimental groups, such as control versus drug-treated or wild-type versus knockout.
14. The MitoTracker Green signal will fade if left exposed to light for a prolonged period of time. Protect MitoTracker aliquots and labeled sperm suspensions from

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Fig. 4. (*continued*) plane of the image. (C) An egg with a bound sperm, with inset showing a close-up of the sperm DNA. (The egg DNA is out of the plane of focus.) (D) An egg with a recently fused sperm, with inset showing a close-up of the sperm DNA. (The egg DNA is out of the plane of focus.) Note the difference in appearance (slight flaring) at the posterior end of the sperm nucleus as compared to the bound sperm in (C). (E) Eggs with sperm at a later stage of decondensation, with inset showing a close-up of the sperm DNA. (The egg DNA is out of the plane of focus.) The flare at the posterior region of the sperm nucleus has become more pronounced. (F) An egg with two fused sperm. The egg has exited from metaphase II arrest and is progressing in to anaphase (labeled “ana”). The sperm heads are marked with an asterisk and an arrowhead; the one marked with the arrowhead is somewhat beyond the plane of focus.

light and view labeled sperm by fluorescence microscopy as soon as possible (preferably on the same day the experiment is performed).

15. Labeling of eggs with DAPI (or Hoechst [13]) allows for the detection of sperm–egg fusion by observing the transfer of the DNA-staining dye from the egg cytoplasm to the sperm nucleus once the sperm has fused with the egg. Alternatively, sperm–egg fusion can be detected simply by staining the fertilized eggs with a DNA-specific dye, as sperm head morphology changes upon sperm–egg fusion and with the initiation of sperm nucleus decondensation. This method is easier, although recognizing the earliest stages of sperm decondensation requires some practice; stages of sperm nucleus decondensation are illustrated in Fig. 4.
16. It is important to wash the eggs thoroughly so that no DAPI is left in the culture medium to stain the sperm.
17. Insemination conditions are selected based on the fertilization end point of interest, the desired rate of fertilization for control eggs, and the tolerance for polyspermy. A 15-min insemination protocol has been used to assess sperm–egg adhesion before any decreases in egg membrane receptivity to sperm that can occur approx 60 min after fertilization (14–16). Inseminations of 45 to 60 min routinely are used to examine sperm–egg adhesion and fusion. Typical sperm concentrations used to inseminate ZP-free eggs are in the range of 10,000 to 250,000 sperm/mL. In our experience (with eggs from CF-1 females and sperm from CD-1 males), a 60 min insemination with 25,000 sperm/mL (i.e., a sperm:egg ratio of 25:1) will result in 70 to 80% of the egg fertilized with low levels of polyspermy. A 60-min insemination with 100,000 sperm/mL (i.e., a sperm:egg ratio of 100:1) will result in 90 to 100% of the eggs fertilized with 10 to 50% of the eggs being polyspermic (fertilized by more than one sperm). The emission of the second polar body, indicative of exit of the egg from meiotic arrest, will be apparent at approx 60 to 90 min after insemination.
18. Experimental manipulations of sperm or eggs can be performed at several points in this protocol. The gametes can be pretreated with antibodies to surface molecules or with pharmacological reagents before and/or during insemination (Fig. 1). If a drug has reversible effects, then it must be present during the insemination; this then should be taken into account when interpreting results, as both the eggs and sperm would have been exposed during this time period.
19. It is critical that these washes are kept consistent between experimental groups for accurate assessment of sperm adhesion to the egg membrane. All washes of all experimental groups should be performed by the same person using the same pipet, preferably by an individual who does not know which experimental group is which to avoid bias.
20. For experiments in which it is desired to aggressively remove the majority of bound sperm, eggs can be washed more forcefully in W-B containing 0.05% PVA.

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## Collagen Gel Contraction Assay

Peter Ngo, Punitha Ramalingam, Jonathan A. Phillips,  
and Glenn T. Furuta

### Summary

Mucosal tissues undergo contraction and relaxation on a continuous basis. In its normal state, the pliable intestinal tract is characterized by a rhythmic pattern of contractions controlled by its intrinsic neuronal innervation. In chronic inflammatory diseases such as Crohn's disease, the intestine can become stiff and fibrotic, losing much of its normal motility. Although muscle fiber contraction accounts for much of this activity, contraction of nonmuscle tissue is constantly occurring in events associated with chronic inflammation, such as wound healing, scar formation, and tissue remodeling. However, the physiological and pathological mechanisms defining these events are not well defined. Tissue contraction is a dynamic event characterized by both intracellular and extracellular events. A number of cells, such as fibroblasts, epithelial cells, lymphocytes, and eosinophils, normally reside within the gastrointestinal tract. Additionally, the extracellular matrix is composed of a complex infrastructure that includes collagen and other molecules. The manner in which these two components interact is not certain, but the use of recent model systems has provided insights into these processes. The collagen lattice contraction assay provides a model for tissue contraction that takes advantage of the finding that cell-populated collagen hydrogels contract over time in a predictable, consistent manner. This model allows for investigation of the influence of specific agonists on the rate and extent of matrix contraction.

**Key Words:** Contraction; collagen hydrogel; collagen lattice; collagen gel.

### 1. Introduction

Mucosal tissues are composed of several molecular and cellular components. Extracellular matrices can be remodeled by resident cells, such as fibroblasts, to form scar tissue and to contract. In vitro models of tissue wound healing, remodeling, and contraction offer investigators a highly controlled and flexible environment to study the influences of a variety of factors contributing to matrix contraction. Because collagen is the principle extracellular matrix molecule

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found in injured tissue, various models have employed collagen as a matrix for imitating the wound environment.

In 1979, Bell et al. (1) took advantage of the solubility of collagen type 1 in acidic solutions to create cell-populated collagen hydrogels. Measuring the dimensional changes of these collagen gels allows investigators to evaluate the influence of certain variables on the rate and extent of matrix contraction and remodeling in a three-dimensional system (2–4).

## 2. Materials

### 2.1. Cell Culture and Counting

1. Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Invitrogen Co., Grand Island, NY).
2. Fetal bovine serum (Gibco).
3. Solution of penicillin–streptomycin (10,000 U/mL penicillin G sodium and 10,000 µg/mL streptomycin sulfate in 0.85% saline; Gibco).
4. Solution of trypsin (0.25%) and EDTA from Gibco.
5. Trypan Blue stain (Gibco).
6. Hy-Lite Counting Chamber (Fisher, Pittsburgh, PA).

### 2.2. Collagen Lattices

1. Rat tail collagen type 1 (Sigma, St. Louis, MO); store at 2 to 8°C.
2. NaOH (1 M; Sigma).
3. Acetic acid, Glacial 99.9% (J.T. Baker, Phillipsburg, NJ).
4. 0.22-µm syringe-driven filter units (Millipore Corp. Bedford, MA).
5. 200-µL pipet tips (ESP 200; Continental Lab Products, San Diego, CA).
6. 24-well cell culture cluster plates (Corning, Corning, NY).

### 2.3. Image Acquisition/Analysis

1. Canon A70 Digital Camera 3.2 megapixel (or equivalent).
2. ImageJ Software v. 1.32 (National Institutes of Health, Bethesda, MD). This software is available for download in the public domain at <http://rsb.info.nih.gov/ij/>.
3. Portable Lightbox (Apollo, Court, NY).

## 3. Methods

This method defines preparation of collagen solutions, optimization of gel solidification conditions, quantification of gel contraction, and interpretation of data. This model system permits the study of specific agonists or inhibitors on contraction of different cell populations in a three dimensional system.

### 3.1. Preparation of Collagen for Use in Collagen Gels

1. Acetic acid solution (0.2%) is made from 100% glacial acetic acid and water (see Note 1).
2. Filter sterilize the solution with a 0.2-µm filter and cool to 4°C.

3. Type 1 collagen is mixed with 0.2% acetic acid solution under sterile conditions to make 6 mg/mL of collagen solution (100 mg collagen dissolved into 16.7 mL acetic acid solution in 50-mL conical tube).
4. Gently agitate the collagen solution at 4°C. This process takes 2 to 5 d and collagen must be completely in solution before proceeding (*see Note 2*).
5. After the collagen is entirely dissolved into solution, dilute with equal volume of filter-sterilized water to make a 3 mg/mL collagen solution in 0.1% acetic acid.
6. Equilibrate with gentle shaking at 4°C for 1 d.
7. Collagen solution should be stored at 4°C.

### 3.2. NaOH Titration of Collagen

Perform this assay whenever using a new batch of collagen to optimize solidification. After identifying the optimal amount of NaOH to add to the collagen/media mixture, use the same quantity for all subsequent gels.

1. Use eight clear-colored 1.6-mL Eppendorf tubes for pH titration of gels.
2. Add 0.4 mL of cell specific media or DMEM to each Eppendorf.
3. Add 0.2 mL of collagen solution (3 mg/mL in 0.1% acetic acid) to one tube containing media. Immediately add 1  $\mu$ L of 1 M NaOH and pipet mixture up and down three times with 1-mL pipet. DO NOT DELAY (*see Note 3*).
4. Repeat the above step with increasing amounts of 1 M NaOH solution (1–8  $\mu$ L) to determine the amount of NaOH needed to produce a well-solidified gel with neutral pH (*see Note 4*).
5. Allow the mixture to solidify for 20 min. The final collagen concentration should be 1 mg/mL. Compare rigidity and color of gels titrated with different volumes of NaOH to determine which volume of NaOH produces a well-solidified gel with neutral pH. The least amount of NaOH needed to turn the phenol red media indicator a light pink color will produce the most rigid collagen gels.

### 3.3. Preparing Cells Before Suspension in Collagen Gels

Populating collagen gels with cells requires careful attention to cell concentrations. Determining optimal cell concentration requires performance of a cell concentration curve. Below is a protocol that provides uniform distribution for a final population of  $1 \times 10^5$  cells/mL of Caco2 epithelial cells. During this portion of the procedure cells can be resuspended in media containing agonist/antagonists to determine effects on collagen gel contraction.

1. A confluent layer of Caco2 cells should be detached from the culture vessel using warmed Trypsin-EDTA solution (0.25% trypsin + EDTA-4Na in Hank's Balanced Salts Solution; *see Note 5*).
2. Suspend cells into complete culture media (~10mL media per 75-cm<sup>2</sup> vessel). DMEM with 10% fetal calf serum and 1% penicillin–streptomycin was used for Caco2 cells (*see Note 6*).
3. Count cells using Trypan Blue stain and counting chamber.

4. Centrifuge the remaining cell suspension at 500g for 10 min.
5. Remove supernatant and add fresh warm culture media ( $\pm$  experimental variable) to achieve a concentration of  $1.5 \times 10^5$  cells/mL (*see Note 7*). Cells should be resuspended at 1.5 times the final desired cell concentration as cells are further diluted with addition of collagen solution.
6. Mix cells gently into suspension.

### **3.4. Pouring Cell Populated 1 mg/mL Collagen Gels**

Creating consistent, homogeneous, cell populated collagen gels requires practice. Because of the rapid solidification of the collagen gels, it is important to calculate the necessary volumes of all reagents and ensure that adequate quantities are readily available before starting the protocol. The following is a protocol using 24-well plates as molds for 500- $\mu$ L collagen gels.

1. Collagen gels should be prepared under sterile conditions.
2. Add 0.66 volumes of well-mixed cell suspension ( $\pm$  experimental variable) to a sterile tube.
3. Add 0.33 volumes of 3 mg/mL collagen solution to the cell suspension (*see Note 8*).
4. Quickly add the appropriate volume of 1 M NaOH (*see Subheading 3.2.*) and mix the solution up and down three times.
5. Immediately transfer 500  $\mu$ L of the mixture to a 1.9-cm<sup>2</sup> well (*see Note 9*).
6. Allow gels to solidify, covered at room temperature for 20 min.
7. Gently add a minimum of an equal volume (500  $\mu$ L) of culture media ( $\pm$  experimental variable) to each well.
8. Dissociate the gel from its mold by gently running the tip of a 200- $\mu$ L pipet tip along gel edges being careful not to shear or tear gels.
9. Resuspend gels by gently pulling the edges of the gel away from the mold using the pipet tip (*see Note 10*). Gently swirl plate to make sure that gel is free from the plate.
10. Replace 24-well plate into incubator at 37°C, humidified 5% CO<sub>2</sub>.

### **3.5. Documenting Contraction**

Contraction occurs in a time dependent manner. The rate and degree of contraction depends on the cell type and concentration, and the presence of any contraction agonists or inhibitors in the media. The concentration of serum in the media also affects contraction. **Figure 1** illustrates contraction of Caco2 populated collagen gels at 24 h.

1. At predetermined time-points, remove the 24-well plate from the incubator for image acquisition (*see Note 11*).
2. Place the 24-well plate on top of a lightbox.
3. Using a digital camera at a fixed distance above the gels, obtain an image at each of the time-points (*see Note 12*).
4. Return the gels to the incubator.

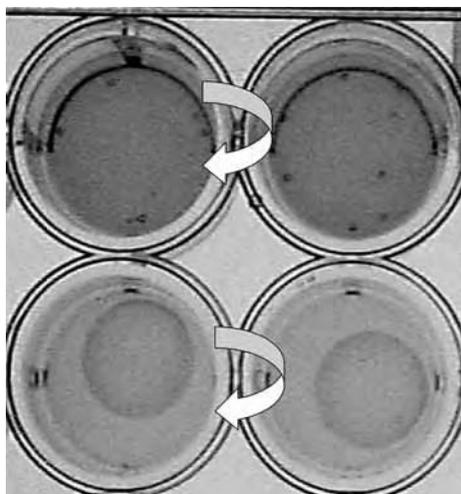


Fig. 1. Photograph of collagen gels taken at 24 h. The top two wells contain acellular collagen gels showing no contraction. The bottom two wells contain Caco2-populated gels displaying uniform, symmetric contraction.

### 3.6. Interpretation of Data

The degree and rate of contraction of gels exposed to various conditions can be evaluated by calculating the gel surface area from acquired images. To standardize comparison, results can be reported as a percent of initial surface area for each given time point. **Figure 2** shows results at 24 h from a collagen gel contraction assay with 3T3 fibroblast cell concentration curve. Increasing cell concentrations result in increased collagen lattice contraction.

1. Images can be analyzed with National Institutes of Health ImageJ software.
2. Trace the outline of each collagen gel and calculate the surface area according to ImageJ software instructions (*see Note 13*).
3. Report the surface area at each time point as a percentage of initial gel surface area.

### 4. Notes

1. Unless stated otherwise, all solutions should be prepared in water that has resistivity of 18.2 M $\Omega$ -cm and total organic content of less than five parts per billion. This standard is referred to as “water” in this text.
2. Gentle agitation with a shaker may be used to aid in dissolving collagen, but forceful agitation or repeated inversion may disassociate collagen fibrils, leading to poorly solidifying gels.
3. A collagen lattice will begin to form immediately when collagen, media, and NaOH are mixed together. Reagents and equipment (NaOH, collagen solution, and 1-mL

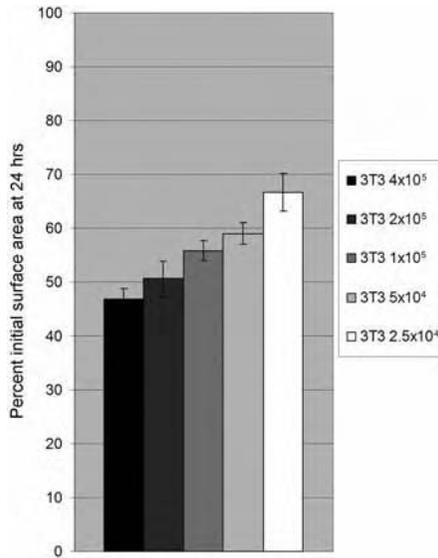


Fig. 2. Results from 3T3 fibroblast collagen gel contraction assay with 3T3 cell concentration curve. Increasing 3T3 concentrations result in increased collagen gel contraction. Results represent means  $\pm$  standard deviation of mean of four lattices.

pipet set to 500  $\mu$ L of volume) should be readily available for immediate use. Repeated pipetting or agitation after the gel has begun to solidify will shear collagen and produce a poorly solidified gel.

4. After mixing, gels should be a homogeneous color. A nonhomogeneous gel indicates inadequate mixing.
5. Trypsin not only aids in lifting cells from culture vessels but also breaks up aggregates of cells to produce a more even distribution of cells within gels.
6. Contraction of collagen gels is dependent on the presence of serum in media and gels. Gel contraction increases in a concentration dependent manner with increasing serum so consistent serum concentrations should be used for all conditions within a particular assay (4). Media containing 10% fetal bovine serum yields consistent results.
7. This step allows resuspension of cells at desired concentrations. In addition, cells can be resuspended in solutions containing contraction agonists or inhibitors. This step should be performed for all cell populations as it also removes trypsin from the suspension which may interfere with collagen gel contraction.
8. When making each 500- $\mu$ L collagen gel, it is advisable to mix 400  $\mu$ L of cell suspension and 200  $\mu$ L of collagen solution; the additional volume allows for easier transfer of mixtures into molds without incorporating bubbles in gels. Multiples of these volumes can be used to create experimental replicates.

9. Be careful not to introduce bubbles into the mixture as these will solidify in the gel. For replicates, a 5-mL stripette works well to both mix and quickly dispense 500- $\mu$ L of mixture into each well. Drawing up more than the needed volume into the stripette will prevent air bubbles from incorporating into the last poured gel.
10. All gels should be freed from well walls in the same manner. Running the pipet tip around edges of detached gels should cause the gel to turn easily within the well, confirming that it is not adherent to the well bottom.
11. Depending on the rate and degree of contraction with each specific cell type and concentration, photographs should be obtained at different time points, that is, 0, 6, 12, 24, and 48 h after gels are poured typically yields an adequate initial time course.
12. Using a Canon Powershot A70 3.2 megapixel digital camera, a distance of 40 cm with 3X optical zoom yields good quality images of the 24-well plate and collagen gels. It is best to obtain several photos at each time point to ensure an image with good resolution is obtained. With increasing cell concentrations and with increasing contraction the gel edge is easier to visualize allowing for easier analysis of contraction. Alternatively, a flatbed digital scanner can be used to obtain images for analysis.
13. Adjusting the contrast or brightness of each image prior to analysis with ImageJ software may allow easier surface area calculation. To increase accuracy in outlining gels, images can be magnified using ImageJ software without affecting surface area calculations. The outline of wells in the 24-well plate provides an optimal reference for initial surface area of each gel.

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## Methods to Assess Tissue Permeability

Juan C. Ibla and Joseph Khoury

### Summary

An essential requirement for adequate organ performance is the formation of permeability barriers that separate and maintain compartments of distinctive structure. The endothelial cell lining of the vasculature defines a semipermeable barrier between the blood and the interstitial spaces of all organs. Disruption of the endothelial cell barrier can result in increased permeability and vascular leak. These effects are associated with multiple systemic disease states. The mechanisms that control barrier function are complex and their full understanding requires a multidisciplinary approach. In vivo permeability data often complement molecular findings and add power to the studies. The interaction of multiple cell types and tissues present only on mammalian models allow for testing of hypothesis and to establish the physiological significance of the results. In this chapter, we describe methods that can be used systematically to measure the permeability characteristics of several organs.

**Key Words:** Permeability assay; vasculature; edema; inflammation; Evan's blue; fluorescence; bioparticles; water content; wet/dry ratio.

### 1. Introduction

Mucosal surfaces provide a physical barrier between the environment and the internal layers of many organs. The dual role of maintaining close contact, yet separated biological compartments requires multiple interactions between endothelia, epithelia, and *trans*-membrane adhesion domains. This highly adapted cellular hierarchy denotes a specific anatomical location within an organ (e.g., polarized epithelia) and assigns individual cell types vital tasks for organ function. The endothelial lining of all organs is charged to interact with the systemic circulation, transduce soluble messages, and control regional blood flow and organ development (1,2). Insight into the mechanisms that regulate these natural barriers is necessary for the understanding or normal physiology and disease progression. It is well established that abnormal barrier function is

present in a multitude of disease states and that available methods can be used to estimate the severity of diseases and response to treatment (3). Several methods have been developed to study endothelial permeability in the vasculature, both in vivo and in vitro (4). These include blood clearance and diffusion methods that can be performed both invasively and noninvasively in animal and human subjects (5). Experimentally, tissue permeability methods can be accomplished with equipment available in standard laboratory settings and the results obtained are easy to interpret and highly reproducible.

## 2. Materials

### 2.1. Miles Assay for Vascular Permeability

1. Mice.
2. Phosphate-buffered saline (PBS; Gibco BRL, Invitrogen, Carlsbad, CA).
3. Evan's blue (Sigma, St. Louis, MO).
4. Ligand, cytokine, growth factor, or condition being tested.
5. Syringe with needles.
6. Surgical dissecting equipment.
7. Photographic equipment.
8. Formamide (Sigma).
9. Water bath at 55°C.
10. Spectrophotometer at 450 nm and 590 to 620 nm (SpecraMax 250, Molecular Devices, Sunnyvale, CA).
11. 96-well plate or cuvette for spectrophotometer (Corning Costar, Acton, MA).

### 2.2. Fluorescent-Labeled Molecules

1. Mice.
2. PBS.
3. Fluorescein isothiocyanate (FITC)-dextran, molecular weight 4 to 40 kDa (Sigma), or fluorescent-labeled microspheres (Molecular Probes, Eugene, OR).
4. 1-mL syringe and 23-gage needles.
5. 1.5-mL microcentrifuge tubes (Eppendorf, Westbury, NY).
6. Fluorescent plate reader (Cytofluor 2300, Millipore, Framingham, MA).
7. Fluorescent microscope (Olympus, B.H2, Melville, NY).
8. Plates for fluorescence (Corning Costar).

### 2.3. Labeled Bioparticles

1. Fluorescent bacteria commercially labeled with fluorescein, Alexa.
2. Fluor (488/594) and Texas Red dye among others (Molecular Probes).
3. Sterile PBS or normal saline.
4. 2 mM sodium azide (Sigma).
5. Vortex (Bio-Rad, Hercules, CA).
6. Hemocytometer (Fisher Scientific, Pittsburgh, PA).

Normal Vascular Permeability



Increased Vascular Permeability



Fig. 1. Photographs representing normal and increased vascular permeability in mice. C57Bl6 male mice were injected with 0.2 mL of Evan's blue via tail vein. Animals were then subjected to 4 h of normobaric hypoxia (8% O<sub>2</sub>, 92% N<sub>2</sub>) or room air conditions. Postmortem macroscopic examination of intra-abdominal viscera displayed significant differences between experimental and control groups. (Please *see* color insert following p. 50 for a color version of this figure.)

7. Microscope (Olympus BH2).
8. Mice.

## 2.4. Water Content in Tissues

1. Microcentrifuge tubes (Eppendorf).
2. High-fidelity electronic balance (Mettler-Toledo, P.M 400, Columbus, OH).
3. Speed-Vac (Vaccufuge, Eppendorf).
4. Tissue.

## 3. Methods

### 3.1. Miles Assay for Vascular Permeability

The classical method in which vascular permeability can be measured *in vivo* is by the Miles Assay for Vascular Permeability, also known as the Evan's blue dye method (6). Evan's blue is a marker that specifically binds to albumin, allowing for a quantification of vascular leakage into the extravascular tissues. The accumulated dye can be quantified by the use of a spectrophotometer. Results are often evident at the macroscopic level, as depicted in Fig. 1.

1. Reconstitute, Evan's blue to a final concentration of 0.5% in PBS.
2. 0.2 mL of Evan's blue is administered by tail vein injection per mouse.
3. The stimulation of choice is then administered with a subset of mice not receiving the stimulation as controls.
4. The mice are then sacrificed.
5. Dissection of the organs of interest are performed and photographed as necessary.
6. Evan's blue concentration is quantified by eluting 50 mg of individual organs in 0.5 mL of formamide at 55°C from 2 h to overnight.
7. Aliquots of the eluted dye are then measured by absorbance at 610 nm with subtraction of reference absorbance at 450 nm.

### 3.2. Fluorescent-Labeled Molecules

Fluorescent dyes are linked to either dextran or polystyrene microspheres that could be used as tracers for permeability. These tracers may then be visualized either directly *in vivo* by aid of intravital microscopy or in tissue sections. Several dyes and molecule sizes may be used to assay both epithelial and endothelial permeability. Although the initial method employed by Rudolph and Heymann (7) used radiolabeled microspheres, growing concerns over environmental and health issues as well as cost of handling, disposal and half-life of the radiolabel have brought to the development of nonisotope fluorescent or colored molecules. The method we describe here uses FITC-dextran (8), but it may easily be adapted for various fluorescent-labeled microspheres (*see Note 1*).

1. Reconstitute FITC-dextran in, PBS to a concentration of 80 mg/mL.
2. Administer FITC-dextran to each animal 60 mg/100 g body weight (*see Note 2*).
3. Expose animals to condition of interest for appropriate amount of time (2–6 h).
4. Collect whole blood by cardiac puncture in anesthetized animals using the syringe and needle.
5. Transfer blood to a microcentrifuge tube, let sit on ice for 1 h, spin at 77,000g for 5 min, and collect supernatant (serum).
6. On a fluorescence plate, make serial dilutions of FITC-dextran in PBS (range from 10 to 0.015  $\mu\text{g/mL}$ ), to unknown wells, add 60  $\mu\text{L}$  1:3 diluted serum in PBS per well (triplicate).
7. Read plate at excitation wavelength 485 nm and emission 530 nm.
8. Measurements are recorded as nanograms of FITC-dextran per microliter of serum.

### 3.3. Labeled Bioparticles

The dynamic interaction of cellular components within a tissue can be analyzed *in vivo* by the kinetics of labeled bioparticles during exposure to critical physiological conditions. This technique utilizes a series of fluorescent labeled, heat or chemically killed bacteria of a variety of sizes and antigenic

characteristics. These methods have been employed to study a diverse group of parameters including epithelial permeability, phagocytosis and opsonization. Results can be obtained by fluorescent microscopy, quantitative spectrophotometry and flow cytometry (9,10). We have used these methods to study bacterial translocation in transgenic mice exposed to mucosal barrier disruptive conditions (hypoxia).

1. Reconstitute labeled bacteria (100 mg,  $3 \times 10^8$  *Escherichia coli*) to 20 mg/mL in sterile PBS or normal saline (see **Note 3**).
2. Add 2 mM sodium azide to bacterial suspension and store at 4°C. Without the addition of sodium azide, bacterial suspension should be used within 1 d.
3. Vigorously vortex the solution  $3 \times 15$  s at the highest setting.
4. Count the number of bioparticles per milliliter using a hemocytometer in a phase-contrast or fluorescence microscope.
5. Dilute bioparticles to  $4 \times 10^8$  per 0.5 mL.
6. Vortex aliquots of bioparticles  $3 \times 15$  s at the highest setting.
7. Using a 1-mL syringe, inject bioparticle suspension into mice by oral/esophageal injection.
8. Expose animals to desired experimental conditions.
9. Harvest target organs and serum.
10. Fluorescence can be quantified directly in serum or by eluting organ aliquots in formamide at 55°C from 2 h to overnight.
11. Dissected organs can be fixed in formalin embedded and cut for microscopy.

### 3.4. Water Content in Tissues

Tissue inflammation is often characterized by cellular infiltration and accumulation of extracellular water (edema). Quantifying water content in tissues can be a useful measurement to determine the integrity of vascular and epithelial barriers. This parameter can often be combined with other permeability assays to corroborate the effect of experimental conditions or pharmacological interventions (11,12). We describe a simple and highly accurate method to measure water content in tissues using dry to wet weight ratios. Multiple organs can be assayed simultaneously as shown in **Fig. 2**.

1. After the experimental conditions have been completed, collect 20 to 50 mg of tissue of interest from a representative portion of the organ (see **Note 4**).
2. Obtain wet weight on an electronic high fidelity balance and record (tissue alone and tube containing tissue).
3. Place samples on labeled microcentrifuge tubes (make sure that sample is placed at the bottom of the tube).
4. Place tubes in speed-vac. Set up for vacuum mode at 65°C for 12 to 18 h (see **Note 5**).
5. Obtain dry weight as described previously.
6. Subtract dry value from wet weight.

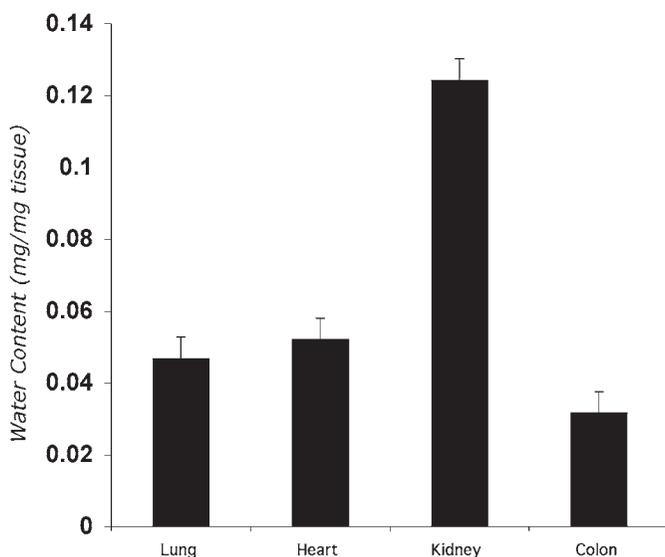


Fig. 2. Vascular permeability profile of different organs in mice. All organs were harvested and water content quantified by wet/dry ratios. Multiple organs were profiled simultaneously. Data are expressed in mg/mg of tissue.

#### 4. Notes

1. If using fluorescent microspheres, fluorescence may be measured directly in serum or in frozen sections of tissues using fluorescence microscopy.
2. For intestinal epithelial permeability, FITC-dextran solution is given by oral gavage. For pulmonary epithelial permeability, FITC-dextran is injected directly into the trachea of anesthetized animals.
3. Bioparticles are provided as lyophilized powders. Upon receipt, they should be stored frozen at  $-20^{\circ}\text{C}$ . In the case of fluorescent-labeled products, these should be protected from light exposure.
4. Smaller amounts of tissue (10–30 mg) from organs with high fat content (intestine) or high cellular density (kidney) can be used, since these tissues will take longer to dry.
5. If tissues from different organs are processed simultaneously, check the vacuum run periodically (every 6–8 h). Lung tissue depending on the amount will typically require less time to dry compared with other organs. Over exposure of the samples may result in protein evaporation, tissue destruction and inaccurate readings.

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## Bacterial–Bacterial Cell Interactions in Biofilms

### *Detection of Polysaccharide Intercellular Adhesins by Blotting and Confocal Microscopy*

**Kimberly K. Jefferson and Nuno Cerca**

#### Summary

Adhesive interactions between bacterial cells coupled with adherence to a solid surface can lead to the formation of a biofilm. The important role of biofilm formation in the pathogenesis of certain types of infection, especially those involving indwelling medical devices, is becoming increasingly apparent. Critical to the development of a biofilm is the elaboration of exo-polysaccharide that contributes to substrate and intercellular adhesion. The synthesis and secretion of large exo-polysaccharides is a metabolically expensive process and is therefore often suppressed under conditions that favor the planktonic mode of growth. One way to identify the environmental cues that cause a given bacterial species to switch to the biofilm mode of growth is to monitor exo-polysaccharide elaboration *in vitro*. The exo-polysaccharide involved in biofilm formation in a number of bacterial species is a polymer of *N*-acetyl-glucosamine. In this chapter, we outline two methods that use wheat germ agglutinin, a lectin that binds to *N*-acetyl-glucosamine, to evaluate extracellular polysaccharide production by a variety of bacterial species.

**Key Words:** Biofilms; exo-polysaccharide; polysaccharide matrix; bacteria; lectins; wheat germ agglutinin; confocal microscopy.

#### 1. Introduction

“United we stand, divided we fall” is one of the most famous quotes of the legendary Greek fabulist Aesop. The truism is as befitting to bacterial consortia as it is to human societal interactions. Interactions between bacterial cells and adherence to a solid surface, mediated in large part by exo-polysaccharide (EPS) intercellular adhesins, result in cohesive, structured communities called biofilms, which are able to withstand many adverse environmental conditions that would destroy individual free-floating bacteria. For reasons that are not completely

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understood, many bacterial and fungal species switch from a planktonic to a biofilm mode of growth under certain environmental conditions or during infection. Many types of infections, including osteomyelitis, endocarditis, and catheter or other medical device-related infections, involve biofilm formation, which is problematic for a number of reasons. Biofilms are tenacious and resilient. Physical forces such as the shear force of blood flow or urine flow across the surface of intravenous or urinary catheters do not wash them away. Biofilms also are resistant to phagocytosis and can tolerate levels of antibiotics 10- to 1000-fold higher than the levels required to inhibit or kill free-floating bacteria. Thus, biofilm infections often are chronic or relapsing, frequently are refractory to antimicrobial chemotherapy, and often necessitate the removal of the colonized tissue or device.

The biofilm EPS matrix is an important intercellular adhesin and is therefore fundamental to biofilm development and structure. EPS also plays a role in protecting the bacteria from adverse conditions and has been implicated in resistance of *Staphylococcus epidermidis* to phagocytosis (1). Elaboration of EPS is a key factor in the switch from planktonic to biofilm growth (2). Antibodies that react specifically to the EPS of certain bacterial species have been produced and used to monitor the effect of different mutations or environmental conditions on EPS production; however, EPS-specific antibodies are not always readily available (3,4). The biofilm EPS elaborated by a number of bacterial species, including *Staphylococcus aureus* and *Staphylococcus epidermidis*, *Escherichia coli*, and *Acinetobacter*, is a polymer of *N*-acetyl-glucosamine referred to as PNAG, PGA, or polysaccharide intercellular adhesin (4-7). The sugar residue *N*-acetyl-glucosamine is a ligand for the lectin wheat germ agglutinin (WGA). Horseradish peroxidase-labeled and fluorescently labeled WGA is commercially available and can be used to detect EPS semiquantitatively by immunoblotting and by confocal microscopy, respectively (8,9). Thus, we have developed a technique that can be used to detect EPS production by a number of bacterial species for which a specific anti-polysaccharide antibody is not readily available.

## 2. Materials

### 2.1. Cell Culture

1. Culture medium is chosen depending on the environmental conditions being tested. A rich medium such as tryptic soy broth + 1% glucose is recommended when it is desirable to maximize biofilm formation.
2. For EPS blots, use 50-mL polystyrene conical tubes.
3. For confocal microscopy, use either 35-mm collagen-coated glass-bottom micro-well dishes (Mat-Tek, Ashland, MA) or 35-mm tissue culture-treated polystyrene wells (Corning, Corning, NY).

## 2.2. Preparation of EPS Extract

1. dH<sub>2</sub>O (distilled water) and ddH<sub>2</sub>O (deionized, distilled water).
2. 0.5 M EDTA (American Bioanalytical, Natick, MA), pH 8.0. Prepare by adding 186.12 mg of EDTA to 900 mL of dH<sub>2</sub>O, add 5 N NaOH dropwise, and mix continuously until the pH reaches 8.0 and the EDTA is completely dissolved.
3. Proteinase K 20 mg/mL (Qiagen, Valencia, CA).
4. 60 and 80°C heat-blocks.

## 2.3. Blotting and Detection of EPS

1. Vacuum manifold slot-blotter or dot-blotter.
2. Nitrocellulose (Amersham, Piscataway, NJ).
3. Tris-buffered saline (TBS). Prepare a stock solution of 1 M Tris-HCl pH 7.5. For 1 L of TBS, combine 100 mL of 1 M Tris-HCl, pH 7.5., 9 g NaCl, and 900 mL of dH<sub>2</sub>O and store at room temperature (RT).
4. TBS + 0.05% Tween-20 (TBST).
5. Blocking solution. Prepare fresh by dissolving 1% w/v bovine serum albumin (Sigma, St. Louis, MO) in TBST.
6. WGA-horseradish peroxidase-labeled lectin from *Triticum vulgare* (HRP; Sigma). Prepare a stock solution by dissolving 1 mg WGA-HRP in 1 mL of ddH<sub>2</sub>O. Store 10- $\mu$ L aliquots at -20°C.
7. Chemiluminescent HRP substrate (ECL kit, Amersham).
8. Plastic wrap.
9. Film cassette.
10. Kodak BioMax MS X-ray film (Amersham).

## 2.4. Confocal Microscopy

1. Phosphate-buffered saline (PBS). To prepare a 10X stock solution, dissolve 1.44 g KH<sub>2</sub>PO<sub>4</sub>, 90 g NaCl, and 7.95 g Na<sub>2</sub>HPO<sub>4</sub> in 1 L of dH<sub>2</sub>O and adjust the pH to 7.5.
2. WGA-Oregon green conjugate (WGA-Green; Molecular Probes, Eugene, OR). Prepare a stock solution by dissolving 10 mg WGA-Green in 1 mL of H<sub>2</sub>O. Store in aliquots of 25  $\mu$ L at -80°C.

## 3. Methods

### 3.1. Preparation of EPS Samples

1. Grow bacteria to stationary phase in 5 mL of medium.
2. Approximately  $5 \times 10^9$  bacterial cells are collected by centrifugation at 8000g. The culture supernatant may be discarded (*see Note 1*) or saved for analysis.
3. Resuspend bacterial cells in 180  $\mu$ L of 0.5 M EDTA by pipetting up and down or by gentle sonication (*see Note 2*) using a probe-type sonicator. Transfer the suspension to a microfuge tube and boil for 5 min to kill the bacteria and to aid in release of EPS from the cell surface.

4. Centrifuge the samples at 18,000g to remove the cells, and transfer 150  $\mu$ L of the EPS-containing supernatant to a fresh microfuge tube.
5. Add 15  $\mu$ L of proteinase K (2 mg/mL final concentration) and incubate the samples at 60°C for 1 h. After protein digestion, heat-inactivate the proteinase K at 80°C for 30 min.

### 3.2. Blotting and Detection of EPS

1. Cut a piece of nitrocellulose to fit into the slot-blot apparatus, and pre-wet with TBS.
2. Sandwich the nitrocellulose between the gasket and top plate of the blotting apparatus and the turn on the clamp vacuum. Fill the wells with TBS and turn on the sample vacuum until the liquid has been drawn through the membrane. Turn off the sample vacuum.
3. A series of 2- to 10-fold dilutions of the EPS samples should be made in TBS. Add the samples and dilutions to the wells of the vacuum manifold and turn on the vacuum until the samples have been completely drawn through the nitrocellulose membrane.
4. Disassemble the vacuum manifold and allow the nitrocellulose membrane to dry thoroughly at RT.
5. Wet the membrane with TBS and block in 25 mL of blocking solution (*see Note 3*) for 1 h at RT with rocking.
6. Add 15 mL of 50 ng of WGA-HRP + 10 mg bovine serum albumin/mL TBST and incubate for 30 min at RT with rocking.
7. Wash the membrane 3 times for 5 min with at least 25 mL of TBST.
8. Rinse the membrane briefly with dH<sub>2</sub>O. Lift the membrane with forceps or gloved fingers and gently shake off excess water. Lay the membrane on a flat surface such as a clean benchtop.
9. Work very quickly from this step forward. Thoroughly mix 2 mL of ECL reagent 1 and 2 mL of reagent 2 and immediately pipet onto the membrane. Surface tension will hold the solution on the blot. Leave the solution on the blot for exactly 1 min.
10. Lift the blot and shake off excess solution. Wrap the blot in plastic wrap and tape to the inside of a film cassette. Take the film cassette to a darkroom and lay a piece of film on the blot under safelight conditions. Close the cassette and expose the film for 1 min. Remove and develop the first film, add a second film to the blot and start a stopwatch to monitor the exposure-time of the second film.
11. Gage the exposure time for the second film after the first film has been developed. A sample blot illustrating results obtained with EPS-positive and EPS-negative strains of staphylococci and *E. coli* is shown in [Fig. 1](#).

### 3.3. Prepare Biofilms for Confocal Microscopy

1. Inoculate 2.5 mL of the medium from liquid cultures and grow biofilms at 37°C with rocking for 18 to 72 h (*see Note 4*) in 35-mm dishes (*see Note 5*).
2. Remove medium from biofilm and rinse with PBS (*see Note 6*).

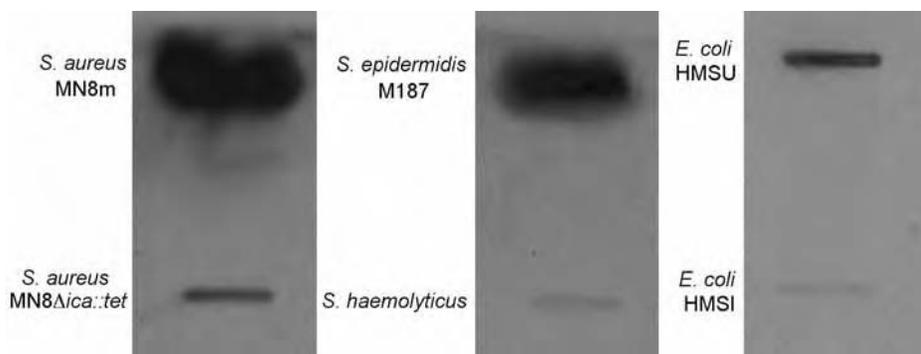


Fig. 1. Analysis of EPS elaboration by blotting. Surface extracts from staphylococci and *E. coli* were blotted onto nitrocellulose and probed with WGA–HRP. Strains are indicated to the left of each blot: PNAG-hyper-producing *S. aureus* strain MN8m, the PNAG-negative *S. aureus* strain MN8 $\Delta$ ica::tet, PNAG-producing *S. epidermidis* strain M187, PNAG-negative staphylococcal species *S. haemolyticus* (strain M176), *pga*-positive, *E. coli* clinical isolate (strain HMS-U) from a urinary tract infection, and a *pga*-negative, *E. coli* urinary tract infection isolate (strain HMS-I).

3. Dilute WGA–Green in PBS (final concentration 0.1 mg/mL) and add 1 mL to the biofilm. Incubate in the dark for 15 min (*see Note 7*).
4. Pipet off WGA–Green solution and rinse the biofilm with 2 mL of PBS.

### 3.4. Detection of EPS by Confocal Microscopy

1. Biofilms should be observed by confocal microscopy using an immersion objective (Achromplan  $\times 63/0.95W$ ).
2. The bacterial cells are detected by the refraction of light in the red spectrum (*see Note 8*) and WGA–Green is detected by fluorescence in the green spectrum, using a single channel analysis (*see Note 9*).
3. Excitation wavelengths are set at 633 nm with a output power of 70% for the detection of bacterial cells and 488 nm with a output power of 10% for the detection of WGA–Green. The excitation beam splitter is HFT UV/488/543/633.
4. The filter used to detect the light refracted by bacterial cells is LP650, and the filter for detection of light emitted by WGA–Green is BP 505-530. The beam splitter is NFT 545.
5. The pinholes of both channels are adjusted to obtain equivalent stack sizes.
6. A fast scan is performed to adjust the detector gain and detector offset of both channels. The color palette is set to Range Indicator, and the detector gain is adjusted to minimize background color intensity (an indication of minimum saturation of the detector gain). The detector offset should also be adjusted to minimize color intensity if background pixels.
7. A fast scan is performed to determine the top and bottom of the biofilm.

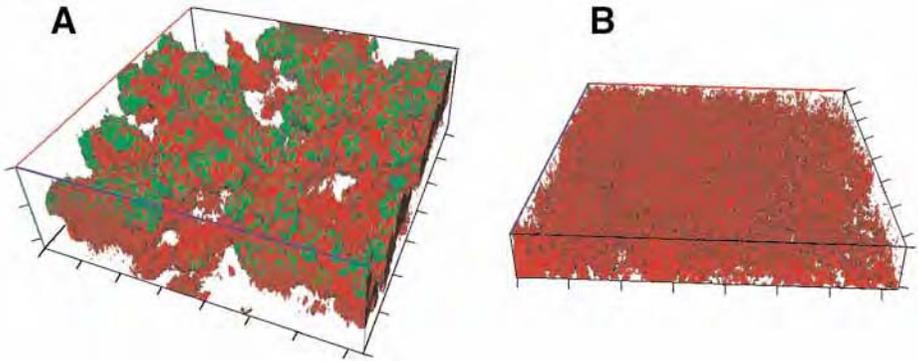


Fig. 2. Use of confocal microscopy to analyze EPS elaboration. The intercellular adhesion (*ica*) locus in *S. aureus* encodes the proteins necessary for PNAG synthesis so *ica*-positive and *ica*-negative mutants were used as positive and negative controls. Biofilms produced by (A) the strong PNAG-producing *S. aureus* strain MN8m and (B) the PNAG-negative *S. aureus* strain MN8 $\Delta$ *ica::tet* were stained with WGA–Green and imaged by confocal microscopy. Biofilm cells are presented in red and PNAG in green. Each bar segment represents 20  $\mu$ m. (Please see color insert following p. 50 for a color version of this figure.)

8. Once the z-settings have been set, a full resolution scan is done. Sample images of EPS-positive and EPS-negative strains of *S. aureus* are shown in [Fig. 2](#).

#### 4. Notes

1. EPS may be secreted into the culture fluid; therefore, the investigator may wish to reserve the supernatant and blot it onto the nitrocellulose membrane. However, noncell-associated EPS does not appear to contribute to biofilm development ([10](#)).
2. EPS-producing bacteria may be mucoid and difficult to resuspend. Sonication may be used to disrupt cell clusters but should be at low power to prevent release of cell wall components that may contain *N*-acetyl-glucosamine and could cross-react with WGA–HRP.
3. Skim milk contains sugars that can interact with WGA, and should not be used as a blocking agent.
4. In some bacterial species, biofilm maturation requires several days of growth ([11](#)). If bacteria are allowed to grow for more than 18 h, then the medium should be replaced every 12 h.
5. Because of variations in surface proteins and hydrophobicity among different bacterial species, glass or tissue culture-treated polystyrene may provide a better substrate for adherence and biofilm formation. This must be determined experimentally for each species. Most bacteria will not, however, adhere to polystyrene that has not been tissue culture-treated.

6. Loosely adherent biofilms should be washed very gently or the initial washing step may be skipped.
7. The 15-min incubation time should be adhered to as longer incubation times can actually reduce the level of fluorescence.
8. Fluorescent labeling of bacterial cells can produce artifactual results and certain markers can interfere with the binding or detection of WGA–Green. The detection of unlabeled bacterial cells by refracted light overcomes this problem.
9. If there is a chance that emission or reflected light spectra could overlap, then multi track analysis should be used, instead of single track.

## Acknowledgments

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## A Biochemical Method for Tracking Cholera Toxin Transport From Plasma Membrane to Golgi and Endoplasmic Reticulum

Heidi E. De Luca and Wayne I. Lencer

### Summary

Asiatic cholera is a rapidly progressing disease resulting in extreme diarrhea and even death. The causative agent, cholera toxin, is an AB<sub>5</sub>-subunit enterotoxin produced by the bacterium *Vibrio cholera*. The toxin must enter the intestinal cell to cause disease. Entry is achieved by the B-subunit binding to a membrane lipid that carries the toxin all the way from the plasma membrane through the *trans*-Golgi to the endoplasmic reticulum (ER). Once in the ER, a portion of the A-subunit, the A1 chain, unfolds and separates from the B-subunit to retro-translocate to the cytosol. The A1 chain then activates adenylyl cyclase to cause disease. To study this pathway in intact cells, we used a mutant toxin with C-terminal extension of the B-subunit that contains *N*-glycosylation and tyrosine-sulfation motifs (CT-GS). This provides a biochemical readout for toxin entry into the trans Golgi (by <sup>35</sup>S-sulfation) and ER (by *N*-glycosylation). In this chapter, we describe the methods we developed to study this trafficking pathway.

**Key Words:** Retrograde transport; ganglioside; lipid trafficking; cholera toxin; retro-translocation; *N*-glycosylation; tyrosine sulfation; endoplasmic reticulum; *trans*-Golgi network.

### 1. Introduction

Cholera toxin (CT) typifies the structure and function of the AB<sub>5</sub> toxins, all of which must enter the cytosol of host cells by traveling backwards from the plasma membrane (PM) to the endoplasmic reticulum (ER) and retro-translocating to the cytosol (1,2). This pathway is unusual because membrane proteins rarely move backwards from the PM to the ER (3,4) and because it is dependent on toxin binding to a membrane lipid, ganglioside GM1 for CT, and not a membrane protein (5).

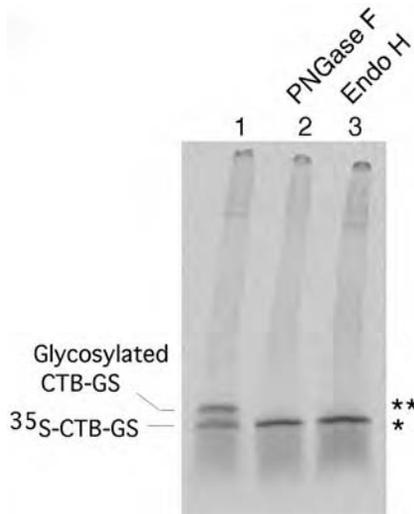


Fig. 1. Phosphorimage showing sulfation (marked by \*) and *N*-glycosylation (marked by \*\*) of CT-GS B-subunit 3 h after apical and basolateral application to T84 cells (see **Subheading 3.1.**). Immunoprecipitates were divided into three equal samples and treated with PNGaseF (**lane 2**), EndoH (**lane 3**), or mock treated (**lane 1**) before analysis by SDS-PAGE. (Reprinted with permission from **ref. 5.**)

To study the toxin pathway, we modified an approach originally developed by Rapak and Johannes (6,7). It uses a mutant toxin tagged with tyrosine-sulfation and *N*-glycosylation motifs and exploits the subcellular localization of the enzymes that catalyze these reactions in native cells. Post-translational tyrosine-sulfation of nascent proteins occurs in the *trans*-Golgi network (TGN) and *N*-glycosylation occurs in the ER (6,8,9). As such, measurement of these modifications on CT gives a biochemical measure of toxin transport from PM through TGN to ER (Fig. 1). In addition, because the ER-localized mutant CT is modified by *N*-glycosylation, the ER fraction of toxin can be isolated by affinity purification using lectins that bind the oligosaccharide (Fig. 2; [5]).

Two methods are discussed in this chapter. One describes the measurement of toxin transport from PM to TGN to ER, as reported by glycosylation and sulfation, and the other describes the affinity purification of the toxin fraction localized in ER. The sulfation–glycosylation assay depends on immunoprecipitation of the B-subunit and subsequent detection of the <sup>35</sup>S-sulfated fraction of this B-subunit by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. *N*-glycosylation is detected by an *N*-glycanase-sensitive shift in apparent molecular weight as observed by SDS-PAGE. In some cells, only a very small fraction of toxin moves through

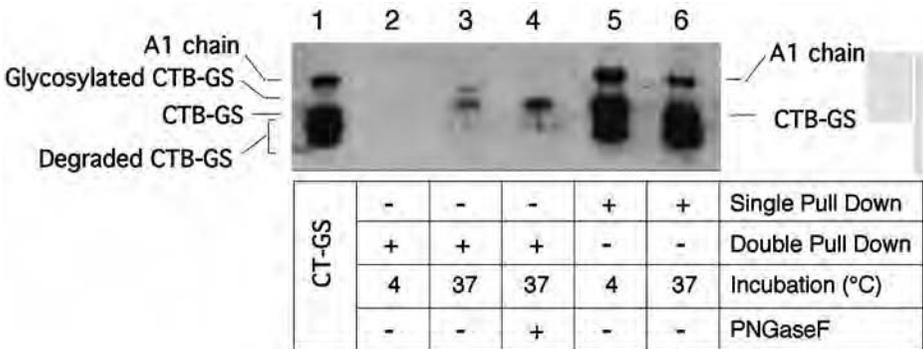


Fig. 2. Immunoblot for A- and B-subunits after double affinity precipitation (*see Subheading 3.2., lanes 2–4*) or single precipitation by GM1 beads alone (*lanes 5 and 6*) in Vero cells. **Lane 4** shows the same sample as in lane 3 treated with PNGaseF. **Lane 1** shows purified CT-GS as control. (Reprinted with permission from **ref. 5.**)

the Golgi and into the ER. In this method, because only the fraction of toxin that traveled through the Golgi is radiolabeled, the assay is extremely sensitive and specific for Golgi/ER transport.

The second method, isolation of the ER glycosylated toxin, describes a technique for affinity isolation of the small fraction of the glycosylated B-subunit. This method depends on two sequential affinity precipitations. The first one isolates all the *N*-glycosylated proteins with a glucose/mannose specific lectin, and the second one isolates the subfraction of toxin B-subunit from this collection of *N*-glycosylated proteins. The double-affinity precipitation method proved to be highly specific and allowed us to determine the conformation of toxin in the ER. Since protein disulfide isomerase can interact only with the proteolytically activated CT A-subunit to unfold and dissociate the A1 chain from the B-subunit, the CT-GS toxin used in this assay is activated by trypsin incubation before use.

## 2. Materials

See **Table 1** for a summary of buffers used in these assays.

### 2.1. Sulfation and Glycosylation Assay

1. Hank's Balanced Salt Solution (HBSS) without sulfate: Dissolve dry HBSS without calcium chloride, magnesium sulfate, phenol red, and sodium bicarbonate (Sigma, St Louis, MO) in 900 mL of water. Adjust to 1 mM magnesium chloride, 1 mM calcium chloride, and 10 mM HEPES final concentration. Adjust the pH to 7.4, bring volume to 1 L with water, and store at 4°C for up to 4 d (*see Note 1*).
2. HBSS with sulfate: dissolve dry HBSS without phenol red and sodium bicarbonate (Sigma) in 900 mL of water. Adjust to 10 mM HEPES final concentration, adjust the pH to 7.4, bring volume to 1 L with water, and store at 4°C for up to 4 d.

**Table 1**  
**Summary of Buffers Used in the Assays**

Buffer	Buffer name	Composition	Protocol steps
A	Lysis stock buffer	20 mM Tris-base, 150 mM NaCl, 5 mM EDTA, 20 mM Triethanolamine, pH to 8.0 with HCl	
B	SDS-based lysis buffer	0.5% (w/v) SDS, 0.2% (w/v) bovine serum albumin in lysis stock buffer (Buffer A)	<b>Subheading 3.1.2., step 1</b>
C	Triton dilution buffer	2.5% (v/v) Triton X-100 (Sigma) in lysis stock buffer (Buffer A)	<b>Subheading 3.1.2., step 3</b>
D	Mixed micelle buffer	1% (v/v) Triton X-100, 0.2% (w/v) SDS, 8% (w/v) sucrose in lysis stock buffer (Buffer A)	<b>Subheading 3.1.2., step 10</b>
E	Sucrose final wash buffer	8% (w/v) sucrose in lysis stock buffer (Buffer A)	<b>Subheading 3.1.2., steps 10 and 11</b>
F	TN lysis buffer	10 mM Tris-base, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, 1.75% (w/v) NOG, 0.2% (w/v) gelatin, 0.1% sodium azide	<b>Subheading 3.2.2., steps 5 and 6</b>
G	ConA-binding buffer	10 mM Tris-base, pH 7.4, 6 mM CaCl <sub>2</sub> , 6 mM MnCl <sub>2</sub> , 6 mM MgCl <sub>2</sub> , 0.5 M NaCl, 0.2% (w/v) gelatin	<b>Subheading 3.2.3., step 1</b>
H	TN wash buffer for ConA beads	10 mM Tris-base, pH 7.4, 0.5 M NaCl, 1% (v/v) Triton X-100, 1.75% (w/v) NOG, 1 mM CaCl <sub>2</sub> , 1 mM MnCl <sub>2</sub> , 1 mM MgCl <sub>2</sub> , 0.1% (w/v) sodium azide in water	<b>Subheading 3.2.3., steps 2–4</b>
I	TN wash buffer for GM1 beads	10 mM Tris-base, pH 7.4, 0.15 M NaCl, 1% (v/v) Triton X-100, 1.75% (w/v) NOG, 1 mM CaCl <sub>2</sub> , 1 mM MnCl <sub>2</sub> , 1 mM MgCl <sub>2</sub> , 0.1% (w/v) sodium azide in water	<b>Subheading 3.2.3., step 6</b>

SDS, sodium dodecyl sulfate; NOG, octyl  $\beta$ -D-glucopyranoside; TN, Triton-NOG; ConA, Concanavalin A.

3. <sup>35</sup>S-Sodium Sulfate (Sulfur-35 Radionuclide ~1000 Ci/mMole, Perkin Elmer, Boston, MA; *see Note 2*). All radioactive precautions must be taken when working with any kind of radioactivity, including <sup>35</sup>S. We routinely use a radioactive beta shield when working with this nuclide, in addition to gloves, goggles, and a lab coat. All radioactive waste must be disposed of properly and according to each institution's guidelines.
4. Sepharose CL-4B (Sigma) is washed three times in phosphate-buffered saline (PBS) and resuspended to 50% slurry in PBS/0.02% sodium azide before use. Wash sepharose by spinning at 3000g to pellet, remove supernatant, add wash buffer to fill tube, and rotate 1 to 2 min at 4°C. Spin again to pellet beads and repeat wash twice more. After the final wash, remove as much wash buffer as possible with a gel loading tip or small gage needle, and add wash buffer to create 50% slurry of the sepharose (*see Note 3*).
5. Protein A sepharose (Amersham Biosciences, Piscataway, NJ) is washed three times in PBS and resuspended to 50% slurry in PBS before use. Wash sepharose by spinning at 3000g to pellet, remove supernatant, add wash buffer to fill tube and rotate 1 to 2 min at 4°C. Spin again to pellet beads, and repeat wash twice more. After the final wash, remove as much wash buffer as possible with a gel loading tip or small gage needle, and add wash buffer to create 50% slurry of the sepharose.
6. Lysis stock buffer (Buffer A): 20 mM Tris-base 150 mM NaCl, 5 mM EDTA, 20 mM triethanolamine, pH 8.0. The stock buffer can be stored for 3 mo at 4°C.
7. SDS lysis buffer (Buffer B): 0.5% (w/v) SDS, 0.2% (w/v) bovine serum albumin in Lysis stock buffer (Buffer A). Buffer B must be kept at room temperature; otherwise, the SDS will precipitate out of solution. It can be stored at 4°C but must be warmed to room temperature to redissolve SDS crystals. Add protease inhibitors just before use (*see Note 4*).
8. Triton dilution buffer (Buffer C): 2.5% (v/v) Triton X-100 in Buffer A.
9. Mixed micelle buffer (Buffer D): 1% (v/v) Triton X-100, 0.2% (w/v) SDS, and 8% (w/v) sucrose in Buffer A.
10. Sucrose final wash buffer (Buffer E): 8% (w/v) sucrose in Buffer A.
11. Rubber cell scraper (Fisher Scientific, Pittsburgh, PA).
12. 2X Laemmli sample buffer (Bio-Rad, Hercules, CA): add dithiothreitol or 5% (v/v) β-mercaptoethanol to sample buffer immediately before use. β-mercaptoethanol should be handled in a fume hood with gloves because of its toxicity and strong odor.
13. 10 to 20% Tris-HCl ready gel and gel running and transfer apparatus including cells, electrodes, and power supply (Bio-Rad). Precautions should be taken to avoid any electric shock stemming from the power supply unit. Carefully read the instructions of the power supply before use. Always ensure the power supply is off before connecting or disconnecting the electrodes to reduce the risk of electric shock.
14. Gel fixation buffer: 30% (v/v) methanol, 7% (v/v) acetic acid in water.
15. Rabbit anti-CT B-subunit antibody (3–5 mg/mL [*10,11*]) can be aliquoted and stored at –80°C.
16. CTB-GS and CT-GS protein (0.1–3 mg/mL [*5*]) aliquoted and stored at –80°C.

17. Radioactive containment box (see **Note 5**).
18. Gel-drying apparatus.
19. PhosphoImager.
20. Six-well tissue culture plate, with 35 mm diameter wells (Fisher Scientific).

## 2.2. Capture and Isolation of ER Glycosylated Toxin

1. *N-p*-Tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Sigma).
2. Soybean trypsin inhibitor (Sigma): We prepare a stock solution of 0.7 mg/25  $\mu$ L in water just before use.
3. Triton-NOG lysis buffer (Buffer F): 10 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA; 1% (v/v) Triton X-100, 1.75% (w/v) octyl  $\beta$ -D-glucopyranoside (NOG), 0.2% (w/v) gelatin. Store at 4°C and use within 2 d. Add protease inhibitors just before use.
4. Concanavalin A (ConA)-binding buffer (Buffer G): 10 mM, Tris-base pH 7.4, 6 mM CaCl<sub>2</sub>, 6 mM MnCl<sub>2</sub>, 6 mM MgCl<sub>2</sub>, 0.5 M NaCl, and 0.2% (w/v) gelatin. Store at 4°C and use within 2 d. Add protease inhibitors just before use.
5. ConA sepharose beads (Amersham Biosciences, Piscataway, NJ) are prewashed three times in a 1 : 1 solution of Buffer F:Buffer G. Wash sepharose by spinning at 3000g to pellet, remove supernatant, add wash buffer to fill tube, and rotate 1 to 2 min at 4°C. Spin again to pellet beads, and repeat wash twice more. After the final wash, remove as much wash buffer as possible with a gel loading tip or small gage needle, and add wash buffer to create 50% slurry of the sepharose.
6. Triton-NOG wash buffers (Buffers H and I): Start with a stock buffer of 10 mM Tris-base, pH 7.4; 1% (v/v) Triton X-100, 1.75% (w/v) NOG, 1 mM CaCl<sub>2</sub>, 1 mM MnCl<sub>2</sub>, and 1 mM MgCl<sub>2</sub> in water. For ConA bead wash buffer (Buffer H), add 0.5 M NaCl. For GM1 bead wash buffer (Buffer I), add 0.15 M NaCl. Store solutions at 4°C and use within 2 d. Add protease inhibitors just before use.
7. 0.2 M methyl- $\alpha$ -D-mannopyranoside (Sigma) is dissolved in Buffer H.
8. PBS: Prepare 10X stock with 1.48 M NaCl, 14 mM KH<sub>2</sub>PO<sub>4</sub>, 81 mM Na<sub>2</sub>HPO<sub>4</sub>, 26.8 mM KCl, adjust pH to 7.4, and bring volume to 1 L. Filter sterilize into an autoclaved bottle, and store at room temperature. Prepare working 1X solution by diluting one part 10X stock with nine parts water.
9. GM1 beads are prepared with lyso-monosialoganglioside GM1 (Matreya, Pleasant Gap, PA) and Dynabeads<sup>®</sup> M-270 Carboxylic Acid (DynaL, Oslo, Norway) according to Dynal's manufacturer instructions for bead coupling. The coupled beads are then washed in PBS three times before they are added to the sample. The beads are easily washed using the capture magnet and successive removal and addition of 1 mL of PBS. After the washes, bring the beads up to the original volume in PBS.
10. Peptide: *N*-Glycosidase F (PNGase F, Glycerol free) and associated buffers (New England Biolabs, Beverly, MA).
11. SDS loading buffer (6X): Mix 7 mL of 4X Tris-HCl/SDS pH 6.8 (0.5 M Tris-HCl containing 0.4% (w/v) SDS), 3 mL of glycerol, 1 g of SDS, 0.93 g of dithiothreitol, and 1.2 mg of bromophenol blue. Bring the volume to 10 mL with water, divide into 0.5-mL aliquots, and freeze at -80°C.

12. SDS running buffer: Make a 10X stock of 250 mM Tris, 1.92 M glycine, and 1% (w/v) SDS in water. DO NOT adjust pH. For a working stock, mix one part 10X buffer with nine parts water. Store 10X and 1X buffers at room temperature.
13. Transfer buffer: 200 mL of methanol, 3 g of Tris-base, 14.4 g of glycine are added to 800 mL of water. Mix and store at 4°C to chill before use. This solution is made fresh for every gel, but a 10X stock can be made with the salts and then diluted with methanol and water before use. The solution should be cooled to 4°C before use to help minimize any temperature increase during the transfer.
14. Tris-buffered saline (TBS): Make a 10X stock of TBS with 500 mM Tris 2 M NaCl, pH 7.4 in water. Prepare a working stock of 1X TBS by adding one part 10X TBS to nine parts water. Both 10X and 1X stocks can be stored at 4°C for 4 mo.
15. TBS with Tween-20 (TTBS; Sigma): Prepare a working stock of 1X TTBS by adding 1% (v/v) Tween-20 to 1X TBS. Both 10X and 1X stocks can be stored at 4°C for 4 mo.
16. Blocking buffer: Add 5% (w/v) of nonfat dry milk to TTBS. Stir 30 min at room temperature and filter into a vacuum flask using a büchner funnel and a piece of filter paper. Store up to 3 d at 4°C.
17. Anti-rabbit IgG (whole molecule)–peroxidase antibody produced in goat (secondary antibody, Sigma).
18. Enhanced chemiluminescent detection kit (Pierce Biotechnology, Rockford, IL).
19. Round tissue culture dishes, 60-mm diameter. The actual growth area diameter of these dishes is approx 50 mm (Fisher Scientific, Pittsburgh, PA).

### 3. Methods

The mutant cholera toxin used in the following assays was developed by Jobling and Fujinaga (5). The C-terminal motifs were specifically designed to provide maximum opportunity for glycosylation and sulfation and to exert minimal effect on normal cholera toxin function in the cell. Theories regarding optimal placement of these motifs, linker lengths, and conformation were considered when designing the mutant toxin. Functional tests revealed a minimal change in toxicity of the mutant toxin compared to the wild type.

The following methods can be modified to fit other cell or biological systems. Depending on the binding affinity of the toxin to the cell receptor, the conditions and incubation times should be modified to achieve maximal saturation binding at steady state. Although there are no specific cleavage sites within the linker, the appended tag is subject to nonspecific proteolysis. In some cell or in vivo systems, the addition of protease inhibitors is crucial to maintain the full-length tag to allow affinity precipitation with the lectin. To control for the isolation of the ER glycosylated toxin, a single affinity precipitation with GM1 alone is conducted with a fraction of the lysate. This will reveal, by immunoblot, the entire B-subunit toxin that was taken up by the cell during the incubation and control for any problems that may have occurred during the toxin uptake steps.

### 3.1. Sulfation and Glycosylation Assay

#### 3.1.1. Depletion of Cell Sulfate and Toxin Incubation

1. Seed T84, Vero, Cos, or Y1 cells into a six-well plate and grow to 90 to 100% confluency.
2. Wash each well three times with 4 mL of 37°C HBSS without sulfate. This is easily done by aspirating off the media with a 5-mL pipet, gently adding the HBSS wash, and then repeating this process twice.
3. Add 4 mL of HBSS without sulfate to each well and incubate at 37°C for 30 min in a CO<sub>2</sub> incubator.
4. Aspirate off the HBSS without sulfate and replace with 4 mL fresh HBSS without sulfate. Incubate for 1 h at 37°C in a CO<sub>2</sub> incubator.
5. Aspirate the HBSS without sulfate and replace with 1 mL of 0.5 mCi/mL <sup>35</sup>S-sodium sulfate in HBSS without sulfate. Incubate the cells for 30 min at 37°C in a CO<sub>2</sub> incubator using a radioactive containment box to prevent contamination.
6. Add CTB-GS or CT-GS to a final concentration of 20 nM (or appropriate) and incubate for desired time (30 min to overnight) at 37°C in CO<sub>2</sub> incubator, using a radioactive containment box.
7. Aspirate off and properly dispose of <sup>35</sup>S toxin solution.
8. Wash cells three times with 3 mL of 4°C HBSS with sulfate. Add wash buffer to cells by gently pipetting against the wall of the well and gently aspirate off wash buffer. This technique helps prevent the cells from being washed off the well bottom.

#### 3.1.2. Immunoprecipitation With CT antibody

1. Remove the last wash buffer from the wells and add 600 µL of room temperature Buffer B.
2. Using a rubber cell scraper, scrape the cells off the well bottom and collect in a 2-mL screw cap tube (*see Note 6*).
3. Heat the tube to 100°C for 5 min, then transfer the tube to ice, and add 600 µL of ice-cold Buffer C. This is an appropriate stopping point for the assay. If desired, after the addition of Buffer C, the samples can be stored at -80°C for later processing.
4. To fracture the DNA, put the sample tubes in an automatic vortex at 4°C, and vortex at maximum speed for 20 min. Pass the sample through a 25-gage needle five to six times to disrupt the DNA, and transfer the sample to a new tube.
5. Clarify the DNA by adding 30 µL of 50% Sepharose CL-4B slurry. Tumble sample for 5 min, spin at maximum speed (16,000g) for 3 min, and transfer the supernatant to a new tube. Repeat this clarification step one more time.
6. Preadorb the sample by adding 15 µL of 50% protein A bead slurry. Tumble sample for 15 min at 4°C, spin at maximum speed for 1 min, and transfer the supernatant to a new tube.
7. Dilute the anti-CTB stock antibody 1:30 in PBS and add 100 µL of this to each tube. Tumble at 4°C for 1 h.

8. Add 15  $\mu\text{L}$  of 50% protein A bead slurry to each tube and tumble at 4°C for 1 h to overnight.
9. Spin the sample at maximum speed for 1 min. Carefully remove the supernatant and store at -80°C (in case of recapture).
10. Wash the protein A bead pellet in 1 mL of ice-cold Buffer D. Add 1 mL of buffer to the pellet, incubate on ice for 2 min, and spin at maximum speed for 1 min. Remove the supernatant and repeat the wash four times for a total of five washes, then repeat the wash step once more with Buffer E.
11. Remove as much Buffer E as possible with a gel-loading tip or small gauge needle and then add 20  $\mu\text{L}$  of 2X Laemmli sample buffer to the bead pellet. Use a 30-gage needle to make a hole in the top of the tube (to avoid explosion), and boil for 5 min.
12. Spin at maximum speed for 5 min and carefully remove the supernatant. This is the final sample and can be stored at -80°C until further processing.

### 3.1.3. SDS-PAGE and Phosphoimaging

1. Run the samples on a 10 to 20% gradient SDS-PAGE gel with approx 6–12  $\mu\text{L}$  per lane for a phosphoimage. (For a Western blot, load 2–3  $\mu\text{L}$  per lane. For more detailed SDS-PAGE and Western blotting instructions, *see Subheading 3.2.5.*)
2. Incubate the gel in fixation solution at room temperature for 1 h with gentle shaking.
3. Soak the gel in water at room temperature for 30 min with gentle shaking. During the 30-min incubation, change the water four times.
4. Dry the gel down onto a piece of filter paper using a vacuum driven gel-drying apparatus (or other quick gel-drying method).
5. Expose the dried gel in a PhosphorImager plate for 3 d to 2 wk and then read the plate on a PhosphorImager (*see Note 7*).

## 3.2. Isolation of ER Glycosylated Toxin

### 3.2.1. Nicking Toxin With Trypsin Treatment

1. Incubate 12  $\mu\text{g}$  of CT-GS with 6  $\mu\text{g}$  of TPCK treated trypsin for 30 min at 37°C. We usually add 6  $\mu\text{L}$  of a 1 mg/mL solution of TPCK trypsin to 12  $\mu\text{g}$  of CT-GS in PBS to a final volume of 180  $\mu\text{L}$  for the incubation.
2. After the incubation, add 0.56 mg of soybean trypsin inhibitor to stop the trypsin digest. Bring the final volume to 12 mL with HBSS with sulfate to yield a solution of 10 nM CT-GS.

### 3.2.2. Toxin Incubation and Cell Lysis

1. Seed cells on a 6 cm<sup>2</sup> dish and grow until 90 to 100% confluent. For Vero cells, we typically seed them in the plates and allow them to grow for 2 to 3 d to adhere to the plate.
2. Wash the adherent cells three times with cold HBSS with sulfate. This is easily done by gently aspirating off the media, adding 2–3 mL of HBSS, and then aspirating off HBSS. Take care to do these steps gently because any turbulence may lift the cells off the plate.

3. Add 10 nM of the nicked CT-GS to the plate. This is usually done in a 1-mL volume. Incubate the cells for the desired time, realizing that even if the uptake incubation is for a short time, the internalization incubation needs to be 3–5 h at 37°C for glycosylation to occur (see **Note 8**).
4. After the incubation, wash the adherent cells four times with HBSS with sulfate. The first wash should be done with 37°C HBSS, and the subsequent three with 4°C HBSS. This will help to release any CT-GS bound to membranes, thus giving a cleaner pulldown.
5. Add 1 mL of Buffer F to each dish and let sit 10 min on ice. Scrape off the cells with a rubber cell scraper, and collect in a 1.5-mL Eppendorf tube. Lyse cells by pipetting up and down with a 1-mL tip seven to eight times.
6. Spin the lysate at 6000g for 10 min at 4°C to pellet insoluble material. Remove the supernatant (~1 mL) and transfer it to a new tube. Reserve 20 µL of the supernatant to run as “lysate” on the SDS-PAGE gel.

### 3.2.3. Double Pulldown With ConA and GM1

1. Add an equal volume of Buffer G to the lysate. Add 100 µL of ConA beads to the tube. Rotate for 1 h at 4°C (see **Note 9**).
2. Spin the samples at 3000g for 5 min at 4°C. Remove and save the supernatant (in case of recapture). Wash the ConA bead pellet with Buffer H. Add 1 mL of Buffer H to the bead pellet, resuspend the pellet for 1 to 2 min on a mixer or by inverting, then spin at 3000g for 5 min to pellet the beads. Repeat the wash three more times.
3. Use a small-gage needle or gel loading tip to remove as much buffer as possible from the bead pellet.
4. Elute the glycosylated toxin by adding 50 µL of 0.2 M methyl  $\alpha$ -D-mannopyranoside in Buffer H to the beads. Rotate the sample for 1 h at 4°C for maximal elution. Spin the sample at 3000g for 5 min at 4°C. Collect the supernatant and save the beads in case of re-elution. If applicable, combine supernatants into one tube (see **Note 10**).
5. Bring the volume of the eluted sample to 1 mL with PBS (with protease inhibitors). Add 40 µL of GM1 beads. Rotate the samples overnight at 4°C (see **Note 11**).
6. Wash the GM1 beads four times with Buffer I. Each wash should allow the beads to rotate with Buffer I for 1 to 2 min. Using a small-gage needle or gel-loading tip, remove as much of the wash buffer as possible.

### 3.2.4. PNGase Treatment

1. To load sample directly onto an SDS-PAGE gel, add 20 µL 2X Laemmli sample buffer to GM1 beads and boil 10 min. Isolate the supernatant with the capture magnet. Load 3 µL of this sample onto an SDS-PAGE gel for a Western blot.
2. To proceed with a PNGase digest, add 20 µL of 2X denaturing buffer from the PNGase kit and boil 10 min. Using the capture magnet, remove the supernatant. Follow manufacturer’s instructions for PNGase treatment. We typically mix 3 µL of eluted sample together with 2 µL of NP-40, 1 µL of 10X G7 buffer, 1 µL of

PNGase, 3  $\mu\text{L}$  of water, and incubate at 37°C for 2 h. In parallel, prepare and incubate a control digest containing all the reagents of the digest with the exception of substituting water for PNGase. After digestion, add two microliters of 6X sample buffer to the digested samples, and boil 10 min. The entire 12  $\mu\text{L}$  is loaded onto a reducing SDS-PAGE gel and Western blotted for CTB.

### 3.2.5. SDS-PAGE and Western Blot

1. The following SDS-PAGE instructions assume the use of a Bio-Rad Mini Protean Cell or Criterion Cell and Mini Trans Blot Cell or Criterion Blotter. If using other systems, follow the manufacturer's instructions. Load 3  $\mu\text{L}$  of the undigested sample, 12  $\mu\text{L}$  of the digested samples ( $\pm$  PNGase), and 2  $\mu\text{L}$  of the reserved lysates into separate lanes of a 10 to 20% Tris-HCl ready gel (Bio-Rad). Assemble the gel apparatus and fill the gel box with SDS running buffer. Connect the gel box to a power supply and run at approx 30 to 35 mA per gel until the dye front has just reached the bottom of the gel.
2. After the gel is completed, disassemble the apparatus, remove the gel, and place it in a large (9  $\times$  13 in) pan filled with transfer buffer. Open the transfer cassette and submerge the black side of the cassette in the pan. Place one pre-wet sponge onto the black side of the cassette (this should be the interior of the cassette), and layer successively one piece of filter paper, the gel, one piece of nitrocellulose, one piece of filter paper, and one sponge onto the cassette. Using the provided roller, very gently roll across the top of the transfer sandwich to remove any air bubbles. Close the cassette, assemble it the transfer cell with the black side of the cassette toward the black electrode, and fill the cell with transfer buffer. Connect to a power supply and transfer at 100 V for 45 min to 1 h (*see Note 12*).
3. After the transfer turn off the power, disassemble the transfer cell, remove the nitrocellulose blot, and properly dispose of the transfer buffer (*see Note 13*). The nitrocellulose should be placed in a small incubation dish with blocking buffer and gentle shaking for a minimum of 1 h at room temperature.
4. Remove the blocking buffer, add a 1:2000 dilution of CTB antibody in blocking buffer and incubate 1 h with gentle shaking at room temperature. After the primary incubation, wash the blot three times for 5 min each with TTBS. Remove the last wash buffer.
5. Add a 1:2500 dilution of Anti-Rabbit IgG (whole molecule)-Peroxidase secondary antibody in blocking buffer to the blot, and incubate for 1 h at room temperature. After the secondary incubation, wash the blot three times for 10 min each with TTBS, then two times for 10 min each with TBS.
6. Develop the blot with a chemiluminescent substrate for detection of horseradish peroxidase. We use the Pierce supersignal detection kit (Pierce, Rockford, IL).

## 4. Notes

1. When making solutions with water, always use milliQ water with a resistivity of 18 M $\Omega$ . This is assumed for all solutions that are made with "water."

2. When purchasing  $^{35}\text{S}$ -sodium sulfate from Perkin Elmer, be sure to specify “high specific activity” and request the freshest lot. We always plan the experiment to coincide with the production of a fresh lot to ensure the highest activity.
3. To create 50% slurry, we typically determine the volume of beads by filling an identical tube to the same volume of water, measuring this volume, doubling it, and adding enough wash buffer to the tube with beads to reach the doubled volume.
4. We typically use an EDTA-free protease inhibitor tablet from Roche Diagnostics (Indianapolis, IN). The tablets are available in sizes to accommodate 10 or 50 mL. Various protease inhibitors can also be added individually to the solutions.
5. The radioactive containment box is used to prevent radio contamination of the  $\text{CO}_2$  incubator. The box is made by affixing a piece of charcoal paper to a cut out section of the lid of a square (2.4 L) Tupperware<sup>®</sup> container. Charcoal pellets are added to the bottom of the container. The plate of cells with  $^{35}\text{S}$ -sodium sulfate is placed on top of the pellet layer, and the lid is closed. This device helps to prevent  $^{35}\text{S}$  contamination of the incubator.
6. It is necessary to use a screw cap tube because of the subsequent boiling step. The screw cap prevents explosion of the tube which could result in radioactive contamination of the surrounding workspace.
7. Do not use any type of plastic wrap to cover the gel because the  $^{35}\text{S}$  signal is very weak. For T84 cells we find 3 d to 2 wk is necessary to see a signal; 1 to 3 d are enough for Vero, Cos, and Y1 cells.
8. We typically incubate with toxin for 45 min on ice, then discard the incubation buffer containing the toxin, add back 3 mL HBSS+, and incubate for an additional 4.5 h at 37°C. It is also a good idea to run a negative control in parallel, in which the entire toxin incubation is held at 4°C. Because the toxin is not taken up at this temperature, the pulldown should be negative for CT.
9. The volume of the lysate is usually 1 mL; therefore, 1 mL of ConA-binding buffer is added. If the volume of cell lysate differs, simply change the volume of ConA-binding buffer added to make a 1:1 dilution. The bed volume of 100  $\mu\text{L}$  of 50% slurry of ConA beads is 50  $\mu\text{L}$ .
10. A solution of up to 1 M methyl  $\alpha$ -D-mannopyranoside can be used without difficulty. We have found that 0.2 M methyl  $\alpha$ -D-mannopyranoside solution may not always elute the entire toxin sample off ConA beads. The 1-h incubation step can be reduced to 30 min if necessary; however, the best results and highest elution rates are observed with 1 hour incubation. It often is beneficial to repeat this elution step by adding another 50  $\mu\text{L}$  of methyl  $\alpha$ -D-mannopyranoside to the beads, rotating 1 h, spinning at 3000g, and combining this 50  $\mu\text{L}$  with the first elution of 50  $\mu\text{L}$  for a total of 100  $\mu\text{L}$ .
11. An overnight incubation yields the best results for the GM1 pulldown. We suspect it takes some time for the CT to release from the membrane GM1 and bind to the GM1 beads.
12. It is imperative to follow the manufacturer’s instructions regarding cooling of the tank. Typically sufficient cooling is achieved by either adding a frozen ice block to

the transfer cell, or using a cooling coil in the transfer cell. A rise in temperature will cause the gel to transfer poorly, and may also damage the transfer equipment.

13. The methanol in the transfer buffer may be considered hazardous waste in some institutions.

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## Isolation and Culture of Murine Heart and Lung Endothelial Cells for In Vitro Model Systems

Yaw-Chyn Lim and Francis W. Lusinskas

### Summary

The inflammatory response is a critical component of host defense. An important goal of our group has been to understand the endothelial-dependent mechanisms that mediate leukocyte recruitment during an inflammatory response. In this chapter, we present a detailed method for the isolation and in vitro culture of murine vascular endothelial cells from the lung and heart. The endothelial cells are of high purity (85–99%) and retain certain of their functional differences, including constitutive and cytokine inducible adhesion molecule expression and chemokine production. Such endothelial cells, therefore, can be used for various in vitro models including leukocyte adhesion assay or in depth biochemical analyses.

**Key Words:** Inflammation; immunity; leukocytes; in vitro culture; cell heterogeneity.

### 1. Introduction

The endothelial monolayer lining all blood vessels plays an active role in the recruitment of blood leukocytes to tissues and organs in response to infection, injury, and immune responses. It is well documented that during an inflammatory response, the endothelium becomes “activated” by inflammatory cytokines or bacterial endotoxins and regulates the expression of adhesion molecules and the production of chemokines that mediate blood leukocyte adhesion and transmigration (1,2). Recent studies reveal that the endothelium is not homogenous because endothelial cells within various vascular beds and organs exhibit different cellular and biochemical properties that are specifically adapted to meet the requirements of their local environment (3–6).

Leukocyte recruitment is a critical component of the inflammatory and immune response systems. However, when recruitment persists or becomes dysregulated, it can lead to numerous pathological conditions, including allergic reactions,

reperfusion injury, organ rejection, and atherosclerosis and result in massive tissue and organ damage. Many studies have been devoted to the elucidation of the molecular and biochemical signals that mediate these interactions in attempt to formulate effective therapeutic interventions. Much of the knowledge on this complex phenomenon is generated from well-designed *in vitro* studies that use isolated human leukocytes and endothelial cells, such as human umbilical vein endothelial cells, in their model systems.

To study the mechanisms that initiate and propagate the immune and inflammatory responses, investigators increasingly have turned to gene knockout approaches to create mice deficient in one or more molecules implicated in these processes (7–9). However, the ability to set-up useful *in vitro* models using endothelium from these knockout mice remains limited because the methods to isolate and culture murine endothelial cells have met with varying success. This in part is caused by the amount of tissue available from a mouse as well as the difficulties isolating pure populations of endothelial cells. Nevertheless, the availability of a simple, reproducible, and well-tested method to isolate murine endothelial cells would be useful because it would enable us to culture cells from these genetically modified animals for use in various *in vitro* model systems (5,10–12).

In this chapter, we describe a detailed protocol for the isolation, culture, and characterization of murine lung and heart endothelial cells. This protocol has allowed us (and other groups) to generate endothelial cells from different mouse strains and backgrounds for *in vitro* models to study the role of adhesion molecules and chemokines in leukocyte–endothelial cell interactions under different biological conditions (5,13–15). A more detailed characterization of lung and heart endothelial cell phenotypes isolated using this protocol is available (5).

## 2. Materials

### 2.1. Endothelial Cell Isolation and Culture

1. Collagenase solution: Hank's Balanced Salt Solution (HBSS; Biowhittaker, cat. no. 10-543) containing 0.2 mg/mL (~180–200 U/mL) type 1 collagenase (Worthington Biochemical Corporation; cat. no. 4197; *see Note 1*).
2. Base medium: Dulbecco's Modified Eagle's Medium (Invitrogen Corporation, cat. no. 11965-092) containing 25 mM HEPES (Sigma; cat. no. H0887), 20% fetal calf serum, 100 U/100mg/mL penicillin and streptomycin, and 2 mM L-glutamine (Invitrogen Corporation, cat. nos. 600-5140 and 320-5030, respectively; *see Note 2*).
3. Complete culture medium: 80 mL of base medium supplemented with heparin (Sigma, cat. no. H-3933, 100 µg/mL final concentration), endothelial cell growth stimulant (Biomedical Technologies, cat. no. BT-203, 100 µg/mL final concentration), nonessential amino acid (Invitrogen Corporation; cat. no. 11140050), and

sodium pyruvate (Invitrogen Corporation, cat. no. 11360070) at 1X final concentration, respectively.

4. Trypsin–versene mixture (0.5% trypsin–0.02% EDTA) from Invitrogen (cat. no. 610-5300) or Biowhittaker (cat. no. 17-161).
5. Cell dissociation buffer from Sigma (cat. no. C5789).
6. Precoat a 75-mm<sup>2</sup> flask with sterile filtered 0.1% gelatin solution (Sigma; cat. no. G1393) as follows: pipet 4 mL of 0.1% gelatin solution to generously cover the bottom of the flask, leave for 1 min, and remove any excess fluid. Air-dry flask completely (4 h under tissue culture hood) before use. Solution can be reused for coating additional flasks.
7. Dynal M-450 sheep anti-rat immunoglobulin (Ig)G magnetic beads (Dynal, cat. no. 110.07; concentration  $4 \times 10^8$  beads/mL).
8. Purified rat anti-mouse CD31 (PECAM-1, clone MEC13.3, 0.5 mg/mL concentration, Pharmingen cat. no. 557355 or 553370) and rat-anti-mouse CD102 (intercellular adhesion molecule [ICAM]-2, clone 3C4, 0.5 mg/mL concentration, Pharmingen cat. no. 553326).
9. Dynal magnetic bead wash buffer (bead buffer): phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (Sigma, cat. no. A9647).

## 2.2. Endothelial Cell Characterization

### 2.2.1. Flow Cytometric Analysis (i.e., Fluorescence-Activated Cell Sorting)

1. Wash buffer/diluent–PBS containing 1% bovine serum albumin.
2. Anti-mouse CD31 conjugated to APC (Pharmingen, cat. no. 551262) and anti-mouse ICAM-2 (CD102) conjugated to fluorescein isothiocyanate (FITC; Pharmingen; cat. no. 557444). Use at 1:50 (v/v) in wash buffer/diluent.
3. Isotype-matched rat-IgG2a conjugated to FITC or APC (Caltag; cat. no. R2a01 and R2a05, respectively). Use at 1:50 (v/v) dilution.
4. Fixative: 1% formaldehyde (37% stock, Baker Chemical) diluted (v/v) in PBS, pH 7.4.

### 2.2.2. Fluorescent Immunoassay

1. Wash buffer/diluent–PBS containing 1% bovine serum albumin (*see Note 3*).
2. Rat antibodies against mouse ICAM-1 (clone YN1/1.7.4, ATCC no. CRL1878, ascites) and vascular cell adhesion molecule (VCAM)-1 (clone M/K2.7, ATCC no. CRL1909, culture supernatant) were used at 1:200 and 1:50 dilution, respectively. Purified mouse antibodies against mouse E-selectin (clone RME-1) and P-selectin (clone RMP-1) were kind gifts from Dr. Andrew Issekutz (Dalhousie University, Halifax, Canada) and were used at 10 µg/mL final concentration (*see Note 3*).
3. Negative control was rat IgG (Caltag Laboratories; cat. no. 10700; 10 µg/mL) as nonbinding MAb control. An irrelevant nonbinding mouse monoclonal antibody (clone K16/16, IgG1 nonbinding control; hybridoma culture supernatant [16]) was used undiluted.

4. FITC-conjugated anti-rat IgG (Caltag Laboratories, cat. no. M30101) or FITC-conjugated anti-mouse IgG (Caltag Laboratories, cat. no. M30101) was used at 1:80 dilution.

### **2.3. Mice**

Optimal results were obtained using three mice of 7 to 9 d of age. We used mice from either C57/BL6 or the 129 background (Taconics Farm, Germantown, NY). The animals were sacrificed by carbon dioxide asphyxiation as approved by the panel on euthanasia at the American Veterinary Association (*see Note 4*).

### **2.4. Instruments and Disposable Supplies for Organ Isolation and Tissue Dissociation**

All metal instruments were sterilized by autoclaving; sterile disposables were from B-D Falcon.

#### *2.4.1. Animal Dissection*

1. Two pairs of scissors and two pairs of forceps. Stand the instruments in 70% ethanol (to minimize contamination) when not in use.

#### *2.4.2. Tissue Dissociation*

1. Two pairs of scissors.
2. Two pairs of forceps.
3. 6-in. long 14-G metal cannula (Fisher Scientific, 6-in. popper needles; cat. no. 1482516N).
4. 70- $\mu$ m pore size cell strainer (B-D Falcon, cat. no. 352350).
5. 50-mL screw cap centrifuge tubes (B-D Falcon, cat. no.352098).
6. 100-mm diameter cell culture dishes (B-D Falcon, cat. no.353003).
7. 5-mL round-bottom tube with snap-cap (B-D Falcon, cat. no. 352058).

## **3. Methods**

### **3.1. Endothelial Cell Isolation and Culture**

#### *3.1.1. Precoating of Anti-Rat IgG Dynal Beads*

In accordance with the manufacturer's recommendations, our standardized protocol for the Dynal bead coating used 1.25  $\mu$ g of antibody per  $10^7$  beads. The bead concentration was maintained at  $4 \times 10^8$  beads per milliliter.

1. Resuspend an aliquot of the Dynal magnetic beads in 1 mL of PBS + 0.1% bovine serum albumin (bead wash buffer), mix well, and place on Magnetic separator (Dynal MPC-S).
2. Remove supernatant and repeat wash three times.
3. Resuspend beads in original volume with bead wash buffer.
4. Add 5  $\mu$ L (2.5  $\mu$ g) of antibody for every 50  $\mu$ L ( $2 \times 10^7$ ) of beads.

5. Incubate bead suspension on an end-over-end rotator overnight at 4°C or for 3 h at room temperature.
6. Repeat wash (**steps 1 and 2**) four times.
7. Resuspend immunobeads in the original volume with bead wash buffer (*see Note 5*). Store beads at 4°C and use within 1 to 2 wk.

### 3.1.2. Dissection

1. Sacrifice the mice and totally submerge the carcass into 70% ethanol, taking care that the skin and fur of the animal is soaked through.
2. Place two paper towels on a Styrofoam board and pin the mouse supine.
3. Pick up the skin above the pubis with a pair of forceps, make a small incision in the skin with a pair of scissors, and deglove the skin to above the chest.
4. Spray chest/abdomen with 70% ethanol and remove any loose fur from gloves and surgical field.
5. Cut open the anterior abdomen and take down the anterior diaphragm.
6. Grip the rib cage with the forceps and cut along the lateral walls of the chest.
7. Flip the “freed” rib cage back and pin away from the exposed thoracic cavity (*see Fig. 1A*).
8. Switch to a second set of dissection instruments.
9. Grasp the mediastinum and carefully cut the tissues and organs away from the vertebral column. Place the excised tissue (*see Fig. 1B*) into a 50-mL tube containing 30 mL of ice-cold base medium.
10. Repeat dissection procedure with the other two mice.

### 3.1.3. Tissue Dissociation

Transfer the tissues to a sterile hood for the remainder of the protocol.

1. Using fresh instruments, transfer the tissues to a sterile, disposable 100-mm diameter tissue culture dish.
2. Grasp the heart, cut it away from the mediastinal tissue and remove most of the left and right atria to reduce contamination of endothelial cells from large vessels (*see Fig. 1C*).
3. Make a central cut in the organ to open up the ventricles and put it into a fresh tube containing 30 mL of base medium.
4. Dissect out the lobes of the lung carefully; taking care to cut away any visible bronchi and mediastinal tissues (*see Fig. 1D*).
5. Cut the larger lobes of the lung into two and place them into another tube containing 30 mL of base medium.
6. Repeat with the mediastinum from the other two mice and pool the organs in their respective tubes. Label tubes to avoid cross contamination of organs.
7. Gently agitate the tubes (1 min) to wash out excess red blood cells from the organs.
8. Place the hearts on a new, dry 100-mm diameter dish and mince finely with scissors (*see Note 6*).

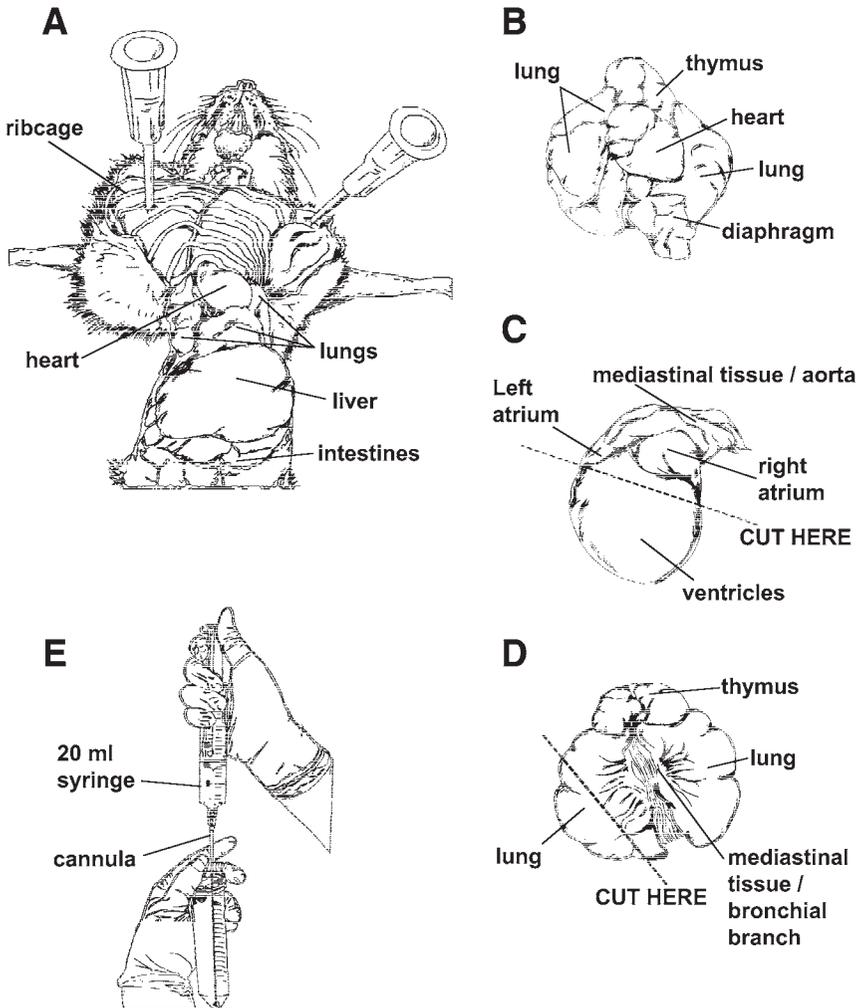


Fig. 1. Harvesting of organs and tissue dissociation. **(A)** The sacrificed mouse is pinned supine onto a styrofoam board and the rib cage is cut open and pinned away from the thoracic cavity; **(B)** the lungs, heart, and mediastinal tissues are carefully removed from the thoracic cavity and placed in a 50-mL tube containing cold base medium; **(C)** the ventricular heart tissue is carefully cut away from the atria and the large vessels; **(D)** the lung lobes are carefully dissected out to minimize contamination by the bronchial tree; and **(E)** the digested tissue is triturated multiple times to break up the cell clumps forming a single cell suspension.

9. Transfer the minced heart tissues to a 50-mL tube containing 15 mL of prewarmed collagenase solution. Wash all the tissue bits off the dish and into the tube of collagenase.
10. Repeat **steps 8 and 9** with the lung tissues.
11. Incubate at 37°C with gentle agitation for 45 min.
12. Attach a 20-mL syringe to a cannula; triturate the cellular suspension 12 to 20 times, taking care to avoid frothing (*see Fig. 1E*).
13. Filter the cell suspension through a 70-mm cell strainer into fresh 50-mL tube and rinse the sieve with 15 mL of base medium.
14. Use a new canula and sieve for each of the different tissues.
15. Centrifuge the cell suspension at 400g (~1300 rpm with a 15-cm diameter rotor) for 8 min at 4°C.
16. Discard supernatant and resuspend the heart endothelial cell preparation in 1 mL of cold base medium. Transfer to 5-mL tube.
17. Repeat **step 16** with the lung preparation using 2 mL of cold base medium.

#### 3.1.4. Cell Sorting: Primary Immunobead Isolation

From this point onward, both the heart and lung preparations are treated identically.

1. Add 15  $\mu$ L of anti-CD31 coated beads for every milliliter of cell suspension.
2. Incubate the tube on an end-over-end rotator for 10 min at room temperature.
3. Place the tubes on a magnetic separator and leave for 2 min. Cell-bound and unbound magnetic beads will be pulled to the magnet side of the tube.
4. Gently, without moving the tube, aspirate the supernatant using a 9-in. glass Pasteur pipet. The beads/cells pellet on the side of the tube should look somewhat foamy if there is a good yield of positively selected endothelial cells.
5. Remove the tube from the magnet and resuspend the pellet in 3 mL of base medium (*see Note 7*).
6. Replace the tube back on the magnetic separator for 2 min; aspirate the supernatant carefully.
7. Repeat washes (**steps 5 and 6**) four times (or more) until the supernatant appears clear.
8. After the last wash, resuspend beads/cell pellet in 3 mL of complete growth medium and transfer to a gelatin coated T75 culture flask. Add 7 mL of complete medium.
9. Culture in a standard CO<sub>2</sub> (5%) incubator.
10. After an overnight incubation, remove the non-adherent cells, wash the adherent cells twice with HBSS and add 10 mL of fresh complete medium.
11. Re-feed cultures every other day with 10 mL of fresh complete medium.

#### 3.1.5. Second Immunobead Isolation

The primary cultures are sorted a second time (second immunobead isolation) at 70 to 80% confluence (usually 7–9 d after plating), to improve endothelial cell purity. These cultures should contain discrete colonies of endothelial cells with the

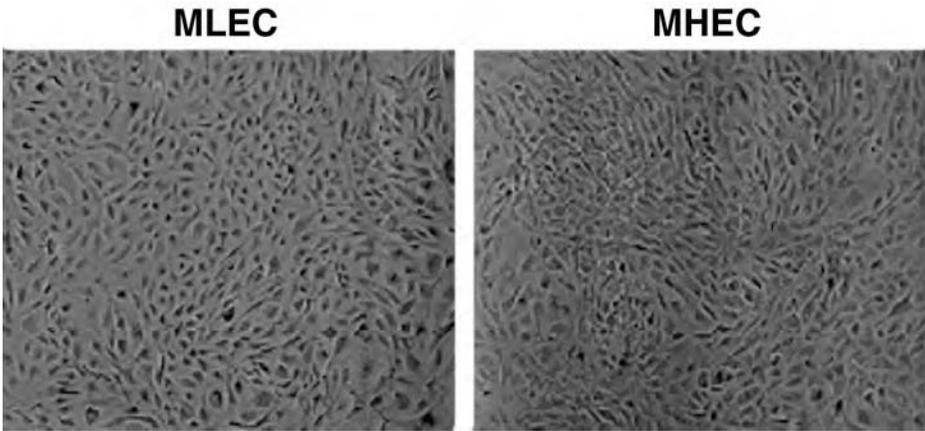


Fig. 2. Confluent mouse lung and heart endothelial cells in culture. Purified mouse endothelial cells were plated out in Dulbecco's Modified Eagle's Medium containing FCS, standard supplements, endothelial cell growth stimulant, heparin, sodium pyruvate, and nonessential amino acids on gelatin-coated 75-mm<sup>2</sup> flask. These cells grow as a monolayer in culture and exhibit the well-described cobblestone morphology.

classical “cobblestone” appearance when viewed by phase contrast microscopy (see [Fig. 2](#)). Routinely, we discard and do not second immunobead sorted cultures that contain less than 70% of “endothelial-like” cells as determined by cell morphology using phase contrast microscopy.

1. Remove culture supernatant and wash the endothelial monolayer twice with 7 mL of Ca/Mg-free HBSS.
2. Add 2mL of trypsin–EDTA, agitate gently, and incubate at 37°C for 3 min.
3. Examine the flask by phase contrast microscopy to confirm that endothelial cells have detached. Monitor this stage carefully to avoid over exposure to trypsin (see [Note 8](#)).
4. Neutralize the trypsin with 10 mL of base medium and transfer the detached cells to a 15-mL tube.
5. Centrifuge at 400g to pellet the cells.
6. Aspirate the supernatant; resuspend the cell pellet carefully in 2 mL of base medium and transfer to a round-bottom 5-mL tube.
7. Add 30  $\mu$ L of anti-CD102 coated magnetic beads (prepared per [Subheading 3.1.1.](#)) and incubate for 10 min at room temperature on an end-over-end rotator (see [Note 9](#)).
8. Place the tube with the cell suspension on a magnetic separator for 2 min to retain cells; remove the supernatant.
9. Resuspend the positively selected cell pellet in 3 mL of base medium and replace on the magnetic separator. Repeat this wash step four times ([steps 8 and 9](#)).
10. After the last wash, resuspend the cells in complete medium and plate onto a gelatin-coated T75 culture flask (see [Note 10](#)).

### 3.1.6. *In Vitro* Cell Propagation

1. The endothelial cell cultures are fed on alternate days with 10 mL of complete culture medium.
2. At confluence, the cultures should be detached with trypsin–EDTA and subcultured at a split ratio of no more than 1:3.
3. The cells should be used in experiments from passage 2 to 4 as follows: The heart endothelial cells (MHECs) retain phenotypic markers (sw>80% positive for VE-cadherin, Tie2, and PECAM-1) at passage 4. In contrast, mouse lung endothelial cells (MLEC) retain these markers only up to passage 3 (5). The purity of cultured MLECs and MHECs will vary from preparation to preparation (see **Subheading 3.3.**).

## 3.2. *Endothelial Lineage Characterization*

### 3.2.1. *Morphological Appearance*

Both murine lung and heart endothelial cells (MLECs and MHECs, respectively) isolated by double-selection method are essentially of mixed vascular bed origin (microvessels, arterial, venous). At confluence, these monolayers exhibit classical cobblestone morphology typical of vascular endothelium (**Fig. 2**). However, overconfluent cultures tend to progressively acquire a more spindly appearance as the cells pack more closely together.

### 3.2.2. *Detection of Endothelial Cell Markers by Two-Color Fluorescence-Activated Cell Sorting*

The lineage and purity of the cell cultures can be confirmed by immunostaining the cells (at subculture 1 or 2) for surface expression of two endothelial cell markers used for the cell isolation, namely platelet endothelial cell adhesion molecule (PECAM)-1 and ICAM-2 or, alternatively, VE-cadherin.

1. Plate isolated endothelial cells in gelatin-coated 60-mm diameter tissue culture plates.
2. Wash the confluent monolayer of endothelial cells twice with HBSS to remove any traces of serum.
3. Add 5 mL of prewarmed nonenzymatic cell dissociation buffer and incubate at 37°C for 15 to 20 min. Monitor the cell detachment progress under a phase contrast microscope. When the cells have all rounded up, gently triturate the media to detach cells and to create single, unicellular suspension (see **Note 11**).
4. Add 8 mL of base medium and transfer the cell suspension to a fresh 15-mL centrifuge tube.
5. Centrifuge at 400g to pellet cells.
6. Resuspend cell pellet to 3 to 5 × 10<sup>6</sup> cells/mL in wash buffer/diluent and aliquot 100 μL of cell suspension into each tube (see **Note 12**).

7. Add 2  $\mu\text{L}$  of anti-PECAM-1 APC and 2  $\mu\text{L}$  of anti-ICAM-2 FITC monoclonal antibodies to tube no. 1.
8. Add appropriate isotype controls to tube no. 2.
9. Incubate on ice for 30 to 45 min (minimum is 30 min).
10. Add 1 mL of wash buffer/diluent to each tube, vortex gently, and centrifuge at 400g.
11. Repeat wash step with PBS.
12. Suspend stained endothelial cells in 200 to 300  $\mu\text{L}$  of fixative (1% formaldehyde in PBS, pH 7.4).
13. Keep tubes containing stained cells tightly capped and covered with aluminum foil at 4°C until analysis.

### 3.2.3. Fluorescent Immunoassay of Constitutive and Inducible Adhesion Molecules

To characterize the ability of these cells to respond to proinflammatory cytokine stimulation, the expression of various constitutive and inducible adhesion molecules on unstimulated and tumor necrosis factor (TNF)- $\alpha$ -stimulated MLECs and MHECs were detected by fluorescent immunoassay. Confluent monolayers in 96-multiwell plates are stimulated with murine TNF- $\alpha$  (120 ng/mL in RPMI + 5% fetal calf serum (FCS) with L-glutamine and antibiotics, 100  $\mu\text{L}$ /well) for 6 or 24 h. Monolayers incubated with medium alone serve as negative (unstimulated) control.

1. Wash wells with 200  $\mu\text{L}$  of wash buffer/diluent twice (*see Note 13*).
2. Incubate triplicate wells with diluted primary antibody or with control rat IgG or murine nonbinding control K16/16 MAb (100  $\mu\text{L}$ /well) for 45 min on ice.
3. Wash five times with wash buffer/diluent.
4. Incubate wells with diluted FITC-conjugated secondary antibody (diluted 100  $\mu\text{L}$ /well) for 45 min on ice.
5. Wash five times with 200  $\mu\text{L}$  wash buffer/diluent.
6. Wash once with PBS.
7. Read fluorescence at 485 nm in a fluorescence microtiter plate.

### 3.3. Quantification and Interpretation: General Conclusions

1. The endothelial cell purity varies from preparation to preparation, but generally twice-sorted isolated MLEC and MHEC cultures that are between 85 and 99% PECAM-1 and ICAM-2 double positive at sc1 or sc2 as determined by fluorescence-activated cell sorting. These cells also are negative for VEGF-R3, thus suggesting that the cultures were minimally contaminated by lymphatic endothelial cells (5).
2. The mean fluorescence  $\pm$  standard deviation (SD) for each adhesion molecule expression is calculated as follows:

Step 1: Calculate the mean background fluorescence level from the mean (triplicate) negative control rat IgG, or K16/16 antibody wells.

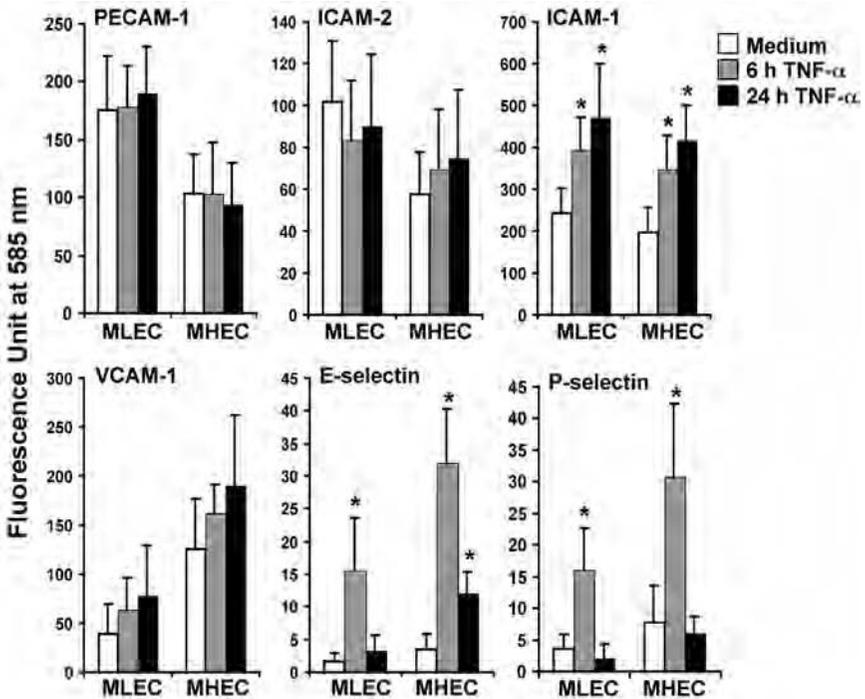


Fig. 3. Expression pattern of adhesion molecules on MLECs and MHECs. Constitutive expression of PECAM-1 and ICAM-2 is not influenced by TNF- $\alpha$  stimulation over the time period studied. In contrast, the expression of ICAM-1, E-selectin, and P-selectin is significantly elevated by continuous exposure to recombinant murine TNF- $\alpha$ . Surprisingly, expression of VCAM-1 on both MLEC and MHEC was not dramatically further increased by TNF- $\alpha$ . Data are mean  $\pm$  SEM from three experiments. (From ref. 5 with permission.)

Step 2: Deduct the mean background fluorescent level from each test well to obtain a corrected mean fluorescence intensity for each well.

Step 3: Determine the mean net fluorescence  $\pm$  SD for each set of triplicate wells.

3. Treatment with TNF- $\alpha$  should not significantly increase the expression PECAM-1 and ICAM-2 on either MLEC or MHEC at any time point (Fig. 3, top panels) because these adhesion molecules are constitutively expressed (endothelial lineage markers) and not induced by TNF- $\alpha$  stimulation.
4. Both MLECs and MHECs show constitutive ICAM-1 expression in culture, which is significantly upregulated by TNF- $\alpha$  after 6 and 24 h of continuous treatment (Fig. 3, top panel).
5. Resting MLECs and MHECs did not express P- or E-selectin, but both molecules were induced after 6 h of treatment with TNF- $\alpha$ . The increased levels of both selectins returned to baseline by 24 h (Fig. 3, bottom panel).

6. VCAM-1 was constitutively expressed at high levels in MHECs, whereas the levels were significantly lower in MLECs. Treatment of MHECs with murine TNF- $\alpha$  for 6 or 24 h did not induce a further increase. MLEC expression of VCAM-1 was increased after both 6 and 24 h of TNF- $\alpha$  treatment, but the increases did not reach statistical significance (**Fig. 3**; [5]).
7. Taken together, these data indicate that endothelial cells from different vascular beds do exhibit differences in their constitutive expression of VCAM-1. In addition, vascular endothelium isolated from lung and heart and propagated in vitro does retain certain of these properties and can be triggered by TNF- $\alpha$  to upregulate adhesion molecules that mediate leukocyte recruitment.

#### 4. Notes

1. Collagenase preparation. Dissolve collagenase in HBSS by incubation at 37°C for 1 h before sterile filtering with 0.22- $\mu$ m filter. Collagenase solution should be prepared fresh and excess solution used within the week.
2. The efficacy of FCS to support endothelial cell growth and proliferation varies between commercial vendors and also from lot to lot from the same vendor. Whenever possible, FCS should be batch tested for endothelial cell growth support before use and optimal FCS purchased in bulk.
3. Add 1% goat serum to the wash buffer/diluent if there is noticeable non-specific background staining. Anti-E-selectin and anti-P-selectin antibodies are also available from BD Pharmingen (clone 10E9.6, Pharmingen cat. no. 553749 and clone RB40.34, Pharmingen cat. no. 553742, respectively).
4. Although our previous studies (5) used mice from 6 to 7 wk of age, our new studies (Lamont and Luscinskas, unpublished observations) show endothelial cells isolated from pups 7 to 9 d of age routinely display better growth and morphology characteristics and longer resection (beyond passage 4) of phenotypic markers as assessed by FACS of VE cadherin.
5. 0.02% sodium azide may be added to the anti-CD31 (or anti-CD102)-coated beads as preservative but must be removed by wash before use in cell purification outlined here.
6. It is not necessary to add any medium to the tissue while mincing because there will be sufficient fluid carry-over from the wash to keep the tissue moist.
7. Avoid the natural tendency to be too gentle with the resuspension steps in **Subheadings 3.1.4.** and **3.1.5.** The cells/beads clumps must be properly “broken up” to get rid of any unbound cells (i.e., nonendothelial cells) caught within the clumps. However, overvigorous pipetting may dislodge beads from the cells!
8. Overexposure to trypsin will affect the viability of cells. If so desired, the detachment of rounded cells can be sped up with a sharp tap on the side of the flask with an open palm.
9. Murine PECAM-1 (CD31) is trypsin sensitive and anti-CD31 beads should not be used for the second-sort.
10. After the second-sort, plate the cells on a single T75 flask. It is important to maintain high cell density plating to avoid large numbers of senescent cells.

11. Alternatively, the monolayers can be detached by brief trypsin-versene EDTA treatment of no longer than 2 min at 37°C.
12. A confluent monolayer of endothelial cells from a T75 flask contains  $1\text{--}3 \times 10^6$  cells. For fluorescence-activated cell sorting analysis, the cells are used at  $0.3$  to  $0.5 \times 10^6$  cells/100  $\mu\text{L}$  reaction volume.
13. Check five random wells under the phase contrast microscope to confirm monolayers remained intact during the wash steps. We used unfixed monolayers in this protocol because the anti-E-selectin (RME-1) and anti-P-selectin (RMP-1) antibodies are not suitable for use on fixed cells.

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## Expression Cloning of Signaling Proteins Regulated by Cell Adhesion

Michelle L. Matter and Joe W. Ramos

### Summary

Many proteins involved in cell–cell and cell–matrix adhesion are regulated by signal transduction pathways and can activate signal transduction on ligation. Adhesion-related signal transduction is important throughout development, hemostasis, immunity, and in diseases such as cancer. Therefore, the identification of the various signaling pathways that are involved is crucial. Expression cloning is an unbiased way to isolate proteins with specific biological functions. This methodology has been adapted for the identification of proteins involved in cell signaling pathways that are mediated by cell–cell and cell–matrix interactions. We have successfully developed and used a novel expression cloning strategy to isolate the integrin-regulated apoptosis signaling protein BIT-1. This screen was based on previous observations that integrin-mediated adhesion upregulates the anti-apoptotic protein bcl-2. Our strategy described in this chapter uses flow cytometry and a reporter construct in which the bcl-2 promoter is linked to enhanced green fluorescence protein. The advantage of using flow cytometry in expression cloning is that it increases the sensitivity of the screen by enabling us to examine function quantitatively at the level of a single cell millions of times in one experiment. The following protocol provides a detailed method for the isolation of proteins that are regulated by cell adhesion.

**Key Words:** Expression cloning; integrin; cell–cell adhesion; cell–matrix adhesion; flow cytometry; EGFP; bcl-2; Bit-1.

### 1. Introduction

Cell surface proteins that mediate cell–cell and cell–matrix adhesion such as cadherins and integrins are regulated by signal transduction pathways and can themselves activate signal transduction in response to adhesion (1–3). One approach that has been used to successfully identify some of the proteins that mediate these signaling events is expression cloning. Expression cloning allows for the isolation of complementary DNAs (cDNAs) that encode proteins with a

specific biological activity. The investigator designs the expression cloning strategy around a robust assay for a function of interest. This can be, for example, a change in adhesion, transcription, protein expression, or cell survival.

A variety of expression cloning strategies have been developed to identify proteins with a particular function. Seed et al. (4,5) developed novel expression cloning methods to clone CD antigens using antibodies to the extracellular face of the antigens. Adams et al. (6) used an expression cloning strategy that relied on a cell adhesion assay to identify the intracellular protein, muskelin, which is involved in the cellular response to thrombospondin-1 adhesion. In collaboration with others, we have used expression cloning screens to clone proteins involved in integrin-related signaling (7–9). Integrins are cell surface receptors that mediate cell–cell and cell–matrix adhesion (1). Expression cloning screens were used to isolate the integrin regulatory proteins CD98 (7) and PEA-15, a protein that also suppresses the Ras mitogen-activated protein kinase pathway (8). Each of these screens relied on measuring integrin activation by flow cytometry with an antibody that binds only active integrin (9).

Recently, in collaboration with Erkki Ruoslahti, Minoru Fukuda, and others, Matter et al. (10) cloned the integrin-regulated signaling protein BIT-1 using expression cloning. This method is the one we describe in this chapter. Integrin-mediated cell adhesion is necessary for the survival of many cell types, including neuronal, endothelial, and epithelial cells (11,12). Loss of cell attachment to the extracellular matrix causes apoptosis in these cells. Integrins suppress apoptosis in attached cells by activating signaling pathways that promote survival and inactivating the ones that promote apoptosis. A number of these pathways have been described and are of varying importance in different types of cells (11). It has been proposed that perturbation of the integrin-activated cell survival pathways is a contributing factor to oncogenesis (11). We have previously shown that matrix-bound integrins promote cell survival in part by activating focal adhesion kinase, which activates phosphatidylinositol 3-kinase, leading to the upregulation of bcl-2 transcription and translation (13). To further examine the mechanisms by which integrins promote cell survival, we used the unbiased expression cloning approach described here.

## 2. Materials

### 2.1. Mammalian Host Cells

1. A COS or CHO cell line. We used CHO-B2Δ cells (these cells contain a shortened  $\alpha 5$  cytoplasmic tail; these cells may be requested from E. Ruoslahti (ruoslahti@burnham.org)).
2. Complete Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Carlsbad, CA) for maintaining cell conditions. To prepare: 10% fetal bovine serum (HyClone, Ogden, UT), 1% nonessential amino acids, 1% glutamine, and 1%

penicillin–streptomycin. Prepare in DMEM and sterilize through a 0.2-micron filter. Store at 4°C (see **Note 1**).

3. L-glutamine (cat. no. G9273, Sigma, St. Louis, MO).
4. Nonessential amino acids (Sigma, cat. no. M7145).
5. Penicillin–streptomycin (Invitrogen).

## 2.2. cDNAs, cDNA Library, and cDNA Preparation

1. 3 M sodium acetate, pH 7.0 (Sigma, cat. no. 52404).
2. Ampicillin (AMP; 25 µg/mL; Sigma).
3. Tetracycline (Tet; 10 µg/mL; Sigma).
4. A cDNA library that has been directionally cloned into a mammalian expression vector (see **Note 2**).
5. Falcon no. 2059 tubes.
6. Glycerol (Sigma).
7. MC1061/P3 cells at a competency of 10<sup>9</sup> or more colony-forming units per µg (cfu/µg) DNA (Invitrogen, San Diego, CA; see **Note 3**).
8. DNA maxi and mini-prep kits (Qiagen, Valencia, CA).
9. Promoterless enhanced green fluorescence protein (EGFP-1; BD Biosciences Clontech, Palo Alto, CA, cat. no. 6086-1).
10. Phenol:chloroform:isoamyl alcohol (25:24:1; Sigma, cat. no. P3803).
11. Lysogeny Broth (LB) Medium (Invitrogen).
12. SOC Medium (Invitrogen).
13. Nitrocellulose membranes (15 cm, BA-85, Schleicher & Schuell, Keene, NH).
14. Polyoma large T-antigen, if not present in cell line (see **Note 2**).
15. 100-mm tissue culture dishes (Falcon).
16. 15-cm plates (Falcon).

## 2.3. Cell Transfection and Cell Isolation

1. LipofectAMINE (Invitrogen) or other appropriate and efficient transfection reagent.
2. Polystyrene tubes (Falcon, cat. no. 2054).
3. 1X PBS (10X solution): 87.7 g of NaCl, 7.1 g of Na<sub>2</sub>HPO<sub>4</sub> (anhydrous), and 6.0 g of NaH<sub>2</sub>PO<sub>4</sub> (anhydrous). Dissolve in 1 liter of double-distilled (dd)H<sub>2</sub>O, pH 7.2. Dilute 1:10 in ddH<sub>2</sub>O for a 1X solution.
4. Hirt's solution: 0.6% sodium dodecyl sulfate, 10 mM EDTA in 5 mL of ddH<sub>2</sub>O.
5. 5 M NaCl (Sigma).
6. Buffer-saturated phenol (Invitrogen).
7. High-performance liquid chromatography-grade H<sub>2</sub>O.

## 2.4. Fluorescence-Activated Cell Sorting

1. Cell dissociation solution (enzyme-free Hank's based Cat S-004-H, Specialty Media Division of Cell and Molecular Technologies Inc., Phillipsburg, NJ).
2. EDTA (10 mM; Sigma).
3. 0.2 mg/mL; Propidium iodide (Sigma).
4. 5% fetal calf serum (Invitrogen).

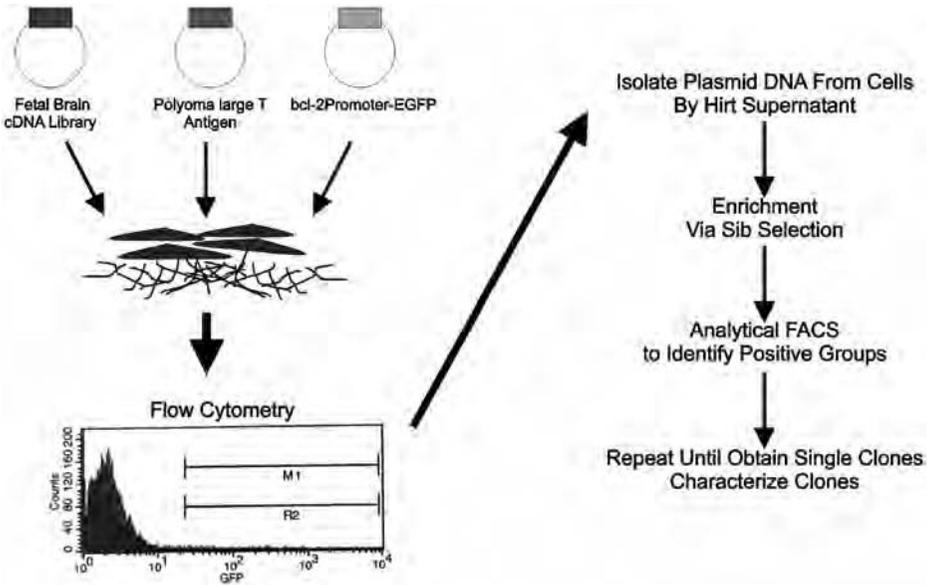


Fig. 1. Expression cloning of transcriptional regulators of bcl-2. CHOΔ cells attached to fibronectin were transfected with a fetal brain cDNA library, the polyoma large T-antigen and the bcl-2 promoter EGFP marker. Cells expressing EGFP were collected by a sterile fluorescence-activated cell sorter and plasmid DNA was isolated by Hirt supernatant. Sibling selection using analytical fluorescence-activated cell sorting analysis to identify positive groups of cells was repeated five times until a single clone that upregulated the bcl-promoter EGFP marker was isolated.

### 2.5. Equipment

1. Fluorescence-activated cell sorter (FACS; FACSTAR, Becton Dickinson, Franklin Lakes, NJ).
2. Flow cytometer (FACScalibur, Becton Dickinson).
3. 42°C water bath.
4. Electroporator.

### 3. Methods

The following strategy describes an expression cloning screen to identify proteins involved in signaling mediated by cell–matrix adhesion. This method is briefly described in [Fig. 1](#). This method also may be adapted to identify proteins in other signaling pathways including cell–cell adhesion. Expression cloning has numerous advantages for the identification of adhesion proteins and proteins that regulate their signaling. It provides an unbiased approach to clone proteins that are involved in adhesion or that may affect adhesion related signal

transduction. Every protein expressed in the cDNA library is tested in the screen and the assay determines the functional relevance of the clones. Moreover, proteins can be isolated that have a functional relationship to adhesion but do not interact directly with specific adhesion proteins. The main disadvantages of expression cloning are that functional alterations that require more than one protein may not be isolated and functions resulting from posttranslational modifications can not be identified.

The expression cloning strategy we describe was successful in isolating the integrin-regulated signaling protein BIT-1 (10). We previously reported that in serum-free conditions cell attachment through the  $\alpha 5 \beta 1$  integrin promotes upregulation of bcl-2 at both protein and transcriptional levels by activating Shc, which activates focal adhesion kinase, which activates phosphatidylinositol 3-kinase (13). Our screening strategy relied on inactivating this integrin-mediated signaling pathway and isolating clones that increased bcl-2 levels in these cells. Because expression cloning relies on the expression of a library of cDNAs, it is essential that it be done in cells that are highly transfectable and that express exogenous protein well. We used a CHO cell line with an  $\alpha 5$  cytoplasmic deletion that inhibits upregulation of bcl-2. These cells were transfected with a fetal brain-derived cDNA library. The cells were also transfected with a plasmid that expressed EGFP (a green fluorescent protein) under the control of the bcl-2 promoter. Thus cells in which bcl-2 transcription was upregulated would fluoresce at 511 nm. This fluorescence was monitored in a flow cytometer, and cells in which bcl-2 transcription was upregulated were isolated (as detected by EGFP expression). This system allows for rapid and quantitative measurement of millions of cells in a single experiment and thus increases the sensitivity of the screen.

The remainder of our expression cloning method involves sibling selection as described herein and was adapted from a method that was used to isolate a novel sulfotransferase using antibody binding and flow cytometry (14). It is also important to note that the cDNAs we isolated were not necessarily specific for integrin-activated signaling. Anything that could generally upregulate bcl-2 might also be cloned. Further experiments are usually required to verify the relevance of a newly identified cDNA. In this way, BIT-1 was identified as a novel regulator of apoptosis by integrins.

### 3.1. Cell Transfection

It is important to have adequate controls in addition to having a sufficient number of experimental samples for sorting the cells and recovery of plasmid cDNA. Controls should include (1) control plate 1 containing co-transfected transfection marker plasmid (promoter bcl-2 EGFP), empty vector plasmid, and the polyoma large T-antigen; and (2) control plate 2 containing co-transfected EGFP

under its own promoter, the empty vector plasmid (pcDNA3.1; *see Note 2*), and polyoma large T-antigen (this control allows assessment of overall transfection efficiency). These two controls allow the baseline fluorescence of the cells to be measured (control plate 1) and the overall efficiency of the transfection (control plate 2). In addition, they are helpful when setting the collection gate on the cell sorter.

1. Twenty-four hours before transfection, plate cells to be transfected at  $2 \times 10^6$  cells per plate in 100-mm tissue culture dishes (at least 20 plates total; *see Note 4*).
2. Transfect cells with 20  $\mu\text{L}$  of LipofecAMINE in 100  $\mu\text{L}$  of serum-free DMEM, according to the manufacturer's direction along with the following plasmids or DNAs:  
Control plate 1: 4  $\mu\text{g}$  of bcl-2 promoter EGFP, 6  $\mu\text{g}$  of vector DNA plasmid (pcDNA3.1; *see Note 2*), and 4  $\mu\text{g}$  of polyoma large T-antigen.  
Control plate 2: 4  $\mu\text{g}$  of EGFP-C1 (this has its own promoter; Clontech), 6  $\mu\text{g}$  of vector DNA plasmid (pcDNA3.1), and 4  $\mu\text{g}$  of polyoma large T-antigen.  
Sort plates: 4  $\mu\text{g}$  of bcl-2 promoter EGFP, 6  $\mu\text{g}$  of cDNA library (fetal brain cDNA library) and 4  $\mu\text{g}$  of polyoma large T-antigen.
3. Incubate for 24 hours at 37°C, in 5% CO<sub>2</sub> and 95% humidity.
4. Wash all plates with 2 mL of serum-free DMEM media.
5. Add 10 mL of serum-free DMEM and incubate cells for an additional 24 h at 37°C in 5% CO<sub>2</sub> and 95% humidity.

### 3.2. Fluorescence-Activated Cell Sorting

1. After 24-h incubation time, aspirate media from the dishes.
2. Rinse with 5 mL of 1X PBS. Remove supernatant.
3. Add 10 mL of Cell Dissociation Solution + 10 mM EDTA to the sort plates and control plates.
4. Incubate for 5 min at room temperature.
5. Collect cells from sort plates and combine into one 50-mL tube (labeled sort cells).
6. Centrifuge the sort cells and the control cells at 120g in a tabletop centrifuge at room temperature to pellet the cells.
7. Aspirate supernatant.
8. Resuspend the sort cells in 1.4 mL of DMEM media by gentle agitation of each tube.
9. Divide the sort cells into six tubes (Falcon, cat. no. 2054) of 225  $\mu\text{L}$  each.
10. Resuspend the control cells separately, each in 200  $\mu\text{L}$  of DMEM.
11. Add propidium iodide (0.1  $\mu\text{g}$  to 0.5  $\mu\text{g}$  per  $1 \times 10^5$  cells) to the samples. Incubate all tubes on ice in the dark for 5 min (*see Note 5*).
12. Wash the cells with 2 mL of cold 1X PBS.
13. Centrifuge the cells for 5 min at 120g at room temperature.
14. Resuspend and combine sort cells in a total of 4 mL of 1X PBS.

15. Resuspend each tube of control cells in 500  $\mu\text{L}$  of 1X PBS.
16. Set the parameters for FACS such that highly transfected control cells from control plate 2 fall within the gate, whereas similarly transfected control cells from control plate 1 do not.
17. Collect the EGFP positive sort cells by FACS, into 1 mL of complete DMEM media (*see Note 6*).

### 3.3. cDNA Recovery From Hirt Supernatant

1. Add an equal volume of 2X lysis buffer (1.2% sodium dodecyl sulfate/20 mM EDTA, pH 7.4) to the tube containing 1 mL of sorted cells.
2. Mix by inversion and incubate for 15 min at room temperature.
3. Add 500  $\mu\text{L}$  of 5 M NaCl, mix and incubate overnight at 4°C.
4. Transfer sorted cells into two sterile microcentrifuge tubes, centrifuge for 20 min at 18,000g at 4°C.
5. Carefully transfer and combine supernatant into a 15-mL Falcon polypropylene tube.
6. Phenol:chloroform extract each supernatant twice (*see Note 7*).
7. Transfer aqueous phase equally into four microtubes.
8. Transfer content of each tube into a Microcon Unit (Microcon 30; Amicon) that is on a filtrate collection tube.
9. Close cap and centrifuge at 18,000g for 10 min (or until there is 10 to 20  $\mu\text{L}$  of retentate remaining).
10. Add 400  $\mu\text{L}$  of high-performance liquid chromatography-quality  $\text{H}_2\text{O}$  to the Microcon unit and spin at 18,000g for 10 min. Repeat this step three times to ensure salt removal because salt can negatively affect the subsequent electroporation step.
11. Remove Microcon unit and invert into a new collection tube (*see Note 8*).
12. Add 8  $\mu\text{L}$  of sterile DI  $\text{H}_2\text{O}$  directly into the six small holes of the Microcon unit and pulse spin at high speed for 5 s (*see Note 9*).
13. Remove unit from each collection tube and discard Microcon unit. Keep plasmid samples on ice.

### 3.4. Electroporation

1. Prepare LB AMP/Tet plates (AMP at 25  $\mu\text{g}/\text{mL}$ ; Tet at 10  $\mu\text{g}/\text{mL}$ ).
2. Take 1  $\mu\text{L}$  of plasmid cDNA and electroporate into MC1061/P3 cells (efficiency is usually from  $1 \times 10^7$  to  $1 \times 10^8$  colony-forming units/ $\mu\text{g}$  DNA).
3. Titer to see how many colonies per 1  $\mu\text{L}$ . Rescue cells in 500  $\mu\text{L}$  of SOC media and grow for 1 h at 37°C.
4. Plate 10, 100, and 250  $\mu\text{L}$  onto 15-cm LB AMP/Tet plates.
5. Place remaining samples at  $-20^\circ\text{C}$  until required.

### 3.5. Sib-Selection of Clones and Detection of Positive Clones

1. Calculate the amount of plasmid DNA required for electroporation into bacterial cells to obtain 500 to 1000 colonies per 15-cm LB AMP/Tet plate. Enough plasmid samples should be electroporated to obtain 8000 colonies, which require using between 10 to 16, 15-cm LB AMP/Tet plates (*see Note 10*).

2. Label plates. Incubate overnight (<16 h) at 37°C.
3. Prepare 16 nitrocellulose membranes and carefully layer over the agar. Incubate upside down for 5 h at 37°C.
4. Carefully remove membrane from each plate. Place each membrane (colony side up) into a labeled 15-cm Petri dish.
5. Prepare 170 mL of LB AMP/Tet (50 µg AMP and 10 µg Tet/mL). Transfer 10 mL of LB AMP/Tet to each plate containing a membrane.
6. Using a sterile cell scraper, scrape off the colonies from each membrane. Transfer culture into a labeled sterile 50-mL centrifuge tube. Secure lid with a piece of tape (do not tightly close lid) and incubate overnight at 37°C.
7. Make a permanent stock of each of the cultures by removing 900 µL of culture into a sterile microfuge tube containing 100 µL of dimethyl sulfoxide. Store bacteria at -80°C.
8. Isolate and purify the plasmids from the remaining 9 mL using a Qiagen mini plasmid kit (*see Note 11*).
9. Dissolve each plasmid pool into 100 µL of 1X Tris-EDTA buffer, pH 7.5.
10. Determine the concentration of plasmid by absorbance at A260 nm and store plasmid preps at -20°C.

### **3.6. Sib-Selection of Clones and Detection of Positive Clones by FAC Analysis**

At this step, the goal is to identify the pools of cDNAs that induce the signal that is being measured. The example described here is the ability of the protein from one of the cDNAs to activate *bcl-2* promoter signaling and thereby turn on EGFP. These isolated cDNAs will substitute for the library in the initial screening process. The cells will have to be collected and subjected to FACS analysis following the same procedure as that described for the library screening method above (*see Note 12*).

1. Co-transfect 8 µg of each pool of DNA into the cells along with 6 µg of activator (*bcl-2* promoter-EGFP) and 6 µg of polyoma large T-antigen. In addition, transfect the two control plates as described previously.
2. Isolate the cells as described previously.
3. Perform analytical FACS to identify the cDNA pools that produce the desired change in the activity of the pathway (here it is the upregulation of *bcl-2* transcription that is visualized by EGFP expression).
4. From positive FACS analysis of EGFP expression prepare DNA by Qiagen miniprep according to standard protocol.
5. Repeat sib-selection on the plasmid pool that showed positive results in the FACS analysis by overlaying a labeled nitrocellulose membrane onto the master plate for that plasmid group. Mark the membrane into eight equal quadrants with a pencil. These marked quadrants should then be traced onto the agar-side (bottom) of the 15-cm LB master plate using a black felt tip pen and labeled 1 to 8 accordingly.

(see **Note 13**). Incubate the master plates with the marked nitrocellulose membranes for 5 h at 37°C.

6. Carefully peel nitrocellulose membrane off the master plates and cut into the eight marked quadrants.
7. Scrape the colonies growing on each quadrant into 10 mL of LB-AMP/Tet using a sterile cell scraper.
8. The plasmids from each quadrant of each master plate are then isolated and purified using a Qiagen mini plasmid kit.
9. Cell transfection is repeated until another sub-pool of positive clones is isolated by analytical FACS.
10. Repeat this procedure until one positive clone is isolated.
11. The plasmid DNA from this clone is then isolated, purified and sequenced (see **Note 14**).

#### 4. Notes

1. During expression cloning experiments using our system, no fetal bovine serum is added to the DMEM.
2. Although there are several options in preparing a cDNA library, we chose to purchase a premade cDNA library from a commercial source. We used a fetal brain cDNA library (Clontech) cloned into pcDNA1, which is no longer available commercially. A replacement vector such as pcDNA3.1 may be used; however, it lacks the polyoma origin of replication and instead contains an SV40 origin. Therefore, the cell line will need to express the SV40 T-antigen to obtain high plasmid amplification. If a murine host cell line is used, then the cDNA encoding the polyoma large T-antigen should be co-transfected. This will promote increased amplification of the desired plasmid (the plasmid should harbor the polyoma origin of replication).
3. The pcDNA1 vector contains the supF suppressor tRNA, which allows the MC106/P3 cells containing pcDNA1 to be resistant to both AMP and Tet. MC1061/P3 cells containing the polyoma large T-antigen (pPSVE1-PyE) are resistant to AMP but not to Tet. This fact allows for the isolation of plasmids derived from pcDNA1 to be obtained and amplified.
4. We transfect 16 sort plates per screen and two control plates.
5. Propidium iodide stains the dead cells and allows them to be removed from the sort gate.
6. We sorted more than  $6 \times 10^6$  cells and collected at least 70,000 cells. Make a note of the number of cells sorted and collected for comparison between multiple screens.
7. Make sure that you no longer see the interface. If it is still apparent, then do one more extraction.
8. It is important to use new tubes at this step.
9. It is important to place the H<sub>2</sub>O directly into the small holes using a P10 pipet.
10. To obtain 8000 colonies, 6  $\mu$ L of plasmid DNA is electroporated into 6X 20- $\mu$ L cells. These plates must be dried for 15 min at 42°C to have a distribution of colonies over the entire plate surface.

11. For a higher yield, use 2X the volume of P1, P2, and P3 solutions when using a pcDNA1-derived cDNA library.
12. Changes in the activity of the signaling pathway may be very low during the initial rounds of screening, but the signal should increase as the individual cDNA clone is isolated.
13. Careful concise labeling is imperative for each of the 16 master plates and subsequent overlays with nitrocellulose.
14. Isolation of a single active clone usually requires three to five rounds of sib-selection. Each subsequent round should demonstrate an enrichment of positive cells and an increase in the FACS signal. We performed five rounds of sib-selection to obtain Bit-1.

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## Assays for the Calcium Sensitivity of Desmosomes

Anita J. Merritt, Anthea Scothern, and Tanusree Bhattacharyya

### Summary

Epithelial cells *in vivo* exist as confluent cell sheets, but this confluence is disrupted if the sheets are wounded, if the cells are undergoing morphogenesis, or if they are taking part in invasion and metastasis. Desmosomes are one of the principal types of adhesive junctions in epithelia and are responsible for maintaining tissue integrity. It is likely that modulation of desmosomal adhesion is required to facilitate cell motility in response to alterations in the tissue architecture. Desmosomal adhesion changes from a calcium-dependent state to a calcium-independent state when cells become confluent. Our laboratory has shown that the  $\alpha$  isoform of protein kinase C is involved in signaling the response of desmosomes to calcium concentration and wounding, in cultured epithelial cells and in mouse epidermis (*in vivo*).

**Key Words:** Desmosomes; calcium dependence; calcium independence; protein kinase C $\alpha$  (PKC $\alpha$ ); desmoplakin; cell adhesion; MDCK cells.

### 1. Introduction

Desmosomes are intercellular adhesive junctions that provide tensile strength to epithelial tissues and cardiac muscle, and are particularly abundant in the epidermis. Their major adhesive molecules are the desmocollins and desmogleins, members of the calcium (Ca)-dependent cadherin family (**1**). The Ca switch assay has been used extensively for the study of desmosome assembly (**2–5**). Cells grown in low Ca medium ( $\leq 0.1$  mM) begin to assemble desmosomes when the Ca concentration is raised to physiological levels ( $\sim 1.8$  mM). Desmosomes can exist in two adhesive states; Ca-dependent and Ca-independent, and the transition between the two is regulated by protein kinase C (PKC) $\alpha$  (**6**). Cells in culture become Ca-independent after they reach confluence, and Ca-independence is the natural state of desmosomes *in vivo*. When confluent cell monolayers are wounded or treated with activators of

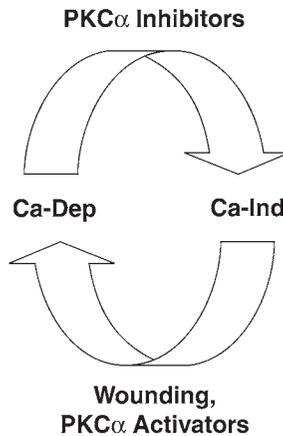


Fig. 1. Diagram to illustrate factors that modulate desmosomes between Ca-dependence (Ca-Dep) and Ca-independence (Ca-Ind).

PKC, cells rapidly revert to Ca-dependence (**Fig. 1**). In this chapter, we illustrate, using the Madin–Darby canine kidney (MDCK) model epithelial cell line, how desmosomal adhesion can be switched between Ca-dependence and Ca-independence. This chapter includes protocols for wounding and treatment with PKC activators/inhibitors. We also will describe how immunofluorescence and electron microscopy can be used to determine the adhesive state of desmosomes and will demonstrate how the assays can be extended to study desmosomal adhesion in mouse epidermis (*in vivo*).

## 2. Materials

### 2.1. Cell Culture

1. Tissue culture hood and incubators at 37°C, 5% CO<sub>2</sub>.
2. T75 tissue culture flasks, 24-well plates, 0.2- $\mu$ m filters, haemocytometer.
3. MDCK II cells ([www.ecacc.org.uk](http://www.ecacc.org.uk)).
4. Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS (Normal Calcium Medium [NCM], Gibco).
5. Ca-free DMEM (Gibco).
6. Phosphate-buffered saline (PBS) or Hank's Balanced Salt Solution (HBSS) without Ca or magnesium (Gibco).
7. Trypsin–EDTA (Gibco).
8. EGTA (Sigma, St. Louis, MO).
9. Fetal bovine serum (FBS; Gibco).
10. Chelex resin (Sigma).
11. Whatman filter paper.
12. Sterile 13-mm circular glass cover slips (Scientific Laboratory Supplies, Nottingham, UK).

13. 100% methanol.
14. Sterile scalpel blades (size 24, Swann-Morton) and cell scrapers (Costar).
15. 50:50 methanol: acetone (store at  $-20^{\circ}\text{C}$ ).
16. Dimethyl sulfoxide (Sigma).
17. Phorbol-12,13-didecanoate, phorbol-12,13-dibutyrate (TPA; Sigma).
18. Gö6976 (Calbiochem).
19. Chelerythrine chloride (Calbiochem).
20. 100-cm<sup>2</sup> plastic dishes (Sterilin).

## 2.2. Immunofluorescence

1. Antibody to desmosomal protein such as 11-5F mouse monoclonal Ab to desmoplakin I and II (7).
2. Rabbit anti-PKC $\alpha$  polyclonal antibody (Sigma).
3. Fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin (Ig)G, and rhodamine red-conjugated anti-mouse IgG (Jackson Labs).
4. PBS tablets (Sigma).
5. 0.5% Triton X-100 (Sigma).
6. Wax pen (DAKO).
7. Gelvatol mounting medium: Gradually add 10 g of gelvatol vinyl alcohol (Fisons) to 20 mL of 0.02 M Na<sub>2</sub>HPO<sub>4</sub>, 0.02 M KH<sub>2</sub>PO<sub>4</sub>, and 0.14 M NaCl at pH 7.2 with 0.04% w/v sodium azide and 1% v/v DABCO. Stir the solution overnight and adjust pH to 6.7. Remove insoluble material by centrifugation at 12,000g for 30 min. Store at 4°C.
8. Microscope slides and 22 × 50-mm glass cover slips (Scientific Laboratory Supplies, Nottingham, UK).
9. Parafilm.
10. Clear nail polish.
11. Poly-L-lysine-coated slides (BDH).
12. Humidified box (*see Note 1*).
13. Fluorescence microscope with FITC and rhodamine red filters such as Zeiss Axiophot or Axioplan.

## 2.3. Wounding of Mouse Epidermis

1. Hair clippers.
2. Scalpel with number 11 blades (Swann-Morton).
3. Marker pen or surgical pen.
4. 70% ethanol.
5. Anaesthesia apparatus.
6. Dissection instruments.
7. Ruler.
8. Aluminium foil.
9. OCT cryoprotectant (RA Lamb Ltd, Eastbourne, UK).
10. Liquid nitrogen.

## 2.4. Conventional Electron Microscopy

1. Ultramicrotome.
2. Platinum wire loops (0.5–3 mm).
3. Molds for embedding.
4. Razor blade.
5. Tungsten-coated glass knives.
6. 100 mesh hexagonal grids of copper.
7. Cacodylate buffer: 1 *M* sodium cacodylate, 250 *mM* sucrose, and 2 *mM*  $\text{CaCl}_2$ , pH 7.5.
8. Paraformaldehyde (PFA) solution: add 8 g of PFA powder to 100 mL of double distilled (dd) $\text{H}_2\text{O}$  in a glass flask. Cover with a glass funnel and an inverted glass Petri dish to form a reflux system. Heat with stirring to 60 to 70°C for at least 30 min. Clear the remaining cloudiness with a few drops of 4 *M* sodium hydroxide solution. Allow to cool and then filter through Whatman paper. Dilute to 4% w/v with dd $\text{H}_2\text{O}$ .
9. PFA in cacodylate buffer: prepare double-strength cacodylate buffer above (i.e., 0.2 *M* sodium cacodylate) and mix 50:50 with fresh 4% aqueous PFA solution. Re-adjust the pH to 7.5.
10. Uranyl acetate stain: prepare a 2% w/v solution of uranyl acetate dissolved in 77% ethanol. Centrifuge 1.5-mL aliquots at 1000*g* for 20 min before use.
11. Lead citrate stain: prepare a 0.3% w/v solution of lead citrate in 0.1 *M* sodium hydroxide. Centrifuge 1.5-mL aliquots at 1000*g* for 20 min before use.
12. Spurr's resin (medium hardness).
13. Ethanol concentrations 70, 80, and 100%.
14. Toluidine blue stain.

## 2.5. Immunogold Labeling

1. Cryoultramicrotome.
2. Tungsten-coated glass knives.
3. Diamond knife.
4. Eyelash probe (*see Note 2*).
5. Platinum loops.
6. 100 mesh nickel hexagonal grids coated with formvar and carbon (Agar Scientific Ltd).
7. Liquid nitrogen storage.
8. Aluminium cryopins (Reichert-Jung Ltd).
9. Light microscope.
10. HEPES buffer: 0.2 *M* HEPES, pH 7.4.
11. PFA in HEPES buffer: prepare double-strength HEPES buffer above (i.e., 0.4 *M* HEPES) and mix 50:50 with fresh 4% aqueous PFA solution. Re-adjust the pH to 7.4.
12. Sucrose/polyvinyl pyrrolidone (PVP [8]): prepare a solution of 2 *M* sucrose in 0.2 *M* HEPES buffer. Heat the mixture with stirring to 60 to 70°C until the sucrose has

dissolved completely. Mix the sucrose solution with a further 100 mL of HEPES buffer and then add 4 mL of 1.1 M sodium carbonate solution. Add 20 g of PVP-10 (molecular weight 10,000) to the solution. Leave the paste overnight at room temperature covered with aluminum foil. The bubbles in the paste will escape leaving a clear solution.

13. Retrieval buffer: 0.2 M HEPES pH 7.4, 2 M sucrose, 0.75% gelatin.
14. PBS.
15. 2% gelatin in PBS poured into sterile Petri dishes.
16. 0.02 M glycine in PBS.
17. Normal goat serum diluted to 5% in PBS containing 1% bovine serum albumin (BSA).
18. Normal goat serum diluted to 10% in PBS containing 1% BSA.
19. 11-5F antibody (7).
20. 0.1% BSA in PBS.
21. Gold-conjugated goat anti-mouse secondary antibody.
22. 2.5% glutaraldehyde in PBS.
23. 2% uranyl acetate oxalate: mix equal volumes of a 4% (w/v) stock of uranyl acetate and 0.3 M oxalic acid. Adjust the pH to 7.5 with 5% ammonium hydroxide. Store in the dark at 4°C and filter through Whatman no. 1 filter paper before use.
24. 2% aqueous polyvinyl alcohol containing 0.2% uranyl acetate.

### 3. Methods

To examine the effect of Ca concentration on desmosomal adhesion, low Ca medium (LCM) must first be prepared by chelation of the Ca from FBS before adding to Ca-free DMEM (LCM) MDCK cells should then be grown on glass cover slips to the required level of confluence before performing the Ca switch assay. To measure the percentage of Ca-dependent and Ca-independent cells, the cells are first stained with a desmosomal antibody, such as one to desmoplakin, before microscopic examination for internalization of desmoplakin staining (Ca-dependent) or cell membrane staining (Ca-independent) and counting the numbers of each type of cell. Ca-dependence also can be induced in confluent (Ca-independent) monolayers by wounding (scraping the monolayers) or by treatment with PKC activators. Similarly, Ca-independence can be induced by treatment with PKC inhibitors. The Ca switch assay also can be used for untreated or wounded mouse skin tissue, in a similar way to MDCK cells. The effects on desmosomes can be investigated further by electron microscopy to see whether desmosomes have normal ultrastructure with intact midlines (Ca-independent) or have split into half desmosomes (Ca-dependent). Because PKC $\alpha$  is an important regulator of the adhesive state of desmosomes, it often is useful to look for colocalization of PKC $\alpha$  with desmosomes and the ideal way to confirm this is by immunogold labeling of desmosomes for PKC $\alpha$  and desmoplakin.

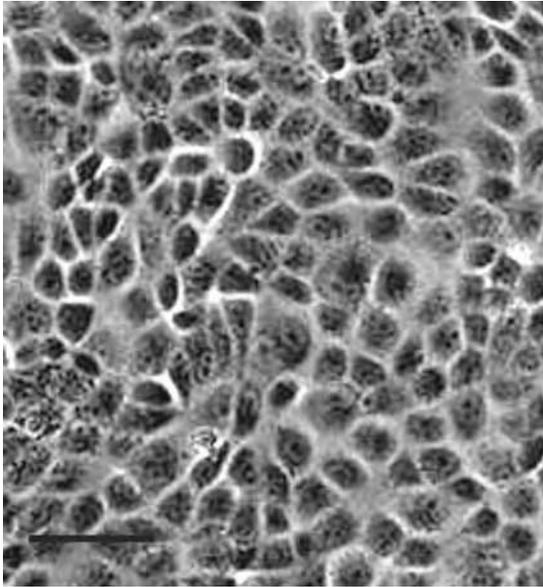


Fig. 2. Phase contrast image of a confluent monolayer of MDCK cells. MDCK cells were seeded at a density of  $4.5 \times 10^5$  cells/well and maintained for 6 d in NCM. The cobble-stone epithelial morphology is readily visible. Scale bar = 25  $\mu\text{m}$ .

### 3.1. Ca Switch Assay for MDCK Cells

#### 3.1.1. Preparation of LCM

1. Stir 20 g of Chelex resin with 50 mL of heat-inactivated FBS (56°C for 30 min) for 2 h (or overnight if more convenient) at 4°C.
2. Decant liquid from beads and sterilize by passing through 0.2- $\mu\text{m}$  filter.
3. Add the chelated FBS to Ca-free DMEM to a concentration of 10% w/v.
4. Add EGTA to a concentration of 3 mM (pH 7.5; see **Note 3**).
5. Store at 4°C until needed for Ca switch assay (see **Subheading 3.1.3**).

#### 3.1.2. Culture of MDCK Cells on Glass Cover Slips

1. Culture MDCK II cells in NCM (DMEM with 10% FBS), until confluent in a T75 flask ( $\sim 1 \times 10^7$  cells/flask). MDCK cells have a characteristic “cobblestone” epithelial morphology when grown as confluent monolayers (see **Fig. 2**).
2. Prepare 24-well plates in which one 13-mm diameter circular glass cover slip can occupy each well (see **Note 4**). The cover slips can be sterilized by autoclaving or by immersing in 100% ethanol (see **Note 5**).
3. Passage the cells with trypsin–EDTA, perform cell counts using a haemocytometer, and seed onto the glass cover slips;  $4\text{--}5 \times 10^5$  cells should be seeded per well to achieve confluence during the next 24 to 48 h.

### 3.1.3. Ca Switch (NCM to LCM; see **Note 6**)

1. Grow MDCK cells (see **Notes 7** and **8**) to the required level of confluence (see **Subheading 3.1.2.**). If the recommended  $4\text{--}5 \times 10^5$  cells/cover slip are seeded, then to guarantee a majority of Ca-dependent desmosomes, cells should be grown to 95% confluence (~1–2 d): if a majority of Ca-independent desmosomes is required, cells should be maintained at confluent density for 6 d. At approx 4 to 5 d, you will get a mix of Ca-dependent and Ca-independent desmosomes.
2. To perform the Ca switch, remove the NCM from the cells and wash three times with Ca-free PBS or HBSS (1 mL per well per wash).
3. Incubate cells with the preprepared chelated LCM (see **Subheading 3.1.1.**) for 90 min at 37°C.
4. Remove LCM, wash briefly in Ca-free PBS or HBSS, and fix with ice-cold 50:50 methanol:acetone for 10 min.
5. Remove fixative, cover plate, and store at –20°C until required for immunofluorescence (see **Subheading 3.3.1.**).

### 3.2. Ca Switch Assay for Mouse Epidermis

1. Sacrifice mouse and remove hair from back skin using clippers.
2. Dissect mouse skin from back, trim fat and dermis tissue, wash in Ca-free PBS, and cut into 1-mm<sup>3</sup> pieces.
3. Submerge tissue in LCM and incubate for up to 6 h in tissue culture incubator (37°C, 5% CO<sub>2</sub>). Incubate control tissue in NCM.
4. Remove medium and wash in Ca-free PBS.
5. Place tissue in aluminum foil molds containing OCT cryoprotectant and freeze in liquid nitrogen.
6. Label molds and store in a –80°C freezer.
7. Cut sections (7 μm) using a cryostat onto poly-L-lysine-coated slides.
8. Air-dry slides for 1 h at room temperature, then cover and place in –80°C freezer until required for immunofluorescence (see **Subheading 3.3.2.**).

### 3.3. Immunofluorescence for Desmoplakin

#### 3.3.1. Cell Monolayers (see **Fig. 3**)

1. If cover slips have been stored in –20°C, allow to reach room temperature for 15 min.
2. Wash cells in PBS, 3 × 10 min.
3. Block nonspecific binding in 5% normal goat serum, 2% BSA, and 0.05% Triton-X-100 in PBS for 30 min.
4. Incubate in mouse anti-desmoplakin antibody (11–5F), at a dilution of 1/50 in PBS, for 1 h at room temperature (see **Notes 9** and **10**).
5. Wash in PBS, 3 × 10 min.
6. Incubate in secondary anti-mouse IgG antibody, such as Donkey anti-mouse IgG FITC at a dilution of 1/100 in PBS, for 30 min at room temperature (see **Note 11**).
7. Wash in PBS, 3 × 10 min, then wash off the PBS by washing with dH<sub>2</sub>O.
8. Mount onto microscope slides with gelvatol and allow to solidify.

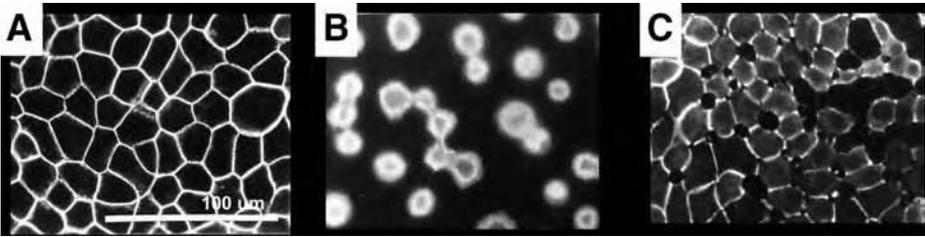


Fig. 3. Immunofluorescence for desmoplakin on MDCK cell monolayers. MDCK cells were seeded at confluent density ( $4.5 \times 10^5$  cells/well) and maintained for 2 d (**A, B**) or 6 d (**C**). Cells were then either left in NCM (**A**), or cells were switched to LCM (**B, C**). Cells were then fixed and stained for desmoplakin using the antibody 11-5F. In (**A**), cells have staining at the cell membranes. In (**B**), the cells have separated and desmoplakin has been internalized, indicative of Ca-dependent desmosomes. In (**C**), the cells have pulled apart, but processes remain connecting the cells and those stain brightly, indicative of Ca-independent desmosomes.

9. To stop drying out, seal the cover slips onto the slides with clear nail polish.
10. When nail polish is dry, wrap slides in aluminium foil and store at  $-20^\circ\text{C}$  until viewing under fluorescence microscope (*see* **Notes 12** and **13**) and performing counts of Ca-dependent and Ca-independent cells (*see* **Subheading 3.6.**).

### 3.3.2. Tissue Sections (*see* **Fig. 4**)

1. Take frozen sections out of the  $-80^\circ\text{C}$  freezer and fix in ice-cold 50:50 methanol:acetone for 20 min at  $-20^\circ\text{C}$ .
2. Draw a ring around each section on the slide using a wax pen to prevent antibody running from one section to another.
3. Stain the sections using the same protocol for monolayers with two exceptions: (a) add 20  $\mu\text{L}$  of diluted antibody per section and (b) incubate slides in a humidified box (*see* **Note 1**).
4. Perform counts of Ca-dependent and Ca-independent cells (*see* **Subheading 3.6.**). Similar protocols can be used for PKC $\alpha$  immunofluorescence (*see* **Notes 14** and **15**).

## 3.4. Wounding

### 3.4.1. Wounding Cell Monolayers

1. Grow MDCK cell monolayers on glass microscope slides within 100-cm<sup>2</sup> plastic dishes. The cells should be seeded at a density of  $1.5 \times 10^5$  cells/cm<sup>2</sup>. Incubate at  $37^\circ\text{C}$  for 6 d.
2. Make a template for wounds by drawing a rectangular grid on paper and place under the slide and view under microscope.

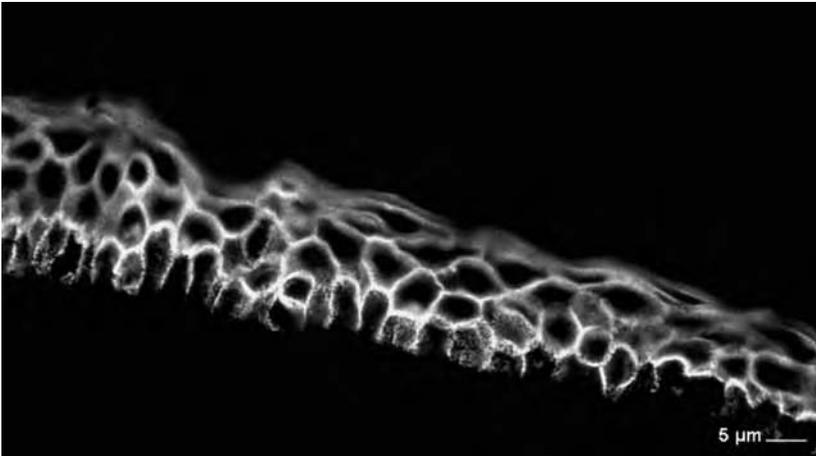


Fig. 4. Immunofluorescence for desmoplakin on mouse epidermis. Immunofluorescence image of 2-wk-old Balb-C mouse skin stained with 11-5F antibody for desmoplakin. Positive staining is evident around the cell membranes of epidermal cells. Scale bar = 5  $\mu\text{m}$ .

3. Create simple wounds by drawing a scalpel blade across the monolayer by hand, tracing the predrawn grid.
4. For multiple wounds, score parallel lines 5 to 20 mm apart, rotate the monolayer 90° and draw a second set of parallel lines.
5. Prepare cell islands by removing cells between alternative sets of parallel score lines using a cell scraper.
6. Place cells in LCM or NCM (for controls).
7. Perform immunofluorescence for desmoplakin (*see Subheading 3.3.1.*), and count the percentage of Ca-independent and Ca-dependent cells (*see Subheading 3.6.*).

#### 3.4.2. Wounding Mouse Epidermis

1. Anesthetize mice using inhalation anaesthetic such as halothane.
2. Sterilize back of mouse with a swab of 70% ethanol.
3. Shave back with electric clippers.
4. Mark 1-cm regions for wounding using a marker pen (*see Fig. 8; Note 16*).
5. Using a scalpel with a no. 11 blade, wound the mice on these marks. The wounds should be of full thickness, cutting through epidermis, dermis, and muscle.
6. Let the mouse recover and give analgesia as required.
7. To harvest the wounds, sacrifice the mice at the appropriate time.
8. Dissect at least 1 cm around the wound area and process for Ca switch assay (**Subheading 3.2.**) conventional electron microscopy (**Subheading 3.7.**), or immunogold labeling (**Subheading 3.8.**) as required.

### **3.5. Treatment of Cell Monolayers With PKC Activators and Inhibitors**

#### *3.5.1. Treatment With PKC Activators to Revert Ca-Independent Desmosomes to Ca-Dependent*

1. Seed MDCK cells at  $4\text{--}5 \times 10^5$  cells/cm<sup>2</sup> on to cover slips and culture for 6 d.
2. Treat the cells with the drug as follows: 5 nM TPA for 1 h; 50 nM phorbol-12, 13-didecanoate for 1 h; and 50 nM phorbol-12, 13- dibutyrate for 1 h. With different batches of cells, the optimal incubation times and concentrations of the drugs may vary slightly.
3. Switch to drug-free LCM for 90 min.
4. Fix the cells and stain for desmoplakin using protocol described in **Subheading 3.3.1**.
5. Calculate the proportion of cells with Ca-independent desmosomes as described in **Subheading 3.6**.

#### *3.5.2. Treatment With PKC Inhibitors to Revert Ca-Dependent Desmosomes to Ca-Independent*

1. Seed MDCK cells at  $4\text{--}5 \times 10^5$  cells/cm<sup>2</sup> on to cover slips and culture for 24 h.
2. Treat the cells with the drug as follows: (a) 10 nM Gö6976 for 1 h; (b) 8.7  $\mu$ M chelerythrine chloride for 15 min. With different batches of cells, the optimal incubation times and concentrations of the drugs may vary slightly.
3. Switch to drug-free LCM for 90 min.
4. Fix the cells and stain for desmoplakin using protocol described in **Subheading 3.3.1**.
5. Calculate the proportion of cells with Ca-independent desmosomes as described in **Subheading 3.6**.

### **3.6. Scoring Cells for Ca-Independence**

1. Select at least 10 fields of view at random.
2. Count the number of cells that remain attached by at least one desmoplakin-staining projection (see **Fig. 3**), using a hand-held counter. (These are Ca-independent cells.)
3. Count the total number of cells using a hand-held counter.
4. Express the number of Ca-independent cells as a proportion of the total number of cells in the fields.
5. Count several duplicates of cultures/tissue for each experimental point.

### **3.7. Conventional Transmission Electron Microscopy Examination of Desmosome Ultrastructure (see **Figs. 5 and 6**)**

#### *3.7.1. Fixation and Embedding*

1. Fix 1-mm<sup>3</sup> pieces of tissue (**Subheading 3.2**. or **Subheading 3.4.2**.) in 2% PFA plus 2% glutaraldehyde in cacodylate buffer pH 7.5 for 2 h at room temperature, changing the fixative after 1 h. Leave in fixative overnight at 4°C.
2. Wash the samples in several changes of cacodylate buffer for 1 h at room temperature.

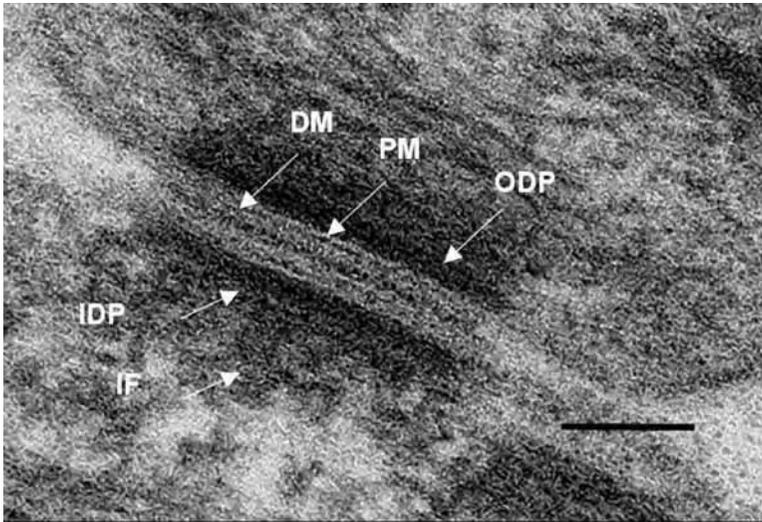


Fig. 5. Electron micrograph showing the ultrastructure of a desmosome. DM, dense midline; PM, plasma membrane; ODP, outer dense plaque; IDP, inner dense plaque; IF, intermediate filaments. Scale bar = 100 nm.

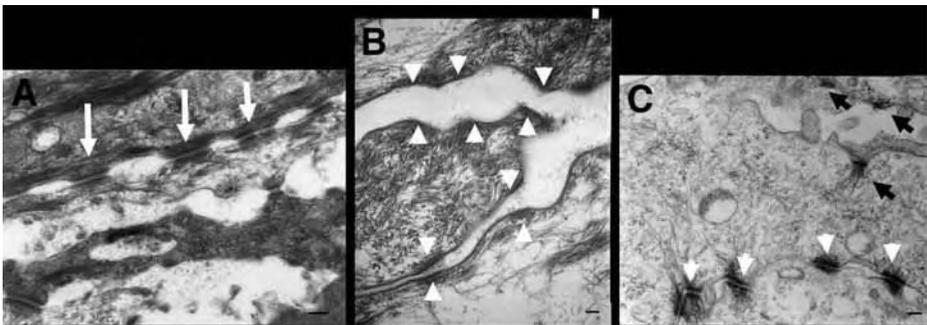


Fig. 6. Electron micrographs showing Ca-independent and Ca-dependent desmosomes after incubation in low Ca medium. (A) Ca-independent desmosomes in mouse epidermis (white arrows) Note the enlarged intercellular spaces. (B) Ca-independent desmosomes in wounded mouse epidermis. White arrows show the desmosomes split into half-desmosomes. (C) Wounded mouse epidermis with Ca-dependent desmosomes (black arrows) and Ca-independent desmosomes (white arrows) further away from the wound edge. Scale bars = 100 nm.

3. Postfix in 2% osmium tetroxide in cacodylate buffer for 1 h at room temperature.
4. Rinse several times in ddH<sub>2</sub>O.
5. Dehydrate through an ethanol series: 70% for 15 min, 80% for 15 min, and 100% three times for 20 min each.

6. Infiltrate tissue with Spurr's resin (medium hardness): 50% resin in dry ethanol overnight, 70% resin in dry ethanol for 1 h, 100% resin in dry ethanol for 3 h three times.
7. Embed tissue in molds containing fresh resin and polymerize at 60°C for 72 h.
8. Embedded tissue can be stored at room temperature before sectioning.

### 3.7.2. Sectioning

1. Trim resin blocks first with a razor blade and then finely trim on ultramicrotome to remove excess resin (*see Note 17*).
2. Cut semithin sections (250–500 nm) and collect on poly-L-lysine-coated glass slides for staining with toluidine blue and examination under the light microscope to ensure that epidermis or wound edges are included in the section.
3. Cut ultrathin sections (50–70 nm) using a diamond knife. The sections will float away from the knife edge on the water reservoir.
4. Collect sections from the water on 100 mesh grids and leave to air-dry (*see Note 18*).

### 3.7.3. Staining

1. Wet grids on drops of ddH<sub>2</sub>O.
2. Float grids on drops of 2% uranyl acetate solution for 30 min at room temperature.
3. Wash each grid three times in ddH<sub>2</sub>O.
4. Incubate on 0.3% lead citrate solution for 5 min.
5. Wash thoroughly in ddH<sub>2</sub>O and then allow to air-dry overnight before examination in the transmission electron microscope (TEM).

## 3.8. Immunogold Electron Microscopy to Localize Desmoplakin or PKC $\alpha$ Within the Desmosome (*see Fig. 7 and Notes 19 and 20*)

### 3.8.1. Fixation and Infiltration

1. Fix 1-mm<sup>3</sup> pieces of tissue (**Subheadings 3.2.** or **3.4.2.**) in 2% PFA in HEPES buffer for 45 min at room temperature.
2. Wash four times with HEPES buffer, 10 min per wash.
3. Infiltrate with sucrose/PVP for 4 h at room temperature or overnight at 4°C (*see Notes 21 and 22*).
4. Mount the infiltrated tissue on freshly cleaned aluminium cryopins and remove any excess sucrose/PVP.
5. Manually plunge freeze the tissue in liquid nitrogen and then store under liquid nitrogen prior to sectioning.

### 3.8.2. Cryoultramicrotomy Sectioning (*see Notes 23 and 24*)

1. Precool the chamber of a cryoultramicrotome to –80°C and place the 45° glass and diamond knives inside to equilibrate.
2. Transfer the frozen tissue from liquid nitrogen to the cryochamber and allow it to equilibrate for 15 min.

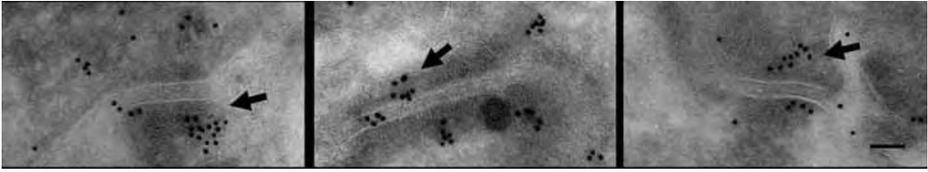


Fig. 7. Electron micrographs of wounded mouse epidermis immunogold labeled for PKC $\alpha$ . Note the localization of PKC $\alpha$  to the desmosomes (black arrows). Scale bar = 100 nm.

3. Using the glass knife, trim the sucrose/PVP block to the smallest possible area.
4. Cut semithin sections for staining with toluidine blue and collect on poly-L-lysine-coated glass slides. Examine under the light microscope to ensure visualization of the epidermis or wound edge.
5. Reduce the temperature of the cryochamber to  $-120^{\circ}\text{C}$  and allow it and the knives to equilibrate.
6. Once the temperature of the tissue has reached  $-110^{\circ}\text{C}$ , ultrathin sections can be cut using the diamond knife.
7. Adjust the cutting speed to allow sections of the required thickness (50–60 nm) to be obtained and to maintain the temperature of the specimen.
8. The sections will form a ribbon on the knife. Use an eyelash probe to arrange them in groups of three or four.
9. Retrieve the sections using a 1.5- to 3-mm wire loop containing a droplet of retrieval buffer. Work quickly to avoid the buffer freezing. The sections will melt instantaneously and adhere to the buffer. Rapidly withdraw the loop (*see Note 25*).
10. Transfer the cryosections to 100 mesh formvar-coated grids.
11. Invert the grids onto a plate of 2% gelatin in PBS and store overnight at  $4^{\circ}\text{C}$ .

### 3.8.3. Immunogold Labeling

1. Immediately before labeling, liquefy the gelatin block by incubating at  $37^{\circ}\text{C}$  for 20 min (*see Notes 26 and 27*).
2. Transfer the grids across three drops of 0.02 M glycine in PBS for 1 h at room temperature (*see Note 28*).
3. Block nonspecific labeling by incubating grids on droplets of 5% normal goat serum plus 1% BSA for 1 h at room temperature.
4. Prepare the monoclonal antibody 11–5F at a dilution of 1/20 in PBS plus 1% BSA.
5. Transfer the grids through three drops of the diluted primary antibody at  $37^{\circ}\text{C}$  (20 min per drop).
6. Wash the grids five times (10 min per wash) in PBS plus 0.1% BSA.
7. Dilute gold conjugated goat anti-mouse secondary antibody 1:200 in 10% normal goat serum plus 1% BSA in PBS.
8. Incubate grids on the secondary antibody for 45 min at room temperature.
9. Wash five times (10 min per wash) in PBS.

10. Post fix in 2.5% glutaraldehyde in PBS for 5 min at room temperature.
11. Wash in five changes of ddH<sub>2</sub>O for 5 min (*see Note 29*).
12. Contrast sections on drops of 2% uranyl acetate oxalate and then wash briefly on three drops ddH<sub>2</sub>O (*see Note 30*).
13. Incubate the grids on 2% aqueous polyvinyl alcohol (PVA)/0.2% uranyl acetate for 15 min (*see Note 31*).
14. Use wire loops to retrieve the grids and draw across filter paper to remove excess liquid.
15. Allow the grids to air-dry before examination in the TEM (*see Note 31*).

#### 4. Notes

1. A humidified box can be made from a plastic sandwich box containing paper towels wetted with distilled water or PBS.
2. An eyelash probe is made by attaching an eyelash to a wooden stick.
3. The Ca concentration of LCM should be less than 0.05 mM, and the Ca concentration of NCM is approx 1.8 mM. The Ca concentration can be measured by atomic absorption spectroscopy if required. *See ref. 10* for details.
4. Each time point in an experiment requires a separate 24-well plate because all cells in one plate should be fixed simultaneously. For each time point, some cover slips should be kept in NCM as controls.
5. If the ethanol method is chosen for sterilizing cover slips, the ethanol should be completely removed before adding cells by either flaming cover slips in a Bunsen flame or allowing to air-dry in the tissue culture hood for 30 min.
6. This method is for a downward Ca switch, i.e. from NCM to LCM. Cells can also be switched from LCM to NCM as described in (4).
7. The Ca switch assay also can be performed with primary keratinocytes (3), Caco-2 colon cells (9), HaCaT cells, and A431 cells (our unpublished data).
8. MDCK cells stably transfected with GFP or YFP-labeled desmosomal proteins can also be used to directly visualize desmosomes during Ca switch assays.
9. Other antibodies than the mouse monoclonal antibody to desmoplakin (11–5F) can be used as a marker for desmosomes. Some excellent antibodies are commercially available from Progen ([www.progen.de](http://www.progen.de)) or Research Diagnostics Inc ([www.researchd.com](http://www.researchd.com)) Requests for our antibody to desmoplakin (11–5F) should be addressed to Professor David Garrod, University of Manchester, UK.
10. To perform the immunofluorescence of the cover slips, cover a large glass plate with Parafilm. Place drops of solutions directly onto the Parafilm and invert the cover slips in the drops. Allow 15 µL for blocking reagent and antibodies and 200 µL for PBS washes. Use forceps to transfer the cover slips from one drop to the next.
11. Nuclei can be counterstained blue by including a 1/1000 dilution of Hoechst 33258 stock (5 mg/mL) with the secondary antibody.
12. Fluorescently stained slides can be kept for up to 2 wk wrapped in foil in –20°C freezer and sometimes longer.

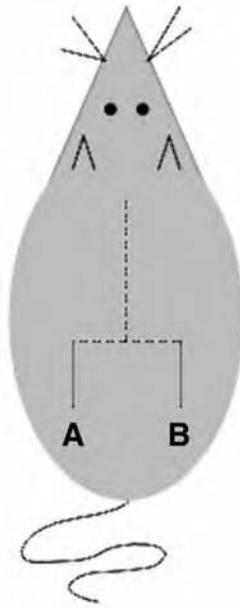


Fig. 8. Diagram to show the regions for wounding mouse epidermis. Wounds were made 3 cm posterior to the animal's neck, measured from the base of the skull, 1 cm long and 2 cm apart. Each mark was 1 cm lateral to the animal's midline. **A** and **B** are 1-cm wound sites.

13. All our microscopy is conducted using a Zeiss Axioplan microscope with a 50W HBO bulb for epi-fluorescence, using  $\times 16$ ,  $\times 40$ , and  $\times 63$  objectives. The filter sets we use are: blue (G365, FT 395, LP420), green (450–490, FT510, 515–565), and red (530–585, FT 600, LP615).
14. To analyze the distribution of PKC $\alpha$  by immunostaining: seed the cells at confluent density and maintain for 6 d to acquire Ca-independent desmosomes then stain exactly as for desmoplakin (DP) but use the anti-PKC $\alpha$  antibody at a dilution of 1/50–1/100, and a goat-anti-rabbit secondary antibody conjugated to cy3 or rhodamine red.
15. To perform double staining for desmoplakin and PKC $\alpha$ , add anti-DP antibody for 1hr, wash in PBS, add anti-PKC $\alpha$  for 1 h, wash in PBS, then add secondary antibodies together for 30 min. We recommend using a FITC-labeled secondary antibody for detecting DP and either rhodamine red or cy3 for PKC $\alpha$ . Take care when analyzing the double staining under the fluorescence microscope, not to get bleedthrough from red channel into FITC channel.
16. The regions for wounding on mouse epidermis were made 3 cm posterior to the animal's neck, measured from the base of the skull, 1 cm long, and 2 cm apart. Each mark was 1cm lateral to the animal's midline. See Fig. 8 and (11) for further details. In the UK, a Home Office licence is required for this procedure.

17. We conduct our sectioning for conventional electron microscopy on an Ultracut ultramicrotome (Reichert-Jung Ltd).
18. An indication of a good section is a silver/gold interference color.
19. Before trying any antibody in immunogold labeling, we strongly recommend trying it on PFA fixed tissue with the immunofluorescence method. If the antibody does not work with this method, it almost certainly will not work in immunogold labeling.
20. Our cryoultramicrotomy protocol is based on that developed by Tokuyasu (8,12–15).
21. The addition of PVP to the sucrose infiltrating solution confers greater plasticity on the blocks than sucrose alone.
22. Tissue sections for immunogold labeling can be embedded in PVA; see ref. 15 for a protocol.
23. The cryoultramicrotome we use is a Leica Ultracut S/FCS.
24. Glass knives are coated with tungsten as described by Roberts (16) and Griffiths et al. (17).
25. To transfer a grid to a droplet from the loop: hold the loop containing the grid perpendicular to the drop and lower into the liquid. The surface tension will release the grid.
26. The immunogold labeling is conducted on drops of each reagent placed on a sheet of clean parafilm.
27. It is very important to keep the back of the grids dry and not to immerse them in reagent. The grids should float on top of the drops.
28. The glycine wash is to quench any free aldehydes in the fixed tissue.
29. It is important to wash the grids in ddH<sub>2</sub>O after postfixing to remove all traces of PBS that would precipitate with the uranyl acetate forming large, electron-dense crystals of uranyl phosphate.
30. The uranyl acetate oxalate step is thought to stabilize membranes against the low pH of unbuffered uranyl acetate.
31. The final PVA embedding step is to protect the cryosections from air-drying artefacts and should be sufficient to leave only a thin film over the section. The addition of uranyl acetate to the PVA adds contrast to the sections.
32. We use Philips 400 and Philips Tecnai Biotwin transmission electron microscopes.

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## Tight Junctions and Cell–Cell Interactions

Markus Utech, Matthias Brüwer, and Asma Nusrat

### Summary

Chronic inflammation in mucosal tissues can influence epithelial barrier function via pro-inflammatory cytokines such as interferon (IFN)- $\gamma$  and tumor necrosis factor- $\alpha$ . Increased mucosal levels of these cytokines have been observed in mucosal biopsies from patients with a chronic inflammatory condition referred to as inflammatory bowel disease. Paracellular permeability across epithelial cells is regulated by tight junctions (TJs), which are the apical most junctions in epithelial cells. Given that pro-inflammatory cytokines modulate the epithelial barrier and that TJs regulate epithelial permeability, we analyzed the influence of IFN- $\gamma$  on TJ function/structure. Our results suggest that IFN- $\gamma$  induced a time-dependent increase in paracellular permeability that was associated with internalization of TJ transmembrane proteins, occludin, junction adhesion molecule A, and claudin-1. In this chapter, we focus on selected methods used to investigate the influence of IFN- $\gamma$  on epithelial barrier function.

**Key Words:** Tight junction, interferon- $\gamma$ ; confocal microscopy; immunofluorescence; transepithelial electrical resistance; paracellular flux.

### 1. Introduction

Epithelial cells lining luminal organs, such as the gastrointestinal tract, interface luminal contents and underlying tissue compartments. Polarized epithelial cells create a highly efficient and selective barrier between different tissue compartments (**1**). Epithelial paracellular permeability is regulated by the apical intercellular junctional complex referred to as the apical junctional complex. The major constituents of this complex are tight junctions (TJs) and subjacent adherens junctions. TJs represent a multiprotein complex consisting of transmembrane and cytosolic components (**2,3**). Three major TJ transmembrane proteins include occludin, members of the claudin family, and the immunoglobulin-like superfamily members, such as junctional adhesion molecule (JAM)-A and coxackie-adenovirus-receptor. These transmembrane proteins affiliate with the underlying

perijunctional F-actin ring via cytoplasmic plaque proteins, primarily *zonula occludens* (ZO) proteins ZO-1, ZO-2, and ZO-3 (2,4,5). Increased permeability of the epithelial barrier is a key contributor to various gastrointestinal diseases such as Crohn's disease and ulcerative colitis (6,7), which are characterized by increased mucosal levels of proinflammatory cytokines such as interferon (IFN)- $\gamma$  (8).

Given the complex composition of mucosal tissues and diverse array of cytokines released in inflammatory states, we have used a reductionistic in vitro cell culture approach to examine the influence of a single proinflammatory cytokine on epithelial barrier function. A model intestinal epithelial cell line, T84 and human recombinant IFN- $\gamma$  was used to analyze the effects of this cytokine on structure and function of the epithelial TJs.

## 2. Materials

### 2.1. Collagen Coating of Permeable Polycarbonate Filter Supports

1. Rat tails (store at  $-80^{\circ}\text{C}$ ; Pet-Freeze Biologicals, Rogers, AK).
2. Hemostat (Daigger, Vernon Hills, IL).
3. 1% (v/v) acetic acid solution in water (see Note 1).
4. Dialysis tubing: Spectra/Por no. 1 (Spectrum Laboratories Inc., Rancho Dominguez, CA).
5. Parafilm "M" (pechiney plastic packing, Chicago, IL).
6. Permeable polycarbonate membrane filters with surface areas of  $0.33\text{ cm}^2$  ( $5\text{-}\mu\text{m}$  pore size) and  $5\text{ cm}^2$  ( $3\text{-}\mu\text{m}$  pore size; Costar Transwell, Corning Incorporated, Corning, NY).

### 2.2. Cell Culture

1. T84 epithelial cells (ATCC, Rockville, MD).
2. Cell medium: 1:1 Dulbecco's Modified Eagle's Medium and Ham's F-12 medium supplemented with 15 mM HEPES, pH 7.5, 14 mM  $\text{NaHCO}_3$ , 0.4% (w/v) penicillin, 0.08% (w/v) ampicillin, 0.9% (w/v) streptomycin, and 6% (v/v) newborn calf serum.
3. IFN- $\gamma$  stock solution (2500 U/mL; kind gift of Genentech, San Francisco, CA).

### 2.3. Transepithelial Electrical Resistance

1. EVOM<sup>TM</sup> Epithelial Voltohmmeter (World Precision Instruments, Sarasota, FL).
2. STX2 electrode set (World Precision Instruments, Sarasota, FL).
3. 0.15 M KCl.

### 2.4. Fluoresceinated Dextran-3 Flux

1. Fluoresceinated dextran (FD)-3 solution: 1% (w/v) fluorescein-labeled dextran 3 kDa (Molecular Probes, Eugene, OR) in Hank's balanced salt solution (HBSS).
2. 96-well plate (NUNC, Rochester, NY).

### **2.5. Cell Preparation for Immunoblotting**

1. Cell lysate buffer: 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4, 1% (v/v) Triton X-100, protease inhibitor cocktail (1:100, Sigma, St. Louis, MO).
2. Cell scraper: 1.3-cm blade, total length 16 cm. (Sarstedt Inc., Newton, NC).
3. Glass homogenizer (douncer; Weathon Inc., Millville, NJ).
4. Protein concentration assay: BCA Protein assay kit (Pierce, Rockford, IL).

### **2.6. Differential Detergent Extraction of TJ Transmembrane Proteins**

1. TX 100 extraction buffer: 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl<sub>2</sub>, 10 mM HEPES, pH 7.4, 1% (v/v) Triton X-100, protease inhibitor cocktail (1:100, Sigma).
2. Running buffer (2X) (pH 8.3): 3% (w/v) sodium dodecyl sulfate (SDS), 0.75 M Tris-HCl, pH 8.8, 20% (v/v) glycerol, and 20 mM dithiothreitol.

### **2.7. SDS-Polyacrylamide Gel Electrophoresis**

1. Separating buffer: 1.5 M Tris-HCl, pH 8.6. Store at 4°C.
2. Stacking buffer: 1 M Tris-HCl, pH 6.8. Store at 4°C.
3. 30% (w/v) acrylamide:bisacrylamide solution (37.5:1; National Diagnostics, Atlanta, GA).
4. TEMED (Bio-Rad, Hercules, CA).
5. SDS solution: 10% (w/v) solution in water (store at room temperature).
6. Ammonium persulfate (AP) solution: 10% (w/v) AP solution in water (prepare solution before use).
7. Running buffer: 50 mM Tris-HCl, 190 mM glycine, 1% (w/v) SDS. Store at room temperature.
8. Prestained protein markers, broad range (6–175 kDa; New England Biolabs, Beverly, MA).

### **2.8. Western Blotting**

1. Transfer buffer: 25 mM Tris-HCl (do not adjust pH), 190 mM glycine, 20% (v/v) methanol.
2. Trans-Blot Transfer membrane, pure nitrocellulose membrane (0.2 μm; Bio-Rad, Hercules, CA).
3. Tris-buffered saline (TBS): 200 mM NaCl, 50 mM Tris-HCl, pH 7.4.
4. TBS with Tween (TTBS): Add 1% (v/v) Tween-20.
5. Blocking buffer: 5% (w/v) nonfat dry milk in TTBS.
6. Primary antibodies are diluted in blocking buffer: polyclonal antibodies: anti-occludin (3 μg/mL), claudin-1 (3 μg/mL), and JAM-A (12 μg/mL) pAbs (Zymed Laboratories, San Francisco, CA).
7. Secondary antibodies are diluted in blocking buffer: horseradish peroxidase-conjugated goat anti-rabbit 0.16 μg/mL (Jackson Immunoresearch Laboratories, West Grove, PA).

8. ECL Western blot detection reagent (Amersham Biosciences, Piscataway, NJ).
9. Hyperfilm ECL film (Amersham Biosciences).
10. Trans-blot paper (Bio-Rad).

### **2.9. Immunofluorescence Labeling of TJ Proteins and Confocal Microscopy**

HBSS+: 0.95% (w/v) HBSS (Sigma) and 10 mM HEPES, pH 7.4.

1. Fixation solution: absolute ethyl alcohol stored at  $-20^{\circ}\text{C}$ .
2. Blocking solution and antibody dilution buffer: 1.5% (w/v) bovine serum albumin (BSA; Sigma) in HBSS+.
3. Primary antibodies: anti-occludin (8  $\mu\text{g}/\text{mL}$ ), claudin-1 (8  $\mu\text{g}/\text{mL}$ ), and JAM-A (8  $\mu\text{g}/\text{mL}$ ) pAbs (Zymed Laboratories).
4. Filter paper: 90-mm diameter (Whatman, Florham Park, NJ).
5. Secondary antibodies: goat anti-rabbit and goat anti-mouse secondary antibodies conjugated to Alexa-488 or Alexa-546 dyes (4  $\mu\text{g}/\text{mL}$ ; Molecular Probes, Eugene, OR).
6. Mounting medium: Slow Fade anti-fade kit (Molecular Probes, Eugene, OR).
7. Cover glasses: 18 mm<sup>2</sup> (Corning Inc., Corning, NY).

## **3. Methods**

Epithelial cells are grown as monolayers on permeable polycarbonate supports. In this system epithelial cells can polarize and form well-developed intercellular junctions and are amenable to analysis by numerous techniques. Functional analysis of paracellular permeability across epithelial monolayers can be determined by measuring the transepithelial electrical resistance (TEER) to passive ion flow and by measuring the rate of paracellular solute flux of tracers from the apical to the basolateral compartment of epithelial cells. Functional studies can be complemented by morphological analysis of TJ protein localization by immunofluorescence labeling/confocal microscopy and by biochemical determination of protein expression and their subcellular compartmentalization. Because such an approach has been widely used to study epithelial TJs, we have highlighted some of the practical aspects of these techniques that we have used to examine the influence of IFN- $\gamma$  on epithelial barrier function. More biophysical explanations and a general overview of these methods can be found in other reviews (9–11).

### **3.1. Collagen Coating of Permeable Polycarbonate Filter Supports**

1. A razor blade is used to make a longitudinal incision through the skin of a rat-tail. The skin is stripped from the underlying tendons, which are extracted using hemostats. Mince and weigh the material extracted. This material can be frozen in 0.75-g aliquots at  $-80^{\circ}\text{C}$ .
2. Soak 0.75 g of collagen in 70% ethanol for 10 to 20 min to dehydrate.

3. Solubilize collagen in 100 mL of 1% (v/v) acetic acid at 4°C. Stir the mixture slowly at 4°C overnight. The next day, the solution will be viscous and some undissolved material will remain.
4. Spin the collagen solution at 25,000g for 30 min at 4°C.
5. Collect the collagen solution, avoiding sedimented material, and dialyze against 2 L water for 24 h; use dialysis tubing, presoaked, for 1 h. Change water three times during dialysis.
6. Remove the collagen from the bag and dilute the solution with water if necessary (a little thinner than honey).
7. Store at 4°C.
8. Before use, dilute stock collagen 1:100 with 70% ethanol.
9. Put 50  $\mu\text{L}$  of diluted collagen on each 0.33-cm<sup>2</sup> filter, and 500  $\mu\text{L}$  on 5-cm<sup>2</sup> filters.
10. Allow to dry before use.

### 3.2. Cell Culture

1. The intestinal epithelia cell line, T84 cells is grown in 5% CO<sub>2</sub> at 37°C within humidified incubators. These cells are passaged when approaching confluence with trypsin/EDTA to provide new maintenance cultures in 162-cm<sup>2</sup> flasks and experimental cultures on different collagen coated filters.
2. For cell culture we plate the cells at 50% density (*see Note 2*).
3. Confluent monolayer of T84 cells in 162-cm<sup>2</sup> flask will yield  $1.7 \times 10^7$  cells. Place  $2 \times 10^5$  cells on 0.33 cm<sup>2</sup> and  $2 \times 10^6$  cells in 5 cm<sup>2</sup>. At such a density, epithelial cells achieve confluence and become polarized after 7 days on 0.33-cm<sup>2</sup> filter and 10 d on 5-cm<sup>2</sup> filters.
4. Before each experiment, measure the TEER of each filter.
5. For IFN- $\gamma$  incubation in 0.33-cm<sup>2</sup> (5-cm<sup>2</sup>) filters, remove 40  $\mu\text{L}$  (80  $\mu\text{L}$ ) of media in the lower chamber and add 40  $\mu\text{L}$  (80  $\mu\text{L}$ ) of IFN- $\gamma$  stock solution basolaterally (*see Note 3*).

### 3.3. Transepithelial Electrical Resistance

1. To determine TEER, plate cells on 0.33-cm<sup>2</sup> filters and let them grow for 7 d (*see Note 4*).
2. The total resistance of the monolayer is defined as follows:

$$R_{(\text{total})} = R_{(\text{monolayer})} + R_{(\text{blank})}$$

3. Measure the blank resistance through a 0.15 M KCl solution and across the membrane support lacking cells.
4. Before measuring TEER incubate the STX2 electrode in 70% ethanol for 10 min and let it air-dry.
5. Measure TEER by dipping simultaneously the electrode in the upper and lower chambers.
6. Calculate the TEER (*see Note 5*).

Record TEER of filters with or without cytokine incubation over a period of 48 h are presented in **Fig. 1A**.

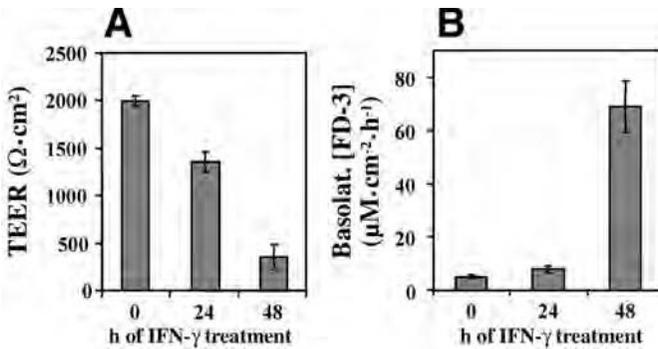


Fig. 1. Influence of IFN- $\gamma$  on epithelia barrier function. Barrier function was monitored by measuring TEER and paracellular flux of FD-3. (A) TEER was measured after basolateral incubation of polarized epithelial monolayers with IFN- $\gamma$ . (B) Paracellular flux of FD-3 in confluent T84 monolayers was measured after basolateral exposure to IFN- $\gamma$ .

### 3.4. Paracellular Flux

1. For flux experiments plate cells on 0.33-cm<sup>2</sup> filters and let them grow for 7 d (*see Note 6*).
2. Check TEER of all monolayers.
3. Wash cells three times with warm HBSS+.
4. Add 200  $\mu$ L of HBSS+ in the upper and 1 mL in the lower chamber.
5. Equilibrate cells in HBSS+ for 10 min on an orbital shaker and keep the cells on a heating stage at 37°C.
6. Aspirate HBSS+ from the upper chamber (avoid disturbing the monolayer).
7. Add 200  $\mu$ L of FD-3 solution to the upper chamber (*see Note 7*). Shake the plate on orbital shaker at 37°C.
8. Remove 50  $\mu$ L from the lower chamber at T = 120 min. Place in 96-well plate (*see Note 8*).
9. Create a standard curve with the FD-3 (serial dilution of mg/mL).
10. Read plate in fluorescence plate reader. Excitation: approx 490 nm, emission: approx 520 nm.
11. Plot standard curve to convert arbitrary fluorescence units to concentration. Flux is expressed as concentration/observed area/time ( $\mu$ M/cm<sup>2</sup>/h).

Paracellular flux of fluorescein isothiocyanate dextran (3 kDa) measurements across epithelial cells exposed to IFN- $\gamma$  is shown in **Fig. 1B**.

### 3.5. Cell Preparation for Immunoblotting

1. T84 cells are grown on 5-cm<sup>2</sup> permeable supports for 10 d and incubated with IFN- $\gamma$  for 48 h.
2. Wash cells twice with HBSS+.

3. Scrape cells into cell lysis buffer by using cell scraper at 4°C.
4. Break cells by using a douncer. Use for every sample the same number of strokes (e.g., 15) at 4°C.
5. Centrifuge cell lysates (1500g, 5 min, 4°C).
6. Determine the protein concentration of each sample and equalize protein concentrations (10 µg/lane).
7. Add the sample buffer and boil the sample for 5 min at 95°C.
8. Analyze equal amount of each fraction by SDS-polyacrylamide gel electrophoresis (PAGE)/Western blotting.

### **3.6. Differential Detergent Extraction of TJ Proteins**

1. T84 cells are grown on 5-cm<sup>2</sup> permeable supports for 10 d and incubated with IFN-γ for 48 h.
2. Wash cells twice with HBSS+.
3. Incubate cells for 30 min with TX-100 extraction buffer. Shake the plate on orbital shaker at 4°C.
4. Carefully remove supernatant.
5. Subject the TX-100-soluble fraction to low-speed centrifugation (1500g, 5 min, 4°C) to remove cellular debris.
6. Add the TX-100-soluble fraction to an equal amount of sample buffer.
7. Scrape the TX-100-insoluble fraction into an equal amount of SDS sample buffer.
8. Boil samples for 5 min at 95°C.
9. Analyze equal volumes of each fraction by SDS-PAGE/Western blotting.

### **3.7. SDS-Polyacrylamide Gel Electrophoresis**

1. These instructions assume the use of a Mini-PROTEAN 3 Electrophoresis System (Bio-Rad).
2. For occludin and JAM-A Western blotting prepare a 1.5-mm thick, 8% gel by mixing 2.5 mL of separating buffer with 2.7 mL of acrylamide/*bis* solution, 4.6 mL of water, 100 µL of AP solution, 100 µL of SDS solution, and 6 µL of TEMED. For claudin-1 Western blotting, prepare a 1.5-mm thick, 12% gel by mixing 2.5 mL of separating buffer with 4.0 mL of acrylamide/*bis* solution, 3.3 mL of water, 100 µL of AP solution, 100 µL of SDS solution, and 4 µL of TEMED. Pour the gel, leaving space for a stacking gel, and overlay it with water. The gel should polymerize in about 30 min.
3. Pour off the water.
4. Prepare the stacking gel by mixing 0.75 mL of stacking buffer with 1 mL acrylamide/*bis* solution, 4.1 mL of water, 50 µL of AP solution, 50 µL of SDS solution, and 10 µL of TEMED. Pour the stack and insert the comb. The stacking gel should polymerize within 30 min.
5. Once the stacking gel has set, carefully remove the comb and use a 3-mL syringe fitted with a 22-gage needle to wash the wells with running buffer.
6. Add the running buffer to the upper and lower chambers of the gel unit and load the samples.

7. Complete the assembly of the gel unit and connect to a power supply. The gel can be run at 30 mA.

### **3.8. Western Blotting**

1. Switch the refrigerated/circulating water bath to maintain a temperature around 4°C.
2. Prechill the transfer buffer.
3. The samples that have been separated by SDS-PAGE are transferred electrophoretically to nitrocellulose membranes. These directions assume the use of a Bio-Rad transfer tank system.
4. Assemble a transfer cassette consisting of the gel and nitrocellulose.
5. Ensure that no bubbles are trapped in the gel, nitrocellulose sandwich.
6. The cassette is placed into the transfer tank such that the gel is between the nitrocellulose membrane and the cathode.
7. Assemble the lid on the transfer tank and activate the power supply. Transfers can be accomplished at 1 A for 1 h at 4°C.
8. Once the transfer is complete the cassette is taken out of the tank and disassembled carefully.
9. The nitrocellulose is incubated in 50 mL of blocking buffer for 1 h at room temperature on a rocking platform.
10. The blocking buffer is discarded and the membrane is quickly shrink-wrapped so that a small aperture is left to fill with 1 mL of primary antibody solution.
11. Air bubbles are smoothed out and the plastic foil is finally sealed.
12. Rotate the membrane for 1 h at room temperature.
13. The primary antibody is then removed and the membrane washed three times for 5 min each with 50 mL of TTBS.
14. The secondary antibody is freshly prepared for each experiment and the membrane is placed in 10 mL of secondary antibody solution for 1 h at room temperature on a rocking platform.
15. The secondary antibody is discarded and the membrane washed twice for 20 min with TTBS and finally with TBS for 20 min.
16. ECL reagent is assembled according to manufacture instructions.
17. After rotating the membrane in 2 mL of ECL reagent by hand for 1 min, the blot is removed, slightly desiccated by using Kim-Wipes, and then placed between the leaves of an acetate sheet protector that has been cut to the size of an X-ray film cassette.
18. The blot is then placed in an X-ray film cassette with film for a suitable exposure time.

Examples of proteins expression and differential detergent extraction results are shown in [Fig. 2](#).

### **3.9. Confocal Immunofluorescence for TJ Proteins**

1. For immunofluorescence labeling confluent T84 cells are plated on 0.33-cm<sup>2</sup> filters.
2. The cells should be rapidly rinsed twice with ice-cold HBSS+.
3. Precooled ethanol solution is then added to inserts for 20 min and stored at -20°C (200 µL in the upper and 1 mL in the lower chamber).

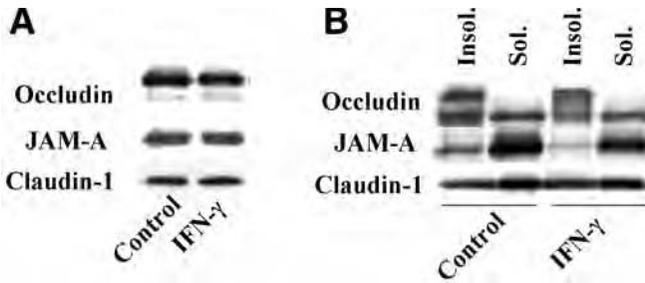


Fig. 2. Influence of IFN- $\gamma$  on protein expression level and differential detergent solubility of TJ transmembrane proteins. (A) Protein expression levels of occludin, JAM-A and claudin-1 in cells incubated with IFN- $\gamma$  (100 U/mL) for 48 h or control were determined. By using SDS-PAGE and immunoblotting for occludin, JAM-A, and claudin-1, no alteration in their overall expression was observed after treatment with IFN- $\gamma$ . (B) T84 monolayers treated with or without IFN- $\gamma$  (100 U/mL) for 48 h were incubated at 4°C with extraction buffer containing 1% TX-100. The TX-100-soluble (Sol.) and -insoluble (Insol.) fractions were analyzed by SDS-PAGE and immunoblotted for occludin, JAM-A, and claudin-1. Significant pools of occludin were TX-100 insoluble, whereas JAM-A and claudin-1 had a significant TX-100-soluble pool. In IFN- $\gamma$ -treated cells, we observed a change in the TX-100 insolubility profiles of JAM-A. The small fraction of JAM-A in the TX-100-insoluble pool was further diminished by cytokine exposure.

4. Ethanol is discarded and the samples are blocked by incubation in blocking buffer for 1 h at room temperature.
5. Disassemble the filters supports and place filters in buffer drops on parafilm in humidity chambers.
6. Transfer filters to 50- $\mu$ L drops containing primary antibody for 1 h at room temperature.
7. After incubation with the respective first antibody the filters are washed three times and incubated with secondary antibodies.
8. The filters are washed three times for 5 min with HBSS+.
9. Filters are mounted in mounting solution on glass slides. Nail varnish is used to seal the cover glasses and slides. The sample can be viewed immediately after the varnish has dry, or be stored in the dark at -20°C for as long as a month.

Confocal images showing internalized TJ transmembrane proteins after 48 h of IFN- $\gamma$  treatment are shown in [Fig. 3](#).

#### 4. Notes

1. Unless stated otherwise, all solutions should be prepared in water with a resistance of 18.2 M Ohm\*cm.
2. T84 cells should not be plated too thinly, otherwise they do not grow properly. A way to increase the density of cells is to trypsinize the cells and to place them in a smaller flask.

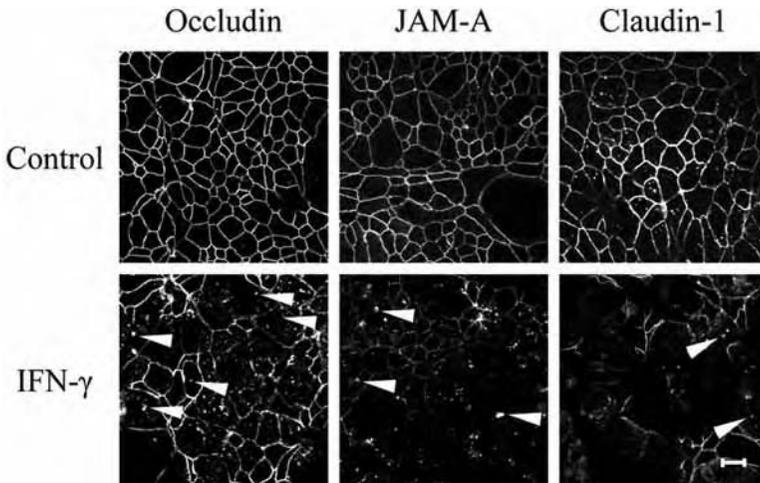


Fig. 3. IFN- $\gamma$  stimuli induce internalization of TJ transmembrane proteins. Localization of occludin, JAM-A and claudin-1 was determined by immunofluorescence labeling/confocal microscopy. In control T84 cells occludin, JAM-A and claudin-1 reveal typical distribution of these proteins in TJs. In T84 cells treated with 100 U/mL IFN- $\gamma$  (for 48 h, occludin, JAM-A, and claudin-1 were internalized from the lateral membrane [arrow-heads]). Scale bar = 10  $\mu$ m.

3. T84 cells express the IFN- $\gamma$  receptors exclusively basolaterally. Furthermore we observed that T84 cells responded to IFN- $\gamma$  if the cells are plated on 5- $\mu$ m pore size filters.
4. Before each experiment check TEER of each filter to maintain equal starting point for each experiment. The TEER should not be lower than 1000  $\Omega$ -cm<sup>2</sup>.
5. The TEER is definite as the resistance of a 1-cm<sup>2</sup> area:

$$\text{TEER} = R_{(\text{monolayer})} \cdot \pi \cdot d^2 \div 4$$

where d = diameter. Resistance readings for 24 mm or larger diameter inserts obtained by using the EVOM with the STX2 electrode should not be converted to unit area resistance. The Endohm is recommended for these large inserts.

6. Fluorescent-labeled dextran instead of radiolabeled solute was used for experiments. A consistent picomolar flux of FD in a range of sizes (3–70 kDa) across T84 epithelial monolayers has been demonstrated. FD flux is time- and dose-dependent (12).
7. Keep 1mL of HBSS+ in the lower chamber without FD-3 solution.
8. Protect plate from light.

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## Simultaneous Immunofluorescence Detection of Cadherin and $\beta$ -Catenin in Mouse Tissue Sections

Nobuko Obara

### Summary

Both cell–cell adhesion and secreted signaling molecules are involved in the regulation of normal tissue development and maintenance.  $\beta$ -catenin seems to link cadherin-mediated cell–cell adhesion and Wnt signaling. Thus, its activity in particular cells or tissues, either during normal development or in tumorigenesis, has become a fascinating topic of investigation. Because the multiple functions of  $\beta$ -catenin intimately relate to its amount and localization within a cell, immunohistochemical detection of this molecule simultaneously with the cell adhesion molecule cadherin should provide helpful information on its potential activities. This detection can be conducted on tissue sections by using appropriate tissue preparation procedures and primary antibodies from different species.

**Key Words:** Cadherin;  $\beta$ -catenin; cell adhesion; Wnt signaling; subcellular localization; immunofluorescence; confocal laser microscopy.

### 1. Introduction

Direct cell–cell adhesion mediated by cell-adhesion molecules, as well as indirect cell–cell interaction mediated by secreted signaling molecules, constitutes the fundamentals of normal tissue development and maintenance (1). Classical cadherins are the best-studied family of cell adhesion molecules that mediate  $\text{Ca}^{2+}$ -dependent cell–cell adhesion (2), and alterations in cadherin function have been implicated in tumorigenesis (3).  $\beta$ -catenin is one of the cytoplasmic components of the molecular assembly required for cell–cell adhesion mediated by cadherin (4), and this molecule also participates in the canonical Wnt signaling pathway (5,6). During embryonic development, the Wnt signaling regulates cell proliferation, cell fate specification, and differentiation. Misregulation of this Wnt signaling pathway caused by defects in Wnt components

leads to abnormality in embryonic development and to a variety of diseases, including cancer in adults (6). Because  $\beta$ -catenin has been presumed to be a key molecule integrating cadherin-mediated cell–cell adhesion with Wnt signaling (5,7,8), the activity of this molecule is an attractive subject to be studied in both normal development and tumorigenesis.

The amount and localization of  $\beta$ -catenin in a cell can be regulated by Wnt signaling. In the absence of Wnt signaling,  $\beta$ -catenin free from cadherin is phosphorylated and targeted for ubiquitination through interaction with axin, adenomatous polyposis coli, and the protein kinases casein kinase-1 (CK1) and glycogen synthase kinase-3 and is then degraded by the proteasome. In this situation, the level of  $\beta$ -catenin in the cytoplasm is kept to a low level. When cells are exposed to Wnt signals, activation of Wnt signaling inhibits  $\beta$ -catenin phosphorylation and, hence, its degradation. As a result,  $\beta$ -catenin accumulates in the cytoplasm and enters into the nucleus, where it interacts with T-cell factor/lymphoid enhancer factor (LEF/TCF) transcription factor to activate Wnt target genes (5,6). In this way, the amount of  $\beta$ -catenin changes depending on the Wnt signaling and its localization within a cell intimately relates to its function. Using confocal laser microscopy, we have demonstrated that abundant nuclear accumulation of  $\beta$ -catenin and upregulation of P-cadherin simultaneously take place in the enamel knot, the signaling center in the developing tooth germ (9). The use of primary antibodies from different species allows simultaneous detection of two or three antigens in general. Provided that the appropriate fixatives and antibodies are used, cadherins and  $\beta$ -catenin can be detected at the same time on a fixed and frozen tissue section, which has the advantage of providing tissue morphology of good quality. For this purpose, we have used periodate-lysine 2% paraformaldehyde (PLP) fixative solution (10).  $\beta$ -catenin colocalized at the cell surface along with cadherins most likely participates in cadherin-mediated cell–cell adhesion, whereas the same molecule accumulated in the cytoplasm or nucleus probably plays another role. Nuclear localization of  $\beta$ -catenin can be distinguished from the cytoplasmic one by use of nuclear counterstaining.

## 2. Materials

### 2.1. Preparation of Frozen Tissues

1. PLP fixative solution: To 50 mL of 200 mM lysine-HCl, add 100 mM dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) until the pH has been adjusted to 7.4 and then adjust the volume to 100 mL with 100 mM sodium phosphate buffer, pH 7.4 (stock solution A). Suspend 8 g of paraformaldehyde (extra pure, Merck) in 100 mL of distilled water and heat in a fume hood on a stirring hot plate at 60°C. Add a few drops of 1 N NaOH to the solution and stir until it clears (stock solution B). Both stock solutions should be stored at 4°C and used within 10 d (solution A) or 1 mo (stock solution B). Just before use, mix three volumes of stock solution A and 1 volume of

stock solution B, and add solid sodium *m*-periodate ( $\text{Na IO}_4$ ) to achieve a 10 mM concentration (do not re-adjust the pH).

2.  $\text{NH}_4\text{Cl}$ -PB: Prepare a solution of 100 mM sodium phosphate buffer (pH 7.4) containing 50 mM  $\text{NH}_4\text{Cl}$ . Store at 4°C.
3. 20% sucrose–phosphate-buffered saline (PBS): To prepare PBS containing 20% sucrose, dissolve 20 g of sucrose in 80 mL of PBS (150 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4), and add more PBS to make a volume of 100 mL. Store at 4°C. To make 10 and 15% sucrose–PBS solutions, mix this solution with PBS (*see Note 1*).
4. Embedding medium for tissue freezing: Tissue-Tek O. C. T. Compound (Sakura Finetek).
5. Tissue-embedding molds (*see Note 2*).
6. Spray freezers (*see Note 3*).

## 2.2. Indirect Immunofluorescence Staining for Cadherin, $\beta$ -Catenin, and the Nucleus

1. Glass slides coated with silane (*see Note 4*).
2. Glass cover slips.
3. Delimiting pen (Dako Cytomation, Denmark; *see Note 5*).
4. Moist chambers for glass slides (*see Note 6*).
5. Staining jars.
6. PBS: 150 mM NaCl in 10 mM sodium phosphate buffer, pH 7.4. Prepare 3 M NaCl and 200 mM sodium phosphate buffer, pH 7.4 (200 mM PB), and store at room temperature. To prepare PBS, mix 50 mL of 3 M NaCl, 50 mL of 200 mM PB, and 900 mL of distilled water.
7. PBS containing 0.1 % Triton X-100 (PBS-TX): prepare 10 % (v/v) Triton X-100 in distilled water. Mix 50 mL of 5 M NaCl, 50 mL of 200 mM PB, 10 mL of 10% Triton X-100, and 890 mL of distilled water.
8. Primary antibody dilution buffer: prepare Tris-buffered saline (150 mM NaCl in 20 mM Tris-HCl, pH 7.4) and supplement it with 1% bovine serum albumin (fraction V) and 0.1 % Triton X-100. Store at –20°C (*see Note 7*).
9. Rat monoclonal antibody against mouse E-cadherin (clone no. ECCD-2, Takara Bio, Otsu, Japan): reconstitute the lyophilized material according to the manufacturer's instruction, aliquot and store at –20°C. Avoid repeated freeze–thaw cycles to prevent denaturing the antibody (*see Note 8*).
10. Rabbit polyclonal anti-human  $\beta$ -catenin (BioSource International, Camarillo, CA, cat. no. AHO0462). Store at 4°C (*see Note 9*).
11. Goat anti-rat immunoglobulin (Ig)G conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR): protect from light and store at 4°C (*see Note 9*).
12. Goat anti-rabbit IgG conjugated with Alexa Fluor 568, highly cross-adsorbed (Molecular Probes, Eugene, OR): protect from light and store at 4°C (*see Note 9*).
13. Igs for negative control staining: normal rat IgG and normal rabbit IgG. Aliquot and store at –20°C (*see Note 10*).
14. Normal goat serum. Aliquot and store at –20°C (*see Note 10*).

15. DAPI: make 100  $\mu\text{M}$  solution in distilled water (stock solution). Protect from light and store at 4°C. To make a working solution, add 3  $\mu\text{L}$  of stock solution to 1 mL of PBS just before use (working concentration is 300 nM).
16. PermaFluor, aqueous mounting medium (Shandon, Pittsburgh, PA): store at 2 to 8°C.

### 3. Methods

In general, fixation before tissue freezing makes it easier to cut frozen sections and preserve tissue morphology of good quality in the sections. In some cases, however, fixation may lead to the loss of antigenicity. The conditions of tissue preparation suitable for immunohistochemistry depend on the nature of the individual antigen determinants. Furthermore, several antibodies are commercially available for a particular molecule and sometimes they react with different antigen determinants. It is thus crucial to choose an appropriate combination of tissue preparation procedures and antibodies to obtain good results. Both of the primary antibodies used here react with the antigens in frozen sections of mouse tissues fixed with the PLP fixative solution without any antigen retrieval procedures.

#### 3.1. Preparation of Frozen Sections

1. To obtain mouse embryos, mother mice are anesthetized with ether and killed by cervical dislocation. After sacrifice of the mother, embryonic tissues are dissected and immediately immersed in the ice-cold PLP fixative solution and fixed for 6 h in the same solution at 4°C. For sufficient penetration of the fixative solution, the size of the dissected tissues should be as small as possible (*see Note 11*).
2. To inactivate residual aldehyde groups, wash the fixed specimens three times for 30 min each in  $\text{NH}_4\text{Cl}$ -PB at 4°C and then overnight in the same solution. Immerse the specimens sequentially in 10, 15, and 20% sucrose-PBS solutions, for 2 h for each concentration, at 4°C (*see Note 12*).
3. Pour a small amount of Tissue-Tek O. C. T. Compound in the embedding mold and put the specimen in it. If necessary, information on the specimens (name of the tissue, date of preparation, orientation of the tissue, and so on) can be marked on the mold before embedment.
4. Quickly freeze the tissues with a spray freezer. Spray the freezing solution in a Petri dish until the bottom of the dish is covered with the solution, and place the embedding mold containing the specimen and Tissue-Tek O. C. T. Compound in it. The clear Tissue-Tek O. C. T. Compound turns white when it is frozen. Immediately put the frozen tissues into a freezer at  $-80^\circ\text{C}$  and store them at the same temperature before use (*see Note 13*).

#### 3.2. Indirect Immunofluorescence Staining and Observation

1. Cut frozen sections at 8  $\mu\text{m}$  with a cryostat at a temperature from  $-25$  to  $-28^\circ\text{C}$  (*see Note 14*). When the tissues stored at  $-80^\circ\text{C}$  are used, they should be placed for a while in the cryostat before cutting to allow equilibration of their temperature.

2. Pick up the sections on silane-coated glass slides, and air-dry them for 30 min at room temperature (see **Note 15**).
3. Draw a circle around the sections with a delimiting pen and wait until the drawn line becomes dry. To rehydrate the sections, immerse the glass slides in PBS for 10 min.
4. Blocking: to make blocking solution, dilute normal goat serum to 10% with PBS-TX. Remove remaining PBS in the circles on the glass slides, and cover the sections with the blocking solution. Place the glass slides in a moisture chamber and incubate them for 10 min at room temperature.
5. Make a mixture of diluted primary antibodies as follows: Add 0.5  $\mu$ L of rat anti-mouse E-cadherin monoclonal antibody (see **Note 16**) and 1  $\mu$ L of rabbit anti-human  $\beta$ -catenin polyclonal antibody (see **Note 17**) to 100  $\mu$ L of primary antibody dilution buffer. The final concentration of each antibody is 10  $\mu$ g/mL. For control staining, make a mixture of purified normal rat IgG and normal rabbit IgG in the same way.
6. Remove the blocking solution from the glass slides and carefully wipe the remaining blocking solution from the line drawn with the delimiting pen (see **Note 18**).
7. Cover the sections on the glass slides with the mixture of diluted primary antibodies (or for control staining, with that of normal Igs) and incubate them for either 2 h at room temperature or overnight at 4°C. In either case, the glass slides should be kept in moist chambers to avoid drying of the sections.
8. Make a mixture of secondary antibodies, Alexa Fluor-conjugated goat anti-rat IgG and anti-rabbit IgG, at a dilution of 1/500 in PBS-TX (see **Note 19**).
9. Wash the sections twice with PBS-TX and then once with PBS for 5 min each time.
10. Incubate the sections with the mixture of secondary antibodies at room temperature for 2 h. The glass slides should be kept in a moisture chamber and protected from the light.
11. Wash the sections twice with PBS-TX for 5 min each time.
12. Cover the sections with DAPI working solution (300 nM in PBS) and incubate for 10 min at room temperature.
13. After a brief wash with PBS, apply mounting medium to the sections and mount cover slips. Because the aqueous mounting medium PermaFluor solidifies and immobilizes the cover slip, sealing with nail varnish is not necessary.
14. The stained sections are examined under a confocal microscope to acquire digital images. Blue, green, and red fluorescence channels give the images of DAPI, E-cadherin, and  $\beta$ -catenin, respectively. Images for each of the three channels can be merged with software when necessary. An example of a triple-stained section illustrating both membrane-associated and nucleus-localized  $\beta$ -catenin is shown in **Fig. 1**.

#### 4. Notes

1. For long-term storage, autoclave the solution at 121°C for 15 min.
2. Instead of commercial embedding molds, cups made from aluminum foil can alternatively be used for the same purpose (**Fig. 2**).
3. Spray freezers used for cutting sections in a cryostat are available from many suppliers dealing in materials for histology and pathology.

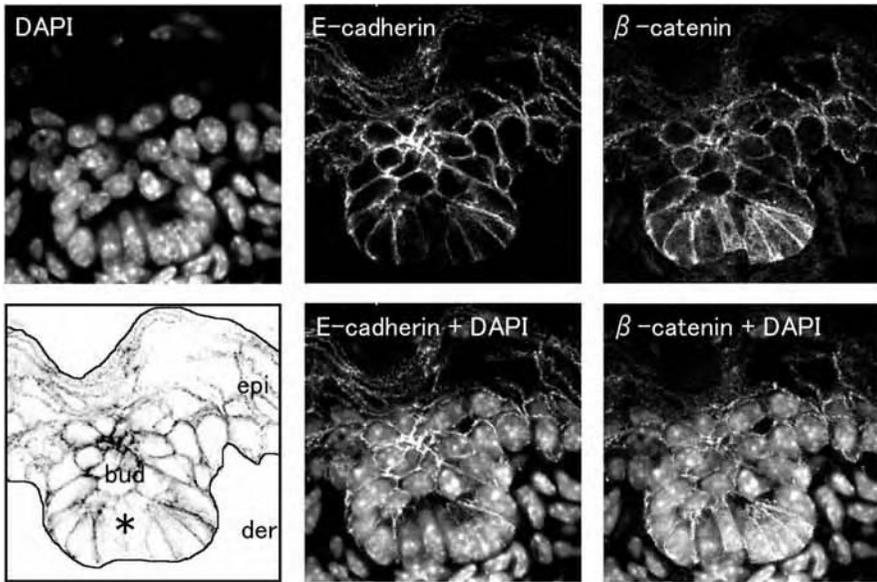


Fig. 1. Simultaneous immunolocalization of E-cadherin and  $\beta$ -catenin in the developing skin of a mouse embryo at embryonic day 17. Images for each of the three elements, i.e., DAPI, E-cadherin, and  $\beta$ -catenin, were acquired separately (upper images) and merged later with Photoshop (Adobe Systems) as shown in lower ones (E-cadherin + DAPI or  $\beta$ -catenin + DAPI). Tissue structure in the images is illustrated in the left lower box. E-cadherin is expressed only in the epidermis (epi) and epithelial bud of the developing hair follicle (bud), whereas  $\beta$ -catenin is expressed in both the dermis (der) and epidermis. At the tip of the epithelial bud (\*), fluorescence indicating E-cadherin expression is rather weak; in contrast, abundant accumulation of  $\beta$ -catenin in the cytoplasm and the nucleus is evident.

4. Glass slides coated with silane can be replaced by ones coated with poly-L-lysine. Both are available from Sigma (Silane-Prep Slides or Poly-Prep Slides). Alternatively, glass slides can be coated in the laboratory by using poly-L-lysine solution (also available from Sigma) according to the instructions provided by the manufacturer.
5. The water-repelling circle drawn with the pen provides a barrier to liquids such as antibody solutions applied to the sections, thus helping to obtain more uniform immunocytochemical staining and allowing a reduction in the amount of reagents used. Similar pens are available also under the designation "PAP pen" from Sigma and some other suppliers.
6. Although some moist chambers designed for immunohistochemistry are commercially available, the use of plastic boxes with some additional laboratory materials is sufficient. An example of such a chamber is shown in [Fig. 2](#).
7. This solution should contain 0.1%  $\text{NaN}_3$  as a preservative when stored at  $4^\circ\text{C}$ .

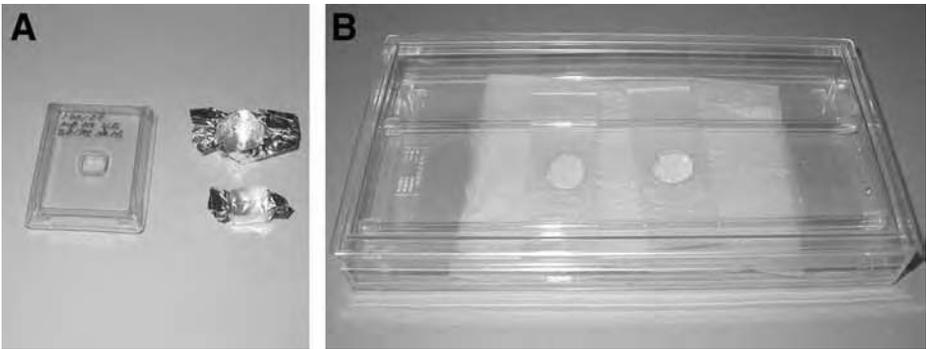


Fig. 2. **(A)** Tissue-embedding molds and **(B)** a moisture chamber. **(A)** A commercial tissue-embedding mold (**left**) and two examples for cups made from aluminum foil (**right**) are depicted. **(B)** An example for a homemade moisture chamber. In a plastic box with a cover, a piece of wet paper wiper is placed, and two glass tubes are aligned in parallel on it. For incubation with antibodies, glass slides bridge the glass tubes, as shown.

8. Reconstituted solution should be supplemented with 0.1%  $\text{NaN}_3$  as a preservative when stored at  $4^\circ\text{C}$ .
9. For long-term storage, aliquot and store at  $-20^\circ\text{C}$ .
10. Both purified normal immunoglobulins for negative control staining and normal sera for immunohistochemical blocking applications are available from many suppliers (Santa Cruz, Dako Cytomation, and so on).
11. The length of fixation should be changed depending on the size and the kind of tissues to be examined. Homogeneous and high-quality fixation can be achieved when the tissue is small enough to allow penetration of the fixative solution within a short time. A thickness of less than 2 mm may be appropriate for most kinds of tissues when they are fixed only by immersion in PLP solution. Alternatively, tissues of postnatal animals can be prepared by perfusion with PLP solution followed by excision and post-fixation with the same solution.
12. The time required for this treatment may depend on the size and the kind of tissues.
13. Alternatively, liquid nitrogen can be used for freezing samples. Pour a small amount of liquid nitrogen into a box made of styrene foam to fill the box with the vapor of liquid nitrogen. Put the embedding molds containing the specimens and Tissue-Tek O. C. T. Compound on a small styrene foam plate, place the plate in the vapor-filled box, and keep it there until the Tissue-Tek O. C. T. Compound has turned white.
14. The appropriate temperature differs depending on the kind of tissues. Also, the temperature should be changed when sections are cut at a different thickness. In general, a higher temperature is required when sections are cut thicker.
15. The dried frozen sections can be stored at  $-20$  or  $-80^\circ\text{C}$  for a few weeks or more. However, the stability of individual antigens may vary greatly from one to another.
16. Original concentration of the rat monoclonal antibody against mouse E-cadherin is 2 mg/mL when it is reconstituted according to the manufacturer's instructions;

and the recommended working concentration for immunohistochemistry indicated in these instructions is 10 µg/mL.

17. Concentration of the undiluted antibody is 1 mg/mL, and the recommended working concentration for immunohistochemistry is 10 to 20 µg/mL according to the manufacturer's instructions.
18. The water-repelling line drawn with the pen is ineffective if it is covered with liquid containing detergent (e.g., Triton X-100).
19. The original concentration of Alexa Fluor-conjugated secondary antibodies is 2 mg/mL, and the recommended working concentration is 10 to 20 µg/mL according to the manufacturer's instructions.

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## In Vitro Neutrophil Transepithelial Migration

Winston Y. Lee, Alex C. Chin, Susan Voss, and Charles A. Parkos

### Summary

Polymorphonuclear leukocyte (PMN) transmigration into tissues is a highly regulated process and plays a central role in host defense. In inflammatory human diseases such as ulcerative colitis and Crohn's disease, the infiltration of intestinal mucosa by large numbers of PMNs contributes to epithelial pathophysiology. The sequence of events that fine-tune PMN migration across epithelial cells is not well-understood. In this chapter, we describe a method to study PMN transmigration across intestinal epithelial T84 monolayers using a modified Boyden chamber system. This in vitro model system consists of three main components: the epithelium, purified PMN, and a chemoattractant gradient. Intestinal epithelial cells are cultured as inverted monolayers on permeable filter supports to facilitate the study of PMN transmigration in the physiologically relevant basolateral-to-apical direction. PMNs are isolated from human blood using dextran sedimentation followed by Ficoll density gradient centrifugation. PMN transmigration is elicited using *N*-formyl-methionyl-leucyl-phenylalanine gradients and is quantified by assaying for myeloperoxidase activity. The advantages of this model are its reductionist approach and the fact that the system can be easily manipulated. Studies using this model system will shed more light on the mechanisms regulating PMN responses in acute inflammatory diseases.

**Key Words:** Neutrophil; PMN; intestinal epithelial cells; T84; chemotactic gradients; fMLP; Boyden chamber; myeloperoxidase; transmigration.

### 1. Introduction

As a first line of defense, polymorphonuclear leukocytes (PMNs) play a critical role in many inflammatory processes. For example, in acute illnesses such as bronchial pneumonia and bacterial enterocolitis and in chronic diseases such as inflammatory bowel disease and asthma, large numbers of PMNs infiltrate through tissues and across mucosal surfaces at sites of inflammation (1–4). Furthermore, in ischemic injuries of the heart, kidney, and intestine, subsequent PMN infiltration can result in reperfusion injury (5–8). In these

examples, the severity of symptoms correlates with the degree of PMN infiltration. In mucosal organs, the presence of PMN transepithelial migration correlates strongly with disease pathophysiology.

For PMNs to exit the circulation and migrate across mucosal surfaces, they must cross vascular endothelium, interstitial matrix (including mesenchymal tissue) and, finally, the epithelium. This process involves a complex series of signaling events between PMN and other cells within the tissue. At sites of inflammation, activated vascular endothelial cells secrete mediators, such as tumor necrosis factor- $\alpha$  and interleukin-8, that are important in PMN recruitment. In addition, the endothelium also expresses surface adhesion molecules that facilitate adhesion of activated PMN (9,10). Selectin-mediated rolling of PMN along the vascular endothelium within the microcirculation at inflammatory sites precedes firm, integrin-mediated adhesion that is followed by extravasation either transcellularly or paracellularly through the vascular endothelium (11,12). Afterward, the movement of subendothelial PMN in the interstitial matrix is guided by gradients of signals derived from invading microorganisms such as bacterial peptides, or agents elicited as cellular responses to injury/invasion such as interleukin-8 (13–16). To migrate across mucosal epithelia, PMNs must first adhere to the basolateral membrane, an event mediated by  $\beta_2$ -integrins (17–19). Subsequently, PMNs migrate along the paracellular space between epithelial cells where a series of ill-defined signaling events facilitate the passage of the PMN across tight junctions and into the lumen. Recent studies have highlighted the importance of junctional adhesion molecule protein family members (20) and CD47-SIRP $\alpha$  interactions (21,22) as mediators of PMN migration across the epithelial paracellular space.

Most of what is known about PMN recruitment, extravasation, and transmigration is derived from studies conducted with modified Boyden chamber systems (Fig. 1). This *in vitro* model of transepithelial or transendothelial migration consists of three components: an epithelial or endothelial monolayer cultured on a permeable support of defined pore size, purified PMNs, and a chemotactic gradient. For epithelial monolayers, cells are grown on such permeable filter supports until confluency is reached, as determined by a number of methods. Freshly isolated PMNs are added to one side of the monolayer and stimulated to transmigrate across the monolayer/filter in the presence of a chemotactic gradient. The utility of this model is its reductionist nature, where the minimal necessary components are assembled to interact under simple conditions. Another utility is the ease with which this system can be manipulated. For instance, antibodies or pharmacological agents can be added to the system to see their effects on transmigration (17,20–23). Cell monolayers can also be stimulated with cytokines and evaluated for alterations in characteristics of PMN

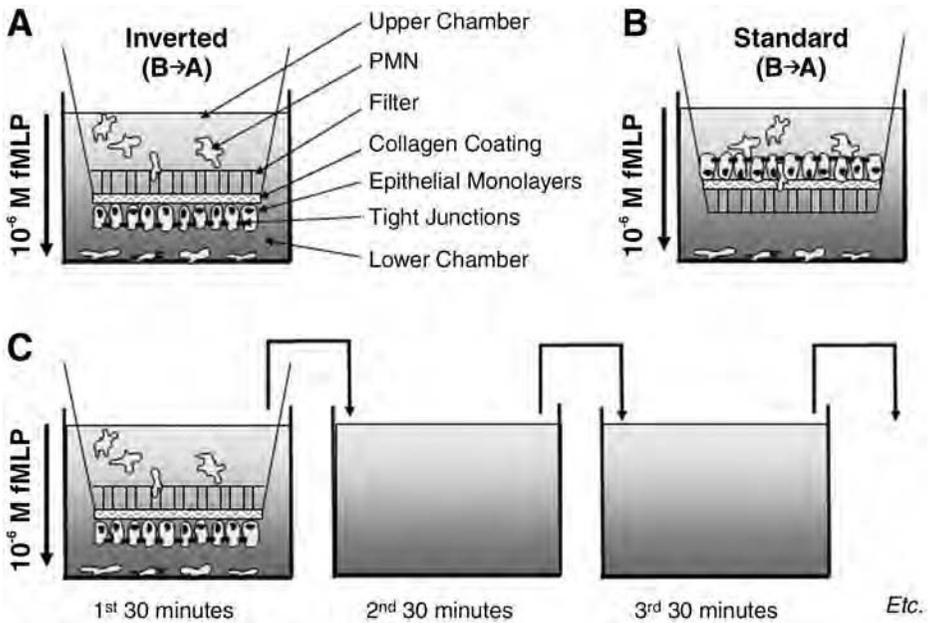


Fig. 1. Illustrations of different setups of the PMN transepithelial migration assay. (A) In this inverted configuration, the apical sides of epithelial monolayer are grown facing the bottom of the well. The different shades of gray represent the chemotactic gradients of fMLP, which dictates the directionality of PMN migration. Therefore, in this setup, PMN will migrate from basolateral side to the apical side of the monolayer (B→A), then detach and settle at the bottom of the well. This setup recapitulates PMN migration across the mucosal surfaces during inflammation. (B) In this “standard” configuration, the apical side of the monolayer is facing away from the bottom of the well. PMN will migrate from the apical side to the basolateral side of the monolayer (A→B), then detach and settle at the bottom of the well. (C) To perform a kinetic assay of PMN transmigration across the epithelial monolayer, the transwell is serially transferred into new wells containing chemotactic gradients of fMLP for each time point needed.

transepithelial migration (24). Although many variations of this system exist, this discussion will focus on the most commonly used transwell setup, in which freshly isolated human PMN transmigrate across T84 intestinal epithelial monolayers in response to *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) gradients.

## 2. Materials

### 2.1. Plating T84 Monolayers

1. Transwell inserts with surface areas of 0.33 cm<sup>2</sup> and pore size of 5 μm placed in a 24-well flat bottom cell culture plate (Costar).

2. T84 cell medium is a 1:1 mixture of Dulbecco's Modified Eagle's Medium and F-12 Nutrient Mixture, supplemented with 0.014 M NaHCO<sub>3</sub>, 0.015 M HEPES, penicillin, streptomycin, and 6% fetal calf serum (Life Technologies/Gibco).
3. 0.05% Trypsin-EDTA (Cellgro).
4. Transepithelial resistance is measured by using an EVOM™ Epithelial Voltometer (World Precision Instruments).
5. Collagen stock can be prepared from rat tail tendon using methods modified from Cereijido and others (25). Dissect the rat tail tendon by peeling off the shiny white layer underneath skin with a hemostat. The tendon is then minced and divided into 75-mg aliquots. At this point, the tendon can be stored at -80°C. Dehydrate the 75 mg of tendon in 70% ethanol for 15 minutes. Solubilize tendons in 100 mL of 1% (v/v) acetic acid at 4°C. Stir the mixture at 4°C overnight. The next morning, the mixture will be viscous, and some undissolved materials will remain. Spin the collagen at 25,000g for 30 min. Dialyze the spun collagen solution against at least 2 L of cold distilled water with two to three changes. The dialyzed collagen will be referred to as the collagen stock and stored at 4°C.

## 2.2. PMN Isolation From Human Blood

1. Sterile 10-mL and 30-mL plastic syringes and 21- and 20.5-gage needles (Becton, Dickinson, and Company).
2. Cell culture grade distilled and deionized water (ddH<sub>2</sub>O) with impedance greater than 18Ω.
3. Anti-coagulation buffer: 4% sodium citrate (Sigma) w/v in ddH<sub>2</sub>O.
4. Normal saline: 0.9% sodium chloride (Sigma) w/v in ddH<sub>2</sub>O.
5. Sedimentation buffer: 2% dextran 500 (Amersham Bioscience) w/v in normal saline.
6. Chilled Ficoll-Paque™ Plus (Amersham Bioscience).
7. Lysis buffer: 0.8% ammonium chloride supplemented with 1.9 mg of EDTA and 0.05 g of sodium bicarbonate per 50 mL of buffer.
8. Hank's balanced buffered saline (HBSS) with calcium (HBSS+) and without calcium (HBSS-; Sigma).

## 2.3. Transmigration and Myeloperoxidase Assay

1. 10<sup>-2</sup> M fMLP (Sigma) stock dissolved in dimethyl sulfoxide. Subsequent dilutions of the stock are done in HBSS+.
2. 1 M sodium citrate in water, pH 4.5.
3. 10% Triton-X 100 in water.
4. 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) buffer: for every 50 mL, add 0.036 g of ABTS, 5 mL of 1 M sodium citrate, 45 mL of water, and 100 μL of 30% w/v H<sub>2</sub>O<sub>2</sub>.
5. Flat bottom 96-well plates (Falcon).
6. Microplate reader with 405 nm filter (Molecular Devices).

### 3. Methods

#### 3.1. Culturing T84 Intestinal Epithelial Cell Monolayers on Transwell Filter Supports

1. The night before seeding T84 cells on transwells, coat the sides of transfilters (*see Note 1*) onto which cells will be seeded with collagen in a 150 × 25-mm Petri dish in a laminar flow hood (*see Note 2*). To do so, dilute collagen stock 100-fold in 60% (v/v) ethanol in water, and transfer 50  $\mu\text{L}$  to each filter. Let the filters dry overnight.
2. As T84 cells reach near confluency in a T162 flask, the cells are first washed with phosphate-buffered saline then detach with trypsin–EDTA. One T162 flask should be treated with 1 mL of trypsin–EDTA and incubated at 37°C for 30 min. To culture monolayers in a normal configuration, in which the apical membranes of epithelial cells face upward, trypsin–EDTA-treated cells are diluted 1:20 in media and 162  $\mu\text{L}$  of the cell suspension is seeded on the collagen coated filter support, which is placed in a 24-well tissue culture dish containing 1 mL of media in each well. To culture monolayers in an inverted configuration, in which the apical membranes of the cells will be facing downward, the trypsin–EDTA elicited cells are diluted 1:10 in media and 81  $\mu\text{L}$  of the suspension is placed on the underside of collagen coated transfilters that are resting upside down in a 15-cm<sup>2</sup> sterile Petri dish (*see Note 2*). The cells are incubated at 37°C overnight to allow for adhesion. Afterwards, the inverted transwells are simply “flipped” and placed into 24-well tissue culture wells filled with 1 mL of fresh T84 medium. The upper chamber is then supplemented with 162  $\mu\text{L}$  of medium.
3. Like many other epithelial cell lines that are maintained in the laboratory, T84 cells will, over time, begin to exhibit aberrant phenotypes, presumably because of genetic drift. To monitor for such “drift,” we routinely measure transepithelial resistance (TER) as T84 cells are becoming confluent in transwells (**Fig. 2A**). Typically, TER increases over time after plating of cells and plateaus between days 7 and 10. For T84 cells, during this 3-d window, TER typically ranges from 800 to 2500  $\Omega\text{-cm}^2$ , which is considered satisfactory (*see Note 3*).

#### 3.2. Isolation of PMNs

1. From experience, we have observed that substantial care must be taken to avoid preactivation of PMNs during the process of isolation. Such preactivation is likely to occur secondary to exposure to trace amounts of microbial-derived products in buffers, glassware, and so on. Assays with preactivated PMNs typically yield altered results. It is recommended to make fresh solutions with sterile water and reagents from dedicated chemical sources each time before isolation.
2. Blood collection and plasma separation: fill a syringe with enough 3.8% sodium citrate so that the final concentration after blood collection will be 0.38%. For example, to collect 27 mL of blood, fill the syringe with 3 mL of 3.8% sodium citrate. After drawing blood, invert the syringe several times, and

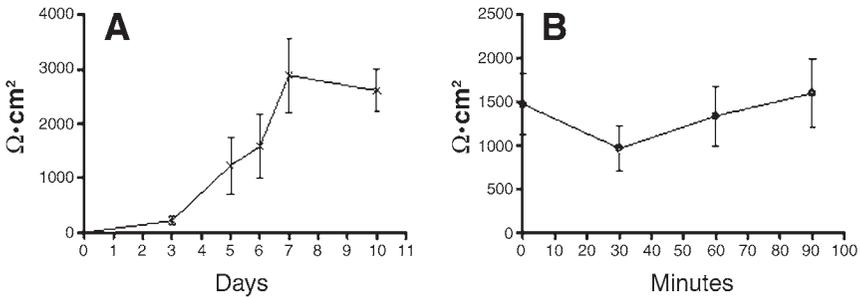


Fig. 2. Measuring TER to monitor the growth of epithelial monolayers. **(A)** TER was measured from the day of seeding epithelial cells onto the permeable support (day 0) to day 10 in the inverted configuration. From day 3 to 7, TER increased rapidly, indicating that epithelial cells were growing rapidly. Then, TER stabilized as the monolayer becomes confluent. It was during the 3-d window that we performed the transmigration assay. **(B)** TER was monitored every 30 min for 90 min after washing epithelial monolayers with HBSS+. Notice how the TER decreased during the first 30 min and then recovered in the next hour. It is recommended to let the monolayers “recover” before performing the transmigration assay.

transfer the blood to a 50-mL conical tube. Centrifuge blood at 162g for 10 min without braking at room temperature, which will separate the blood into three layers: a yellow plasma layer, a thin white buffy coat, and a dark layer of red blood cells at the bottom.

3. Dextran sedimentation of red blood cells (RBCs): from the aforementioned centrifuged blood sample, remove the plasma layer with a clean plastic pipet, taking care not to disturb the white buffy coat. It is not a problem if a small amount of plasma remains on top of the buffy coat. Then, add an equal volume of chilled sedimentation solution and gently invert the tube several times until the dark red layer becomes suspended. Place the conical tube upright at 4°C for 45 min. As the RBCs sediment, forming a thick red layer at the bottom, a pink turbid fluid phase will appear on top. Transfer the pink upper phase to a new 50-mL conical tube and centrifuge at 162g for 10 min at 4°C with the brake turned on. At the same time, aliquot 3 mL of chilled Ficoll into a 15-mL conical tube. After centrifugation, remove the supernatant with a sterile pipet and gently resuspend the pellet in 10 mL of normal saline.
4. Separation of PMN/RBC from monocytes/lymphocytes with a Ficoll gradient: carefully layer the resuspended cell pellet onto the 3 mL of Ficoll using a syringe with a 21-gage needle. After centrifugation at 162g for 30 min at 4°C, four distinct layers will appear. On top there will be a clear fluid phase of approx 8 mL. Immediately below will be a thin cloudy layer consisting of monocytes and lymphocytes. Underneath the thin cloudy layer is a clear Ficoll phase, which overlies a red pellet consisting of RBCs and PMNs.

5. RBC lysis: from the aforementioned Ficoll separation, remove all layers except the red pellet. Gently resuspend the pellet in 50 mL of cold lysis buffer and incubate on ice for no more than 2 min. As RBCs undergo lysis, the suspension will become less turbid. Immediately centrifuge the lysis suspension at 162g for 10 min at 4°C followed by a wash with 50 mL of cold HBSS–.
6. Calculating the yield of PMNs: For each 30 mL of blood drawn, resuspend washed PMN in 1 mL of HBSS–. Mix 10  $\mu$ L of PMN with 990  $\mu$ L of 2% acetic acid. The acetic acid will facilitate in the counting of PMN nuclei, which look multi-lobular, whereas the nuclei of monocytes and lymphocytes will be round (see **Note 4**). Estimate the yield of the cells with a hemacytometer (see **Note 5**).
7. Assessing the state of activation of PMNs: PMNs can easily become primed if at any point during the preparation they are exposed to microbial product contaminants. PMNs from contaminated preparations will migrate across collagen coated filters in significant numbers within the first 30 min of exposure to an fMLP gradient, whereas we have observed that unprimed PMN barely migrate. However, over a time course of 2 h, primed PMNs will migrate significantly less than unprimed PMNs. To perform this assay, place a collagen-coated 5- $\mu$ m pore-sized transwell filter in a 24-well tissue culture plate. Transfer 100  $\mu$ L of HBSS+ and  $10^6$  PMNs in 20  $\mu$ L of HBSS– into the upper chamber; then place 500  $\mu$ L of  $10^{-7}$  M fMLP in HBSS+ in the lower chamber. Incubate for 25 min at 37°C and visually inspect the number of PMNs migrated using a  $\times 50$  field of view. We routinely use this assay before major experiments as a way to evaluate whether PMN preparations are primed or not.

### 3.3. Transmigration of PMNs Across Intestinal Epithelial Cell Monolayers

1. Because epithelial cells are known to secrete growth factors and other cytokines that may modulate transepithelial migration of PMNs, it is important to wash epithelial monolayers with 200  $\mu$ L of warm HBSS+ in the upper chamber and 1 mL in the lower chamber. Immediately after washing, we have observed that the TER may fluctuate; therefore, allow monolayers to “recover” from washing for 45 min at 37°C. Measure the TER (**Fig. 2B**). At this point, the TER should have equilibrated, usually to a lower resistance (see **Note 6**). If kinetic assays are desired, one additional well filled with 1 mL of  $10^{-6}$  M fMLP in HBSS+ should be added per time point for each monolayer. For example, if the experiment involves a kinetic assay with a 30-min time-point over 2 h, it will be necessary to fill four additional wells with chemoattractant.
2. After equilibration of T84 monolayers, remove the HBSS+ from the upper and lower chambers and replace with 100  $\mu$ L and 500  $\mu$ L of HBSS+, respectively. Then, apply the number of PMN needed in a volume of 20  $\mu$ L of HBSS–. For example, if applying  $10^6$  PMNs, add 20  $\mu$ L of PMN suspension at the density of  $5 \times 10^7$  cells/mL. If desired, one can add antibodies or pharmacological agents into the top chamber (see **Note 7**). It is important not to vary volumes of buffers used in upper and lower chambers between experiments. Differences in volumes used can change chemotactic gradients and result in different hydrostatic pressures, which can alter the pattern of transmigration. To initiate transmigration,

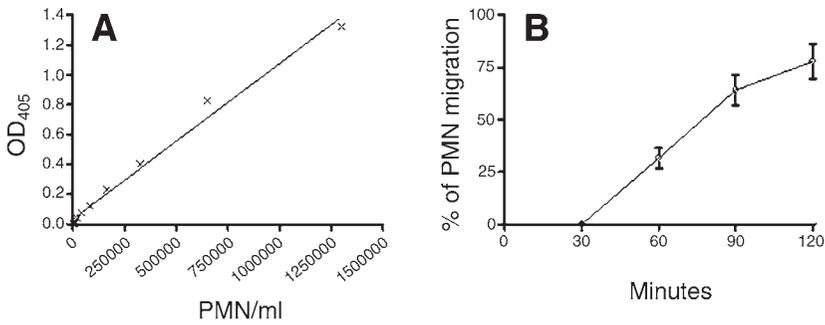


Fig. 3. Using myeloperoxidase assay to quantify PMN in transmigration assay. **(A)** Serial dilutions of PMNs are quantified using myeloperoxidase assays. The OD<sub>405</sub> correlates with the PMN concentration linearly. This linear correlation ( $r^2 = 0.987$ ) allows us to calculate unknown concentrations of PMNs. **(B)** A typical 2-h course of PMN transepithelial migration quantified using myeloperoxidase assays.

add 5  $\mu$ L of  $10^{-4}$  M fMLP to all bottom chambers so that the final concentration of fMLP in the bottom chambers will be  $10^{-6}$  M. Incubate the transwells at  $37^\circ\text{C}$  and serially transfer the monolayers at each time-point into one of the chemo-attractant containing wells that have been prepared for the assay. Swirl the transwell plate lightly before transferring. After the last time point, place the insert in a well with 500  $\mu$ L of HBSS+ (Fig. 1).

- To quantify migrated PMN, the azurophil granule enzyme myeloperoxidase activity is assayed using a simple colorimetric assay. To prepare the standard curve, serially dilute PMN suspension before transmigration (see Note 8). The standard curve should range from  $1.5 \times 10^6$  to  $2 \times 10^5$  PMNs/mL (see Note 9). Add 1 M sodium citrate buffer and 10% Triton-X100 solution in a 1:10 ratio to each well and dilution of the standard. Puncture the filter supports with 100- $\mu$ L pipet tips to solubilize migrated PMNs. Place the plates on a rocking or swirling platform for 15 min at room temperature to permeabilize the cells. Transfer 100  $\mu$ L of PMN lysate from each transwell to a well in a flat bottom 96-well plate. With a multi-channel pipet, aliquot 100  $\mu$ L of ABTS solution to the PMN lysate in the plate. Allow color to develop until the highest OD<sub>405</sub> reading in the standard is approx 2.5. With an OD of 2.5 for the highest reading, the standard curve should be linear. Standard software such as MS Excel® can be used to perform calculations. The standard curve allows both calculation of cell numbers from OD readings and normalization among samples (Fig. 3).

#### 4. Notes

- The pore size of transwell filters should be no less than 3  $\mu$ m in diameter. Otherwise, PMNs will not be able to migrate through the filters.
- T84 intestinal epithelial cells can be seeded either on the top or bottom sides of transwell filters. If T84 cells are seeded on top, the apical membranes will face

upwards resulting in a “standard” configuration. However, with the standard configuration, PMNs will transmigrate across the epithelial monolayers in an apical-to-basolateral direction which is opposite to what occurs in vivo. To recapitulate transmigration in a physiologically relevant direction, T84s can be cultured on the underside of filters in an “inverted” configuration in which the basolateral aspect of the monolayer is facing upwards. Using such inverted monolayers, PMNs will migrate in a basolateral-to-apical direction which is consistent with the direction of PMN transmigration in vivo.

3. When T84 cells or other epithelia are cultured for many passages (typically more than 15 or 20 passages), monolayer properties commonly change as manifested by a faster growth rate and altered barrier function. Epithelial cells may grow and divide through the filter pores and form monolayers on both sides of the transwell filters resulting in artificially high TER ( $>3000 \Omega\text{-cm}^2$ ). Conversely, cells can form poor barriers with low TER values. Thus, when culturing monolayers on permeable supports, if TER values start to drift, it may be worth considering bringing up a new batch of cells.
4. A Trypan Blue exclusion test also can be used to determine the viability of the cells in addition to estimating the yield of the cells in the preparation. However, it is more difficult to distinguish monocytes from PMNs with Trypan Blue. Typically, we obtain more than 99% viability of cells isolated by these methods.
5. For every 30 mL of blood, the yield is generally in the range of  $5 \times 10^7$  PMNs. However, it may be worth considering to maintain a donor log to obtain a reasonable prediction of the expected PMN Yield among individual donors.
6. After the initial wash, TER often will increase by 10% and subsequently decrease as the cells are equilibrating.
7. To test the effects of antibodies on transmigration, generally it is sufficient to add antibody at a concentration of 10 to 20  $\mu\text{g/mL}$  in the upper chamber. The diffusion of antibody to the lower chamber can be assumed to be minimal during the time course of the transmigration assay. However, for pharmacological agents, one should consider adding the agent in both upper and lower chambers. It may be necessary to optimize the assay for each antibody or agent.
8. Once PMNs are exposed to calcium in HBSS+, they are often more difficult to handle because of cell clumping and aggregation. Therefore, it is better to serially dilute PMN in cold calcium free HBSS– before use in transmigration assays.
9. After subtracting the blank OD values from the standard wells, the OD readings of the standards should be proportional to the dilution factor used for the samples. For instance, if  $10^6$  cells give an OD reading of 2.2, then the OD reading of  $5 \times 10^5$  cells should be 1.1.

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## The Blot Rolling Assay

### *A Method for Identifying Adhesion Molecules Mediating Binding Under Shear Conditions*

**Robert Sackstein and Robert Fuhlbrigge**

#### Summary

Adhesive interactions of cells with blood vessel walls under flow conditions are critical to a variety of processes, including hemostasis, leukocyte trafficking, tumor metastasis, and atherosclerosis. We have developed a new technique for the observation of binding interactions under shear, which we have termed the “blot rolling assay.” In this method, molecules in a complex mixture are resolved by gel electrophoresis and transferred to a membrane. This membrane can be rendered semitransparent and incorporated into a parallel-plate flow chamber apparatus. Cells or particles bearing adhesion proteins of interest are then introduced into the chamber under controlled flow, and their interactions with individual components of the immobilized substrates can be visualized in real time. The substrate molecules can be identified by staining with specific antibodies or by excising the relevant band(s) and performing mass spectrometry or microsequencing of the isolated material. Thus, this method allows for the identification, within a complex mixture and without previous isolation or purification, of both known and novel adhesion molecules capable of binding under shear conditions.

**Key Words:** Adhesion molecules; shear stress; shear conditions; flow conditions; parallel plate flow chamber; Western blot; selectins; selectin ligands; blot rolling assay; gel electrophoresis.

#### 1. Introduction

The adhesion of cells to blood vessel walls under shear stress is central to many important physiological and pathological processes. Importantly, shear forces critically influence the adhesion of circulating cells and platelets to vessel walls in physiological cell migration and hemostasis, as well as in inflammatory and thrombotic disorders, cancer metastasis, and atherosclerosis. Although various techniques for analyzing cell adhesion have been described, most involve binding

assays under static conditions and thus do not assess dynamic function. This limitation can introduce bias and exaggerate the relative role(s) of specific adhesion molecules. Notably, certain adhesive receptor–ligand interactions occur preferentially if not exclusively under physiological shear stress (e.g., binding of L-selectin to its ligands) or depend on low affinity and rapidly reversible interactions to serve their functions (e.g., rolling of leukocytes via selectins). Under static binding assay conditions, these types of interactions may be overtly neglected or overshadowed by molecules specialized to form more stable adhesions.

Methods for *in vitro* study of adhesion under shear conditions have been described, including the Stamper-Woodruff (1) and the parallel plate flow chamber (2) assays. However, these methods are constrained in that they require the availability of reagents (e.g., antibodies) that can specifically interfere with the activity of relevant receptors and/or ligands or require purified substrate materials that can be immobilized on the chamber wall. Thus, the applicability of these methods to examine the structure or function of previously uncharacterized ligands is highly limited.

To address these issues, we developed a method for direct real-time observation of adhesive interactions between cells or particles in flow and proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immobilized on membranes (3). This technique, called the “blot rolling assay,” has allowed for the identification of several new glycoprotein ligands for selectins (4,5), and this approach recently has been extended to the study of glycolipid selectin ligands (6). This method allows the real-time assessment and measurement of interaction parameters (e.g., rolling vs firm attachment, specificity, and reversibility with inhibitors) in both physiological and nonphysiological shear conditions, thus permitting a unique user interface for the observation of adhesive events on membrane-immobilized materials. Ligands under investigation can be immobilized directly or segregated by gel electrophoresis (e.g., SDS-PAGE, isoelectric focusing) or other methods before transfer to the membrane. This method also provides for the real-time manipulation of interaction conditions, including wall shear stress, ion requirements, temperature, the influence of metabolic inhibitors, and the presence of activating agents or inhibitors of cell function. Blot-immobilized substrates can be used repeatedly, allowing the *in situ* manipulation of the substrate under continuous direct visualization or direct comparison of different conditions or different cell populations in shear flow. The capacity to observe sequential experimental and control conditions on a single substrate and to observe physiological behaviors and responses to manipulations in real time, provides particularly powerful advantages of this method over conventional static binding assays. Similarly, the ability to observe interaction with individual components of a complex mixture without requiring purification or previous knowledge

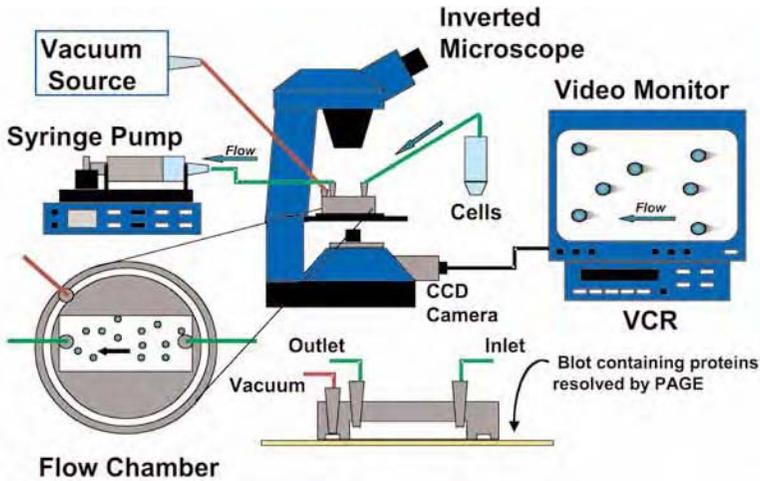


Fig. 1. Diagram of blot rolling assay assembly. The components of the blot rolling assay are shown, including the inverted microscope, flow chamber, CCD camera, syringe pump, vacuum source, video monitor and blot membrane (resting on a Petri dish located on the microscope stage, with the flow chamber positioned on top of it).

of the nature of the components provides advantages over conventional flow based assays. In this chapter, we describe a detailed protocol for performing blot rolling assays of membrane proteins. A video recording of a representative experiment is available at <http://dermatology.bwh.harvard.edu/leukocyte.html>.

## 2. Materials

1. The required components for performing blot rolling assays include an inverted microscope and a parallel plate flow chamber apparatus. **Figure 1** shows the setup used in our laboratories. The parallel plate device we use has internal chamber diameters of  $2 \times 0.5 \times 0.025$  cm. A commercial product with similar properties is available (Circular flow chamber, GlycoTech Corp., Rockville, MD, product no. 31-001). Flow is adjusted by negative pressure regulated by a high-precision, programmable syringe pump (Harvard Apparatus). Wall shear stress ( $\text{dyne/cm}^2$ ) is calculated according to the formula:  $\text{wall shear stress} = 6 \mu Q / wh^2$ , where  $w$  = chamber width (cm),  $h$  = chamber height (cm), and  $Q$  = volumetric flow rate (mL/s).  $\mu$  = viscosity (poise, or  $\text{dyne} \times \text{s/cm}^2$ ; for water at  $25^\circ\text{C}$ ,  $\mu = 0.009$ ), which is adjusted for contribution of 10% glycerol (see **step 8**), according to the equation  $\mu = 1.37 \times \text{medium}$  ( $\mu = 0.0123$  at  $25^\circ\text{C}$ ; see **Subheading 3.4., step 8**, for calculations based on our chamber dimensions). Interaction events can be captured on videotape by using a standard videomicroscopy camera and video recording assembly (**Fig. 1**), for later off-line analysis.

2. PAGE gel system.
3. Sequi-Blot™ polyvinylidene difluoride (PVDF) membrane (Bio-Rad, cat. no. 162-0184).
4. Fetal bovine serum.
5. 0.1% Tween-20–phosphate-buffered saline (PBS) solution.
6. Prestained molecular weight markers.
7. Western Blue stabilized substrate for alkaline phosphate (Promega, cat. no. S3841).
8. Blot rolling medium (H/H medium): Hank's balanced salt solution (Gibco, cat. no. 14170-112) with 10 mM HEPES, pH 7.4. Additions to H/H medium: 2 mM CaCl<sub>2</sub>, 5 mM Na<sub>2</sub> EDTA, and 10% glycerol (American BioAnalytical).
9. Monoclonal or polyclonal antibody reagents for identifying any epitope(s) of interest.

### 3. Methods

#### 3.1. Preparation of Western Blots/Visualization of Protein Bands

1. Material of interest can be prepared for PAGE (with or without SDS) using standard methods. In general, we prepare lysates of cells at  $4 \times 10^8$  cell equivalents per milliliter of lysate buffer. A number of standard detergents have been used for the preparation of lysates, although we have found 1% n-octylglucoside (Roche Molecular Biochemicals, Indianapolis, IN) to provide low nonspecific background binding. Acrylamide gradient gels (e.g., Criterion 4–20%; Bio-Rad) provide improved resolution in higher molecular weight ranges. The amount of protein required will vary with the material under study, but we have found 25 to 100  $\mu$ g of protein per lane to be sufficient for most of our studies. Both reducing and non-reducing gel conditions have been utilized with good results. It is helpful to include prestained molecular weight standards in the lanes adjacent to the material of interest to assist in alignment and localization of regions supporting adherence.
2. When gel electrophoresis is complete, the component proteins are transferred to PVDF membrane using standard transfer methods; although other membrane materials may work as well, we have preferred Bio-Rad Sequi-Blot™, which has 0.2- $\mu$ m pore size and minimal surface irregularities, thus promoting laminar flow over the membrane.
3. After transfer, the blot is placed in a blocking solution to block nonspecific interactions and incubated with gentle agitation (on a rocker platform or rotating platform) for 1 h at room temperature. It is important to place the blot in the dish with the protein side facing up (i.e., the side of the membrane that was facing the gel). The blot is then washed in PBS–0.1% Tween-20 twice for 5 min each with gentle agitation.
4. Immunostaining, if desired, can be performed using standard procedures. Membranes typically are stained with primary antibody for 1 h under gentle agitation, washed twice in PBS–0.1% Tween-20 for 5 min, and then incubated with secondary alkaline phosphatase-conjugated antibody in Tris-buffered saline for 1 h under gentle agitation. The blot is again washed twice in PBS–0.1% Tween-20 for 5 min, rinsed

in PBS followed by Tris-buffered saline (to remove residual PBS and Tween-20), and then developed with alkaline phosphate substrate (i.e., Western Blue™). After developing blots to an appropriate signal-to-background perspective, reactions are stopped with PBS and the blot is washed with PBS twice. Blots to be used for flow experiments may be stored at 4°C. They should be kept in buffer until use. Blots will maintain binding fidelity for several days, though best results are obtained with freshly prepared materials.

5. The membranes are again blocked by incubation in 50% newborn calf serum or fetal bovine serum in binding media for at least 1 h at 4°C and equilibrated in binding media before use in the flow assay.

### 3.2. Preparation of Selectin-Expressing Cells

To assay blots for selectin ligand activity, we typically use human peripheral blood lymphocytes (PBLs) expressing L-selectin or Chinese hamster ovary (CHO) cells permanently transfected to express P-selectin or E-selectin.

1. Human PBLs can be prepared from whole blood by Ficoll density gradient separation. For analysis of L-selectin function on lymphocytes, monocytes may be removed by plastic adherence or other methods to reduce nonspecific binding (e.g., magnetic bead separation). Murine spleen and lymph node T-cells and rat thoracic duct lymphocytes also have been used in the same fashion. E-selectin and P-selectin transfected cell lines are typically split the day before use to achieve log phase growth and are harvested by replacing the media with H/H 5 mM EDTA. The cells can then be released from the flask by manual agitation or scraping. We do not use trypsin to release the cells as this may alter the function of surface adhesion molecules.
2. After counting, the cells are suspended at 10X the concentration desired for use in the flow chamber (e.g.,  $10\text{--}20 \times 10^6$  cells/mL in H/H without  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  and without glycerol) and stored on ice. Clumping of adherent cell populations (i.e., CHO cells) may develop after prolonged storage on ice. This can be remedied by washing again in H/H 5mM EDTA before use.
3. Controls to confirm specificity for selectin binding to blots include use of mock transfected CHO cells and/or preincubation of cell aliquots with function-blocking anti-selectin MAb.

### 3.3. Inverted Microscope Setup

1. Setup will vary with type of microscope. In general, we do not use any filters.
2. **Figure 2** shows the alignment of the parallel plate chamber onto the blot. Begin visualization at low power to align the chamber and identify the area of interest. Many observations are best performed at relatively low magnification (e.g.,  $\times 10$  objective) to allow observation of larger areas and numbers of cells. Observation under increased magnification can be performed to observe individual cell characteristics. Adjust the shutter arm so that the light narrows specifically over area of interest on the blot through the chamber.

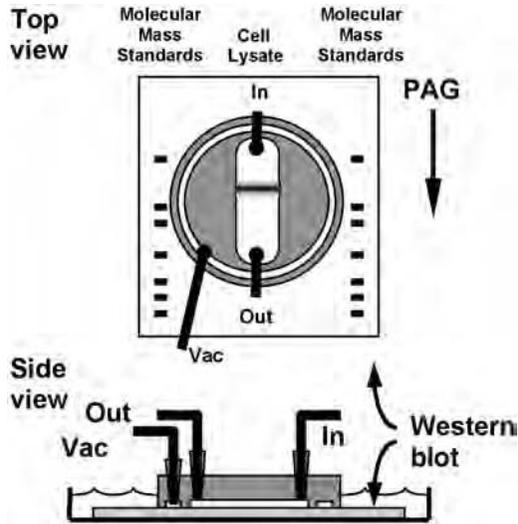


Fig. 2. Blot rolling apparatus. Western blots stained for components of interest are equilibrated in binding media containing 10% glycerol and placed in a Petri dish. A parallel plate flow chamber mounted over the area of interest is held in place by low continuous vacuum pressure (Vac). The inlet (In) is connected to a buffer reservoir and the outlet (Out) to a high precision syringe pump. The apparatus is mounted on the stage of an inverted phase-contrast microscope equipped for video microscopy. Molecular mass(es) of interacting protein(s) resolved in the polyacrylamide gel are estimated by alignment with colored mass standards in adjacent lanes. (From [ref. 3](#) with permission.)

### 3.4. Blot Rolling Assay

1. All media should contain 10% glycerol. Equilibration in dilute glycerol alters the opacity of the PVDF membrane sufficiently to allow transmission of light and the direct visualization of cells interacting with the surface of the blot by standard light microscopy. Assessment of some functions (e.g., integrin activation) requires maintaining the chamber and media at 37°C. This can be achieved with a stage warming device and placement of supply media in water baths adjacent to the apparatus.
2. The chamber should be mounted onto the blot so that the flow channel aligns in parallel over the lane of interest. The flow chamber is secured by application of low vacuum pressure in the same fashion as is used for attachment to glass or plastic surfaces. We have not found it necessary to perform any special maneuvers to maintain an adequate seal on Sequi-Blot PVDF membrane.
3. The flow field is rinsed with media introduced through the 20-mL syringe, attached to the three-way stopcock, to remove bubbles and prepare the blot surface for cell input.

4. Immediately before use, aliquots of cells (e.g., PBL or transfected CHO cells) are diluted 10-fold into binding medium (H/H w/  $\text{Ca}^{2+}$  with 10% glycerol; final concentration is typically  $1\text{--}2 \times 10^6$  cells/mL) at room temperature (or  $37^\circ\text{C}$  as indicated). This minimizes exposure to glycerol and divalent cations, as these may affect cell viability and promote the formation of cell aggregates, respectively.
5. The cell input line is flushed with assay medium and placed into the tube containing cells. Flow is regulated by function of a downstream syringe pump. The use of a programmable pump allows for automation of the assay technique and more reproducible results. We typically initiate flow at a high rate of flow (2 mL/min or approx  $7.5 \text{ dyne/cm}^2$ ) to bring cells through the sample tubing and into the chamber. Upon arrival of cells in the field of view, the flow rate is reduced (typically to  $0.5\text{--}1 \text{ dyne/cm}^2$ ) to allow cells to interact with the blot surface. The flow rate can be adjusted up or down to increase or decrease, respectively, the stringency of binding interactions. One can program step-wise increases in flow (shear stress) without interruption. Maintaining continuous flow while cells are in the chamber minimizes nonspecific binding to the blot surface. This is critical with adherent cell populations, such as CHO cells or monocytes, although lymphocytes typically do not bind when flow is stopped for brief periods ( $<30$  s).
6. Observation of cell interactions with substrate molecule(s) are made in real time and recorded via the camera/video camera record for later analysis. Typically, two types of analyses are performed. First, a “scanning” analysis is performed by moving the microscope stage to view the entire length of the lane in which proteins have been resolved while maintaining a constant shear rate. Typically, tethering and rolling interactions are observed at the leading edge of a “band” on the blot, with rolling in the middle and discontinuation of rolling interactions observed at the trailing edge. Once bands or areas of the blot of interest are identified, we typically perform additional studies comparing control cell preparations (mock-transfected or cells incubated with function blocking antibodies) and varying shear conditions while viewing a fixed area of the blot. In this analysis, tethering is observed at low physiological wall shear stresses ( $0.5\text{--}1.5 \text{ dyne/cm}^2$ ) for short periods (one to several minutes). Bound cells are then subjected to timed stepwise increases in wall shear stress to assess shear resistance as an estimate of relative strength of binding. The dependence of interactions on the presence of calcium (typical of selectin-mediated binding) can be confirmed by perfusion of the chamber with H/H with 10% glycerol and 5 mM EDTA and observing release of bound cells, or by repeating the analysis using H/H with 10% glycerol and 5 mM EDTA for both dilution of the cells and perfusion through the chamber.
7. Antibody inhibition experiments using antibodies to the membrane-bound ligand can be performed *in situ* by first observing interactions on an area of defined interest, then perfusing the chamber with a solution of antibody (e.g.,  $1\text{--}10 \mu\text{g/mL}$  for 30 min) and repeating the observation on the same site. If the antibody blocks binding interactions, the blot is unusable for further studies of that ligand. However, if the applied antibody does not block adhesive interactions, the blot may be reused, i.e., successive rounds of antibodies or reagents may be screened in this fashion.

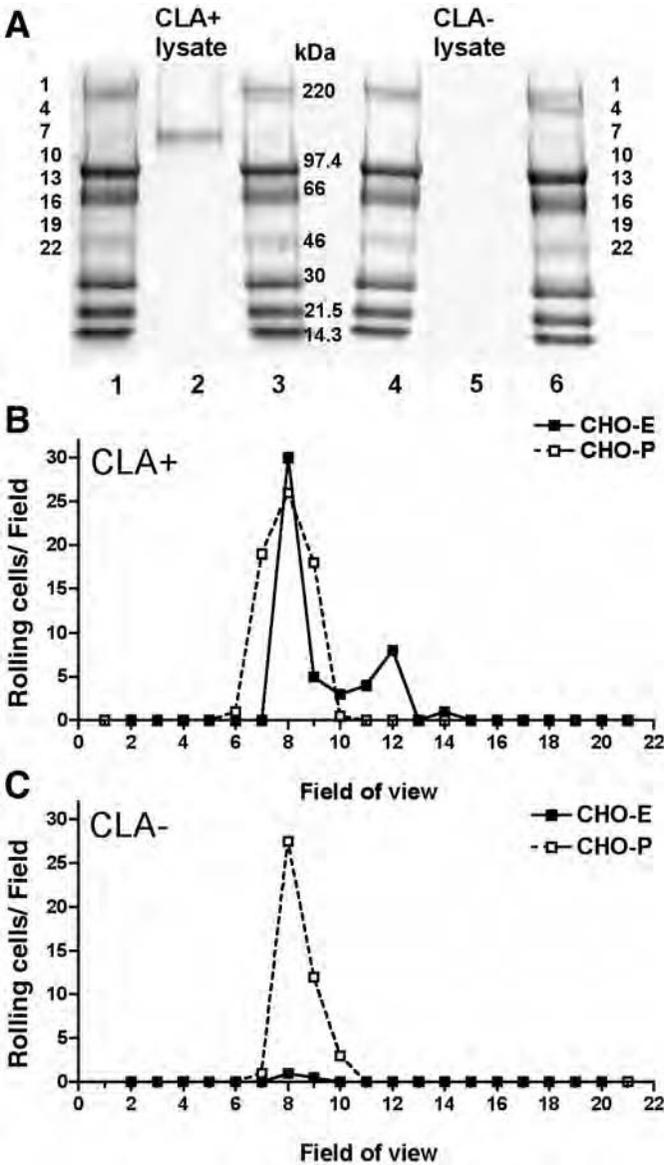


Fig. 3. Cutaneous lymphocyte antigen (CLA) immobilized on Western blots supports both E-selectin- and P-selectin-mediated rolling. (A) Western blots of T-cell lysates stained for CLA expression. Fifty micrograms of cell lysate protein from CLA-positive (left panel) or CLA-negative (right panel) T-cells were subjected to SDS-PAGE, electroblotted onto PVDF membranes, and immunostained with HECA-452 Mab. The single major reactive glycoprotein identified at approx 140 kDa in CLA-positive cells (lane 2) corresponds to the monomer form of CLA/PSGL-1.

8. For selectin-mediated binding, cells must tether and roll in shear flow to be considered specific. Tethering in our studies is defined as reduction of forward motion below the hydrodynamic velocity lasting a minimum of two video frames (0.07 s), and rolling is defined as more than 5 cell diameters of lateral translation below the hydrodynamic velocity. The majority of tethered cells in such studies are observed to roll smoothly across the entire field of view. Nonspecific interactions (i.e., cellular collisions with the substrate that did not lead to tethering and/or rolling) are defined as interactions lasting less than 0.07 s and are not included in the analysis. Nonspecifically bound cells (not rolling and/or not released by perfusion with EDTA) also are discounted from analysis. In general, there is a time-dependent increase in nonspecific (nonrolling) attachments in the absence of flow, though very few cells formed firm (nonrolling) attachments to the blot in continuous shear flow. Tethering rate is calculated as the number of cells that tether per field per time (usually 30–60 s) at a defined shear stress and adjusted to per minute values. As stated above, wall shear stress ( $\tau$ ) values are calculated according to the formula  $\tau$  (dynes/cm<sup>2</sup>) =  $6 \mu Q/wh^2$ , where  $\mu$  is the viscosity of the solution in the chamber (poise),  $Q$  is the volumetric flow rate (mL/s),  $w$  is the channel width (e.g., 0.5 cm for chamber we use), and  $h$  is the channel height (e.g., 0.025 cm for chamber we use [7]). A value of 0.009 poise is used for the viscosity ( $\mu$ ) of water at 25°C (8) and a value of 0.0123 poise is used for the viscosity of 10% glycerol (v/v) in water at 25°C (9). By these values, wall shear stress in 10% glycerol at 25°C is approx 1.37-fold greater than in water at the same temperature.
9. Regions (bands) of interest can be aligned against the molecular weight markers run in adjacent lanes to estimate molecular weight. Additionally, one may subsequently probe the blot with antibody to specific proteins of interest to determine whether band(s) supporting binding represent known proteins. One can excise regions of blot supporting binding and send sample for mass spectrometry and/or protein micro sequencing. This approach was used to identify HCELL, a glycoform

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Fig. 3. (*continued*) Similar blots stained with anti-PSGL-1 mAb show approx equal quantities of 140-kDa PSGL-1 protein in each sample (data not shown). (**B,C**) Rolling cells per visual field ( $\times 10$  objective) at 1.75 dyne/cm<sup>2</sup> wall shear stress. The number of cells observed to bind and roll was observed in overlapping fields of view extending from approx 250 kDa to 40 kDa (identified alongside blot images in **A**). Both CHO-E and CHO-P cells are observed to tether and roll across the area of the Western blot corresponding to CLA/PSGL-1 from CLA-positive T-cells (**B**), whereas only CHO-P cells are noted to form significant rolling adhesions on PSGL-1 from CLA-negative T-cells (**C**). Both CHO-E and CHO-P cells were noted to tether primarily over the area corresponding to CLA/PSGL-1. Mock-transfected CHO cells did not form rolling tethers on any areas of the blots observed (data not shown). Results presented are the means of two independent experiments on a single substrate and are representative of observations on numerous blots of CLA-positive and CLA-negative T-cell. (From **ref. 3** with permission.)

of CD44 that functions as a high affinity L-selectin and E-selectin ligand (4–6). We have also used this method to examine human T-lymphocyte glycoprotein(s) that support E-selectin and P-selectin binding under shear flow (3). **Figure 3** is a representative histogram of a blot rolling assay of T-cell lysates probed with stably transfected CHO cells expressing P-selectin (CHO-P) and E-selectin (CHO-E). A videotape of these studies is available for viewing on the Harvard Skin Disease Research Center web page, under the Leukocyte Migration Core subheading (<http://dermatology.bwh.harvard.edu/leukocyte.html>).

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## Cell–Cell Interaction in the Transcellular Biosynthesis of Novel $\omega$ -3-Derived Lipid Mediators

Nan Chiang and Charles N. Serhan

### Summary

Omega-3 polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) display beneficial actions in human diseases. The molecular basis for these actions remains of interest. We recently identified novel mediators generated from  $\omega$ -3 PUFA during cell–cell interactions that displayed potent anti-inflammatory and proresolving actions. Compounds derived from EPA are designated *resolvins* of the **E** series (RvE1), and those biosynthesized from DHA are denoted *resolvins* of the **D** series (RvD) and docosatriene, such as protectin D1 (PD1), which belongs to the family of protectins. In addition, treatment using aspirin initiates a related epimeric series by triggering endogenous formation of the 17*R*-RvD series, denoted as aspirin-triggered (AT)-RvDs. These compounds possess potent anti-inflammatory actions in vivo that essentially are equivalent to their counterpart generated without aspirin, namely the 17*S*-RvDs. In this chapter, we provide an overview and detail protocols of the biosynthesis and bioactions of these newly uncovered pathways and products that include three distinct series: 18*R*-resolvins of the E series derived from EPA (i.e., RvE1); 17*R*-resolvins of the D series from DHA (AT-RvD1 through RvD4); and 17*S*-resolvins of the D series from DHA (RvD1 through RvD4).

**Key Words:** Resolvin; aspirin; neuroprotectin; resolution;  $\omega$ -3; lipid mediator; inflammation.

### 1. Introduction

Inflammation has emerged as playing a central role in many prevalent diseases, including Alzheimer's disease, cardiovascular disease (**1**), and cancer (**2,3**) in addition to those chronic disorders that are well known to be associated with inflammation, such as arthritis and periodontal disease (**4,5**). In parallel, the beneficial impact of essential  $\omega$ -3 fatty acids was uncovered as early as 1929 (**6**) and was consistently observed in many human diseases for the next several decades (**7–10**). Recently, we identified novel oxygenated products generated from the precursors eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) that

possess potent biological activities within resolving inflammatory exudates (11–13). Hence, the terms Resolvin (Rv; resolution phase interaction products) and docosatrienes were introduced because the new compounds displayed both potent anti-inflammatory and immunoregulatory properties.

These compounds include Rvs of the E series (RvE1) derived from EPA that carry potent biological actions and Rvs of the D series (RvD) from DHA, as well as bioactive members from these biosynthesis pathways carrying conjugated triene structures denoted as docosatrienes (DTs). DTs recently were termed protectin D1 (PD1) because they are both anti-inflammatory (12,13) and neuroprotective (14,15). Unlike other products identified earlier from  $\omega$ -3 fatty acids that are similar in structure to eicosanoids but less potent or devoid of bioactions, these Rvs, DTs, and neuroprotectins evoke potent biological actions in vitro and in vivo (11–15). In this chapter, we focus on the novel compounds derived from cell–cell interactions (i.e., RvE1, RvDs, and aspirin-triggered [AT]-RvDs) and provide detailed protocols for their biosynthesis as well as bioassays.

### **1.1. Novel Lipid Mediators Identified During Resolution of Acute Inflammation: Arachidonic Acid vs EPA and DHA**

We now appreciate that intimate cell–cell interactions within vessel walls, that is, adherent platelets that are studied with polymorphonuclear leukocytes (PMNs), converge on the endothelium and can be visualized by intravital microscopy (16,17) and promote transcellular lipid mediator biosynthesis (18–20). During platelet–leukocyte interactions, arachidonic acid is converted to lipoxins (LX), which are generated to “stop” PMN and stimulate monocytes (21) and macrophages to promote resolution (22). These forms of cell–cell interactions in the vasculature are impinged on by aspirin (23,24). Aspirin inhibits thromboxane production by platelets and prostacyclin biosynthesis in vascular endothelial cells (25,26). During PMN–endothelial and/or PMN–epithelial interactions, aspirin triggers the biosynthesis of 15-epi-lipoxins (aspirin-triggered lipoxin [ATL]; Fig. 1; [23]). Both LX and ATL and their respective stable analogs are potent regulators of transendothelial–transepithelial migration of PMN across these cells and endothelial cell proliferation in vitro and in vivo (27–29).

LX and ATL act in the nanomolar range via specific receptors we identified (30,31) and confirmed by others (32,33), namely LXA<sub>4</sub> receptor. Transgenic mice overexpressing the LXA<sub>4</sub> receptor with myeloid-specific promoter display reduced PMN infiltration in peritonitis and heightened sensitivity to LXA<sub>4</sub> and ATL (34). Transgenic rabbits overexpressing 15-lipoxygenase (LOX) type I generate enhanced levels of LXA<sub>4</sub>, have an enhanced anti-inflammatory status, and are protected from the inflammatory bone loss of periodontal disease (35). Hence, the results of these studies have heightened our awareness that PMN, in addition to their host defense position and the possibility that they can spill

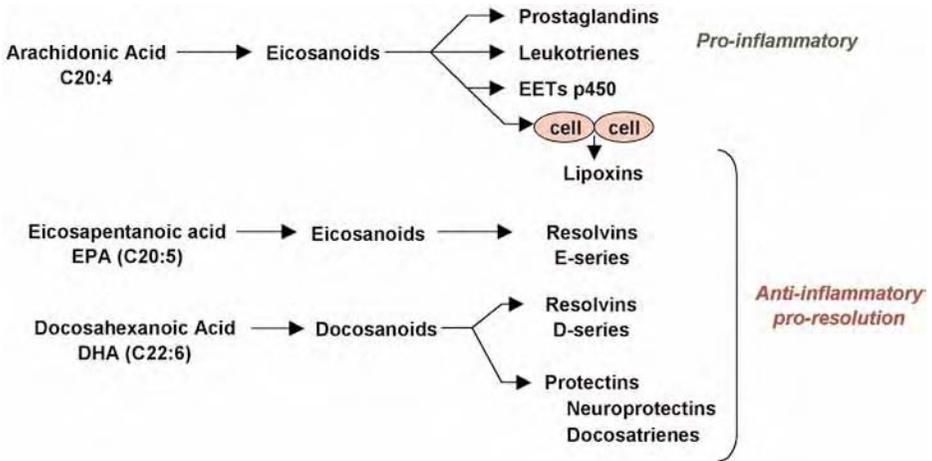


Fig. 1. Essential polyunsaturated fatty acids are precursors to bioactive lipid mediators.

pro-inflammatory agents (36) and mediators such as the classic eicosanoids, prostanoids, and leukotrienes (37,38), which amplify inflammatory responses, also can produce novel protective lipid mediators that actively counter-regulate the inflammation (Fig. 1).

It often is questioned whether essential fatty acids, such as the  $\omega$ -3 EPA or DHA, are converted to potent lipid mediators, as is the case with arachidonic acid. In short, both DHA and EPA are important precursors. In view of the compelling results from the GISSI study, which showed improvements in more than 11,000 cardiovascular patients (39,40), namely, reduction in sudden death by approx 45% by taking almost 1 g of  $\omega$ -3 per day, we recently addressed a potential role of  $\omega$ -3 polyunsaturated fatty acids (PUFAs). The inspection of their methods indicated that all the patients also took daily aspirin, which was unaccounted for in the authors' analysis. Despite very large doses (milligrams to grams daily), most literature regarding  $\omega$ -3 PUFAs suggests beneficial actions in many human diseases, including periodontal (41), anti-inflammatory, and antitumor actions (7,42).

The three major lipoxygenase activities (5-LOX, 12-LOX, 15-LOX) can each convert DHA to various monohydroxy-containing products; however, at the time their in vivo functions were either not apparent or they did not display bioactivity (43,44). Also, DHA can be nonenzymatically oxygenated to isoprostane-like compounds termed neuroprostanes that reflect oxidative stress in the brain (45) or autooxidized to products that are monohydroxy racemates (46) of the compounds that are now known to be enzymatically produced during biosynthesis of the Rvs and DT (12-15). Overall, it is noteworthy that the molecular basis and mechanisms underlying  $\omega$ -3 PUFA's immunoprotective

actions remained to be established and appreciated, and their direct connection to human disease and treatment are still important biomedical challenges (47).

### 1.2. Aspirin Unveils New Directions: AT Lipid Mediators

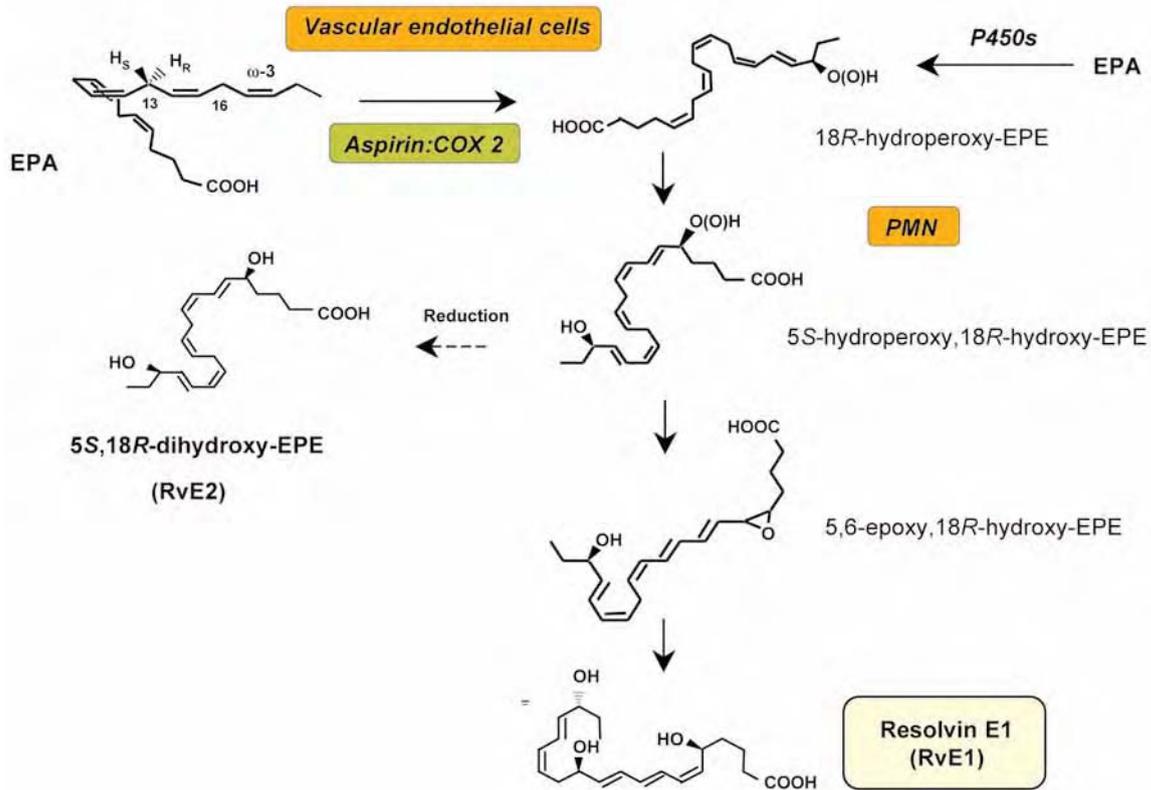
Aspirin is an active ingredient in more than 60 over-the-counter remedies, making it a difficult substance to control for in some human studies. What is the molecular basis for the protective action of  $\omega$ -3, and is there overlap(s) in its actions? To address this in an experimental setting, we used murine dorsal skin pouches (11,12). This model of inflammation is known to spontaneously resolve in rats (48). We adapted this for mice to include both genetics and to set up lipidomics, using liquid chromatography-ultraviolet-tandem mass spectrometry (LC-UV-MS-MS)-based analyses geared to evaluating whether potential novel lipid mediators are indeed generated during the resolution phase of inflammation (11,12). In this pouch (experimental contained inflammation) after 4 h, PMN numbers begin to decrease within exudates (11,12). Exudates were taken at timed intervals, focusing on the period of “spontaneous resolution,” and lipid mediator profiles were determined using tandem LC-UV-MS-MS. We constructed lipid mediator libraries with physical properties (i.e., MS and MS/MS spectra, elution times, ultraviolet spectra, and so on) for matching and to assess whether known and/or potential novel lipid mediators were present within the exudates, and are presently expanding these libraries and the software for their matching (49). If novel lipid mediators were encountered, their structures were elucidated by conducting retrograde analysis for both biogenic enzymatic synthesis and total organic synthesis. This approach permitted assessment of structure–activity relationships as well as the scale-up required to confirm the bioactions of novel compounds identified (11,12).

### 1.3. Anti-Inflammatory and Proresolving Properties

The main bioactive resolvins and docosatrienes as representative members are shown in Figs. 2–4. The bioactions of Rv and DT are listed in Table 1. Direct comparisons of Resolvin E vs the D series (17*R* and 17*S* epimer series) for their ability to regulate PMN *in vivo* were conducted (12,13,35). Both the D and E classes of Rvs are potent regulators of PMN infiltration. 17*R*-RvD series, triggered by aspirin, and 17*S*-RvD series give essentially similar results, indicating that the *S* to *R* switch does not diminish their bioactions. When injected intravenously at 100 ng/mouse, they both gave approx 50% inhibition, and the RvE1 gave approx 75 to 80% inhibition. In comparison, indomethacin at 100 ng/mouse (or  $\sim$ 3  $\mu$ g/kg) gave roughly 25% inhibition (see refs. 12 and 13). In addition, with microglial cells that liberate cytokines in the brain, the Rv D series block tumor necrosis factor (TNF)- $\alpha$ -induced interleukin (IL)-1 $\beta$  transcripts and are potent regulators of PMN infiltration in brain, skin, and peritonitis *in vivo* (13,14).

(text continued on p. 237)

## Biosynthesis of Aspirin Triggered 18R-Resolvin E-series



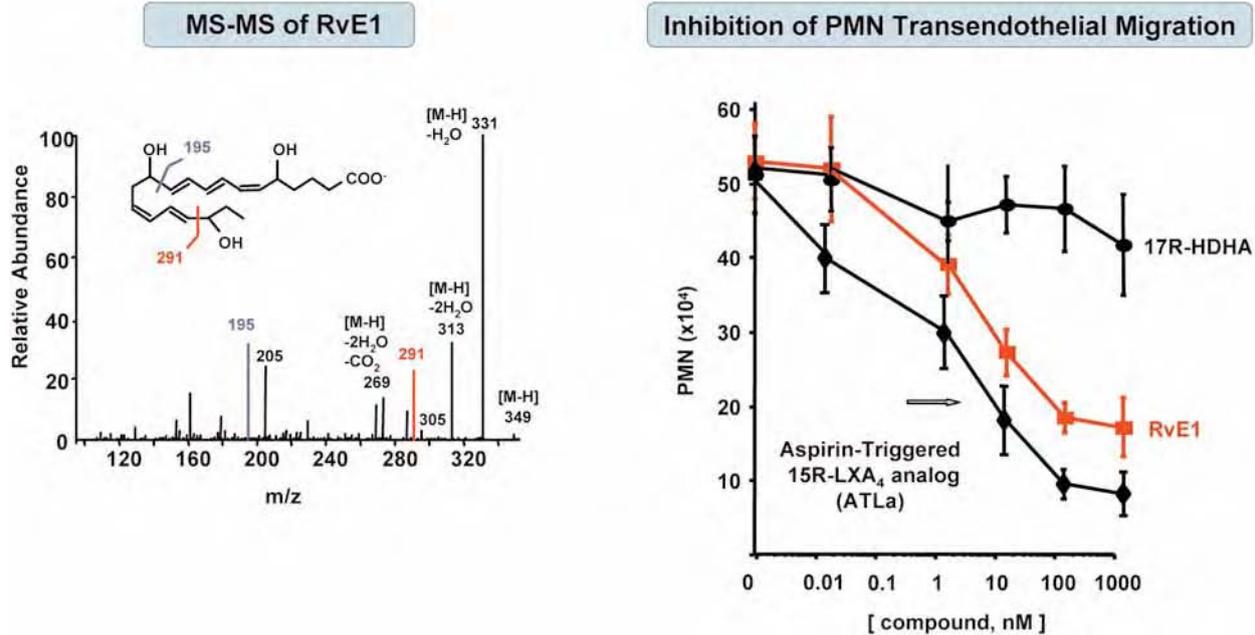
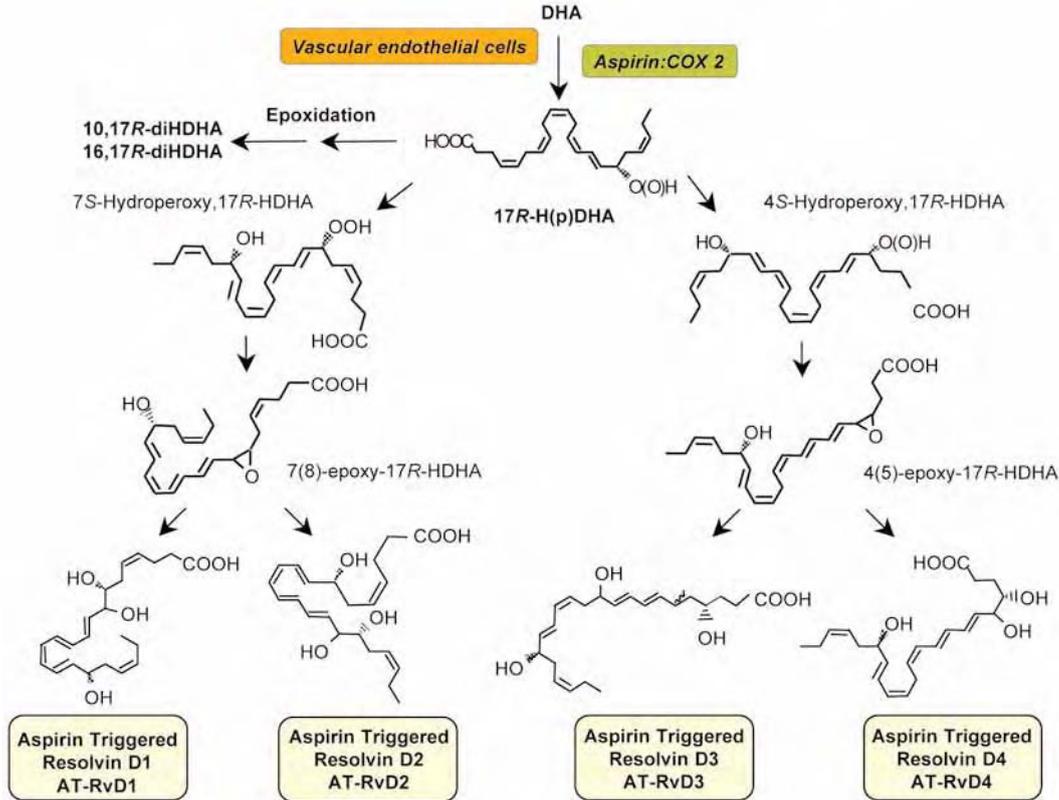


Fig. 2. EPA conversion to RvE1. Biosynthesis of AT 18R-RvE-series. Human endothelial cells expressing COX-2 treated with aspirin transform EPA by abstracting hydrogen at C16 to give R insertion of molecular oxygen to yield 18R-hydroperoxy-EPE, which is reduced to 18R-HEPE. They are further converted via sequential actions of leukocyte 5-LO and lead to formation of RvE1 (12,50). MS-MS analysis of RvE1: murine inflammatory exudate. Selected ion monitoring at  $m/z$  349.5 [M-H]. Structure depicted in the inset giving diagnostic ions at  $m/z$  305, 233, 195, and 291. Inhibition of PMN transendothelial migration. PMN were incubated with isolated compounds [ $10^{-11}$  to  $10^{-6}$  M]: 17R-HDHA, ●; RvE1, ■; or ATL analog used for reference, ◆; transmigration was initiated using an optimal amount of LTB<sub>4</sub> [10 nM] (100%) and HUVEC cocultures (90 min, 37°C). Results are the mean  $\pm$  SEM;  $n = 3$ .

Biosynthesis of Aspirin Triggered 17R-Resolvin D-series



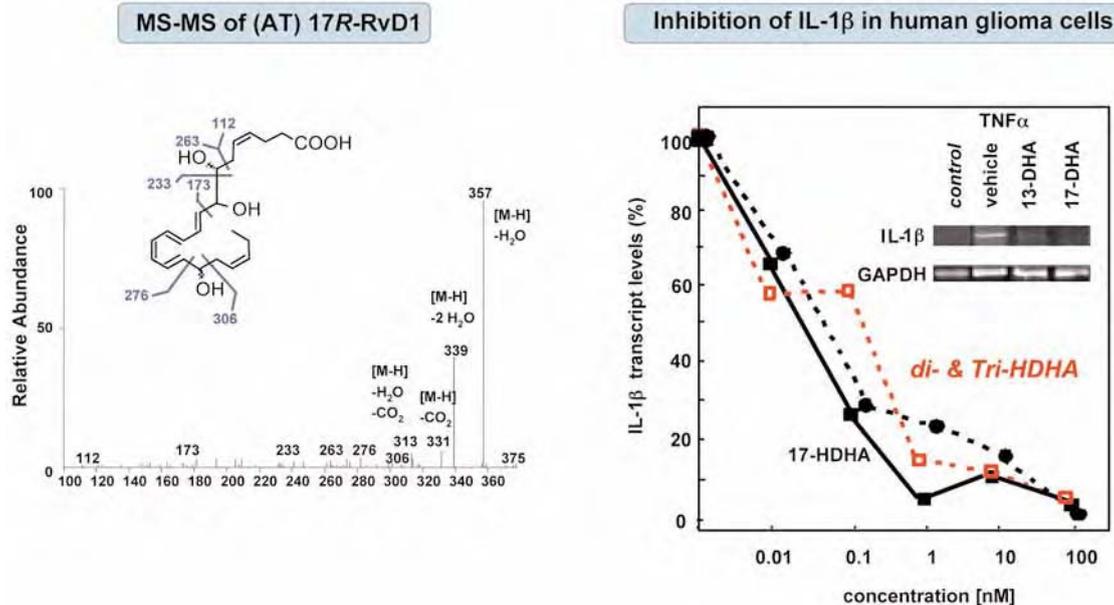
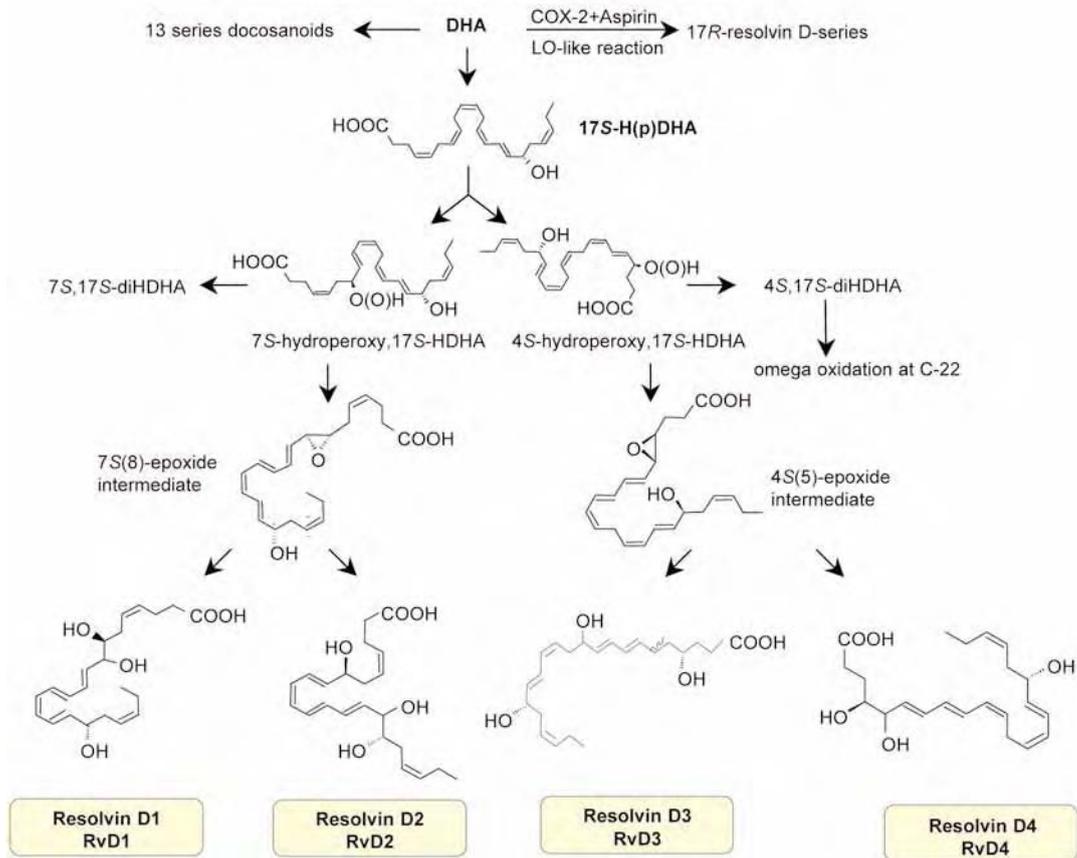


Fig. 3. DHA conversion to AT 17R-RvD-series. Biosynthesis of AT 17R-RvD-series. The 17R series Rvs are produced from DHA in the presence of aspirin. Human endothelial cells expressing COX-2 treated with aspirin transform DHA to 17R-HDHA. Human PMNs convert 17R-HDHA to two compounds via 5-lipoxygenase (5LO [11,12]) that are each rapidly transformed into two epoxide intermediates: a 7(8)-epoxide (left) and the other a 4(5)-epoxide. These two novel epoxide intermediates open to bioactive products denoted 17R series AT-RvD1 through AT-RvD4. Note: stereochemistry depicted in likely configuration based on results with recombinant enzymes (see refs. 12 and 13). MS-MS of (AT) 17R-RvD1. Selected ion monitoring at  $m/z$  375 [M-H]. Structure depicted in the inset giving diagnostic ions at  $m/z$  357 [M-H-H<sub>2</sub>O], 339 [M-H-2H<sub>2</sub>O], 331 [M-H-CO<sub>2</sub>], 313 [M-H-CO<sub>2</sub>-H<sub>2</sub>O], 306, 303, 276, 273, 255 [273-H<sub>2</sub>O], 210, 195, and 180. Inhibition of IL-1 $\beta$  in human glioma cells. DBTRG-05MG cells ( $1 \times 10^6$ /mL) were stimulated with 50 ng/mL of human recombinant TNF- $\alpha$  for 16 hours to induce expression of IL-1 $\beta$  transcripts. Concentration dependence with COX-2 products: 17-HDHA (■), 13-HDHA (●), and di-/tri-HDHA (□). Results are representative of reverse transcription polymerase chain reaction gels of MG cells exposed to 100 nM of 13-HDHA or 17-HDHA and graphed after normalization of the IL-1 $\beta$  transcripts using GAPDH.

### Biosynthesis of 17S-Resolvin D-series



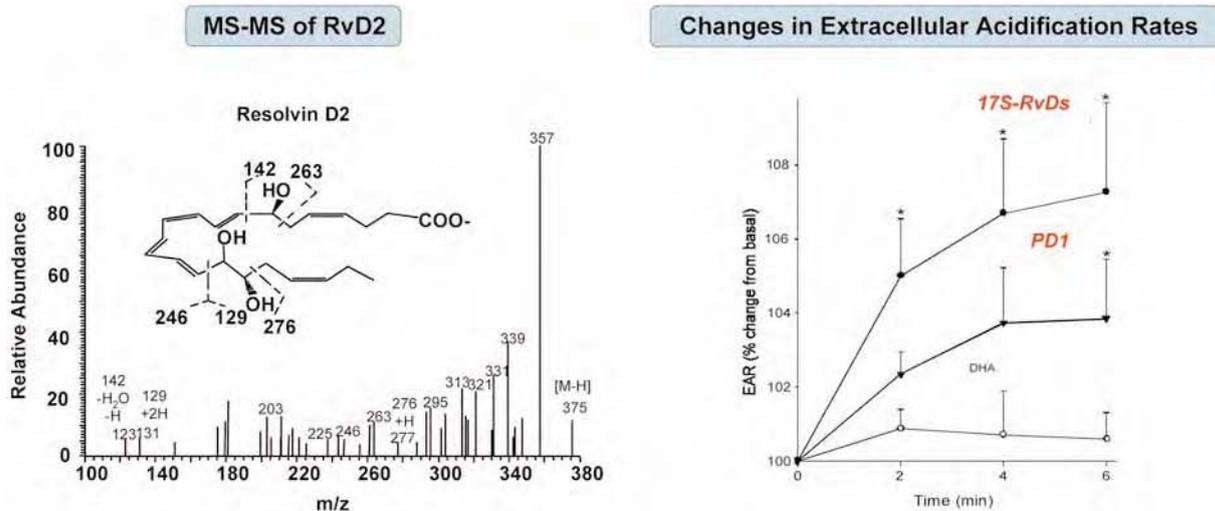


Fig. 4. DHA conversion to 17S-RvD series. Biosynthesis of 17S-RvD-series. These transformations may involve novel enzymes in addition to those that serve in the biosynthesis of eicosanoids (*see Subheading 3.3.* for further detail). The complete stereochemistries for the 17S-RvDs remain to be established and are depicted in their tentative configurations based on biogenic total synthesis, lipidomic analyses and alcohol trapping profiles. MS-MS of RvD2. Selected ion monitoring at  $m/z$  375 [M-H]. Structure depicted in the inset giving diagnostic ions at  $m/z$  123 (142-H<sub>2</sub>O-H), 131(129+2H), 203(246-CO<sub>2</sub>-H), 225 (263-2H<sub>2</sub>O-2H), 246, 263, 277 (276+H), 295 (M-H-2H<sub>2</sub>O-CO<sub>2</sub>), 313 (M-H-H<sub>2</sub>O-CO<sub>2</sub>), 321(M-H-3H<sub>2</sub>O), 331 (M-H-CO<sub>2</sub>), 339 (M-H-2H<sub>2</sub>O), 357 (M-H-H<sub>2</sub>O-CO<sub>2</sub>), and 375 (M-H) for 7S, 16, 17S-triHDHA (17S-RvD2). Changes in extracellular acidification rates. Glial cells: 17S-RvDs evoke ligand operated extracellular acidification. Changes in extracellular acidification rates were analyzed using Cytosensor<sup>®</sup> microphysiometry. Cells were superfused (100  $\mu$ L/60 s) with DHA or 17S-RvDs ( $n = 3$ ). Values are expressed as extracellular acidification rate ( $\mu$ V/s) normalized to baseline (100%), and ligand additions were at 2 min.

**Table 1**  
**Biosynthesis and Biological Activity of Resolvins**

Compound	Biosynthesis	Biological activity
RvE1	Murine exudates ( <b>11</b> )	Inhibit PMN transmigration
	Human blood ( <b>50</b> )	Inhibit PMN infiltration (air pouch and peritonitis)
AT-RvDs	Coincubations of human endothelial cells and PMN ( <b>11</b> )	Inhibit IL-12 production in dendritic cells
	Murine inflammatory exudates ( <b>12</b> )	Inhibit PMN infiltration (air pouch and peritonitis)
	Murine brain stroke ( <b>14</b> )	Inhibit TNF- $\alpha$ -induced IL-1 $\beta$ in human glioma cells
RvD2-4	Human whole blood ( <b>13</b> )	Inhibit PMN transmigration Inhibit PMN infiltration (air pouch and peritonitis)
	Murine exudates	Inhibit TNF- $\alpha$ -induced IL-1 $\beta$ in human glioma cells Changes in extracellular acidification rate

PMN, polymorphonuclear leukocyte; IL, interleukin; TNF, tumor necrosis factor; AT, aspirin-triggered 17R-containing series.

## 2. Materials

### 2.1. Cell Culture

Human glioma cells (DBTRG-05MG cells) and *Sf9* (*Spodoptera frugiperda*) insect cells are from American Type Culture Collection (ATCC; Rockville, MD). Cell culture media and reagents are from Cambrex Biosciences (Walkersville, MD).

### 2.2. Biosynthesis

1. Aspirin and A23187 were from Sigma (St. Louis, MO). EPA was from Cayman Chemical Co. (Ann Arbor, MI) and other synthetic standards, hydroxy fatty acids, and intermediates used for identification were purchased from Cascade Biochem Ltd. (Reading, UK).
2. 17R/S-hydroxy-DHA (HDHA) from Penn Bio-Organics (Bellefonte, PA).
3. IL-1 $\beta$  is from PeproTech (Rocky Hill, NJ).
4. Extract-clean C18 cartridges were purchased from Alltech Associates (Deerfield, IL).

### 2.3. In Vitro Bioassays

1. Recombinant murine and human TNF- $\alpha$  from R&D Systems (Minneapolis, MN).

2. Trizol reagent is from GIBCO BRL Life Technologies (Grand Island, NY).
3. Cytosensor<sup>®</sup> microphysiometer is from Molecular Devices (Sunnyvale, CA).

#### 2.4. Murine Models of Acute Inflammation

1. Male mice of FVB strain (6 to 8 wk) were from Charles River (Wilmington, MA).
2. Zymosan A was from Sigma (St. Louis, MO).

### 3. Methods

#### 3.1. 18R-Resolvin E Series

Resolving exudates in mice contain 18R-hydroxy-eicosapentaenoic acid (HEPE) as well as several related bioactive compounds (**11**). These novel compounds are produced from EPA by at least one biosynthetic pathway operative in human cells. This pathway is shown in **Fig. 2**; blood vessel-derived vascular endothelial cells treated with aspirin convert EPA to 18R-hydro (peroxy)-EPE that is reduced to 18R-HEPE. 18R-HEPE is then released from endothelium and rapidly converted by activated human PMN in the proximity to a 5(6) epoxide-containing intermediate that is further transformed to the bioactive 5,12,18R-tri-HEPE, termed RvE1.

Organic synthesis was achieved and its complete stereochemical assignment was recently established as 5*S*,12*R*,18*R*-trihydroxy-6*Z*,8*E*,10*E*,14*Z*,16*E*-eicosapentaenoic acid (**50**). RvE1 was identified in the resolution phase in mice and appeared as a transcellular biosynthetic product during cell–cell interaction with isolated human cells. More importantly, it proved to be a potent regulator of PMN transendothelial migration and infiltration *in vivo*. Recent results indicate that ChemR23 is a specific receptor for RvE1 (**50**).

##### 3.1.1. Biosynthesis In Vitro and In Vivo

###### 3.1.1.1. HUMAN ENDOTHELIAL CELL INCUBATION WITH PMN

1. Human endothelial cells are incubated with IL-1 $\beta$  (1 ng/mL, 24 h; *see Note 1*) to induce COX-2 (*see Note 2*).
2. Cells are then washed in Hank's balanced salt solution and treated with ASA (500  $\mu$ M; *see Note 3*) for 20 min at 37°C, followed by EPA (20  $\mu$ M).
3. Incubations are terminated with two volumes of ice-cold methanol and then extracted (*see Note 4*) for LC/MS/MS analysis (*see Note 5*) and further incubation.
4. Selected ion monitoring (i.e., at *m/z* 317 and 259) revealed that human umbilical vein endothelial cells (HUVECs) treated with ASA converted EPA to 18R-HEPE. Also, human microvascular endothelial cells (HMVECs) treated with ASA and EPA generated 18-HEPE (10.6 ng/10<sup>6</sup> cells).
5. Human PMNs (30  $\times$  10<sup>6</sup> cells/mL; *see Note 6*) are then incubated (30 min, 37°C) with serum-treated zymosan A (100 ng/mL) and ASA-acetylated-COX-2-derived products from EPA (from **step 3**).

6. Incubations are terminated with two volumes of ice-cold methanol and then extracted for LC/MS/MS analysis.
7. Selected ion monitoring at  $[M-H] = m/z$  349.5 revealed that PMN converted 18R-HEPE to RvE1 (see Fig. 2).

### 3.1.1.2. FORMATION OF RvE1 IN INFLAMMATORY EXUDATES

1. Murine dorsal air pouches are raised on day 0 (see Note 7).
2. On day 6, inflammation is initiated by intrapouch injection of recombinant mouse TNF- $\alpha$  (100 ng/pouch).
3. ASA (500  $\mu$ g) is injected at 3.5 h followed by injection of 300  $\mu$ g of EPA (see Note 8) per pouch at 4 h.
4. At 6 h, pouches are lavaged twice with 3 mL of phosphate-buffered saline (PBS) and the exudate cells enumerated.
5. Exudate cells are then activated by calcium ionophore A<sub>23187</sub> (4  $\mu$ M) at 37°C for 20 min.
6. The supernatants are collected and extracted for LC-MS-MS analysis.

### 3.1.2. Biological Activity In Vitro

#### 3.1.2.1. PMN TRANSENDOTHELIAL MIGRATION

1. HUVECs were used at passages 1 and 2 and isolated by collagenase digestion (0.1% collagenase) and propagated on gelatin-coated (1%) tissue culture plates in RPMI 1640 cell culture medium supplemented with 15% bovine calf serum, 15% NU-serum, 50  $\mu$ g/mL endothelial mitogen, 8 units/mL heparin, 50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin.
2. Human PMN are exposed to RvE1 ( $10^{-11}$  to  $10^{-6}$  M at 37°C for 15 min), washed twice with Dulbecco's phosphate-buffered saline (DPBS), and cocultured with confluent endothelial monolayers grown on 96-well plates ( $4 \times 10^5$  cells in 0.2 mL of DPBS supplemented with 1% BCS) in the presence of agonist LTB<sub>4</sub> ( $10^{-7}$  M, 30 min).
3. After incubation, the medium is aspirated and monolayers washed gently with 1 mL of DPBS to remove adherent cells.
4. The contents of each well are solubilized with 0.1% sodium dodecyl sulfate/0.025 M NaOH.
5. Fluorescence is assessed in a Cytofluor 2300 fluorescence plate reader, and the number of adherent PMN is calculated from the fluorescence of the original PMN suspension.

### 3.1.3. Bioactions In Vivo

#### 3.1.3.1. MURINE DORSAL AIR POUCH: INHIBITION OF PMN INFILTRATION

1. Murine dorsal air pouches are raised on day 0 with male FVB mice.
2. On day 6, mice are anesthetized with isoflurane. Compounds (RvE1 or RvE2, 100 ng; see Note 8 for handling Rvs) or vehicle (0.1% ethanol; see Note 9) were delivered as a bolus injection either into the tail vein in 100  $\mu$ L of sterile saline or locally into pouches in 200  $\mu$ L of sterile saline.

3. TNF- $\alpha$  (100 ng/pouch) is then injected locally into pouches to stimulate PMN infiltration.
4. Inflammatory pouch exudates are collected at 4 h and cells enumerated.

### 3.1.3.2. MURINE PERITONITIS (SEE NOTE 10)

1. Male FVB mice (6–8 wk) are anesthetized with isoflurane.
2. RvE1 (300 ng) or vehicle (0.1% ethanol) alone is administered intraperitoneally in 200  $\mu$ L of sterile saline.
3. Zymosan A (1 mg in 1 mL of normal saline) is then administered intraperitoneally 5 min later.
4. Mice are euthanized with an overdose of isoflurane 2 h later and peritoneal exudates collected by lavaging with 5 mL of sterile saline.
5. Exudate cells are collected and enumerated.

## 3.2. 17R-Series RvDs

In resolving exudates from mice given aspirin and DHA, we found novel 17R-HDHA and several related bioactive compounds (**Fig. 3**). HMECs, also aspirin-treated in hypoxia, generate 17R-HDHA. DHA is converted by human recombinant COX-2, which was surprising, because earlier literature indicated that DHA is not a substrate of cyclooxygenase (**51,52**). However, these investigations were before knowledge of the COX-2 isoform and used organs rich in COX-1. Human recombinant COX-2 converts DHA to 13-HDHA. With aspirin, this switches to 17R-oxygenation to give epimeric AT forms, also in brain (**12,13**), of Rvs (AT-RvD1 through RvD4; see **Fig. 3**).

### 3.2.1. Biosynthesis

#### 3.2.1.1. INCUBATION OF DHA AND ASA WITH ENDOTHELIAL CELLS

1. HUVECs or HMVECs are cultured for transendothelial migration.
2. HUVEC or HMVEC monolayers (1, 2, or 3 passages) are seeded ( $\sim 2 \times 10^5$  cells/cm<sup>2</sup>) on polycarbonate permeable supports precoated with 0.1% gelatin.
3. For hypoxia experiments, plates of HUVEC are treated with TNF- $\alpha$  and IL-1 $\beta$  (both 1 ng/mL), placed in a hypoxic chamber (3 h, 37°C) and returned to normoxia (21 h, 37°C).
4. ASA (500  $\mu$ M, 30 min) was added followed by DHA ( $\sim 5$   $\mu$ M) and calcium ionophore A23187 (2  $\mu$ M; 60 min, 37°C).
5. Incubations are terminated and extracted for LC-MS-MS analyses. Selected ion monitoring at [M-H] =  $m/z$  343 revealed that endothelial cells treated with ASA converted DHA to 17R-HDHA (see **Fig. 3**).

#### 3.2.1.2. INCUBATION OF DHA AND ASA WITH RECOMBINANT COX-2

1. Human recombinant COX-2 is overexpressed in Sf9 insect cells.
2. The microsomal fractions ( $\sim 8$   $\mu$ L) are suspended in 100 mM Tris-HCl, pH 8.0.

3. ASA is incubated ( $\sim 2$  mM,  $37^\circ\text{C}$ , 30 min) with COX-2 before addition of DHA ( $10\ \mu\text{M}$ ).
4. Incubations are terminated and extracted with deuterium-labeled internal standards for LC-MS-MS analysis (see **Note 11**).
5. Selected ion monitoring at  $[\text{M-H}] = m/z\ 343$  revealed that recombinant COX-2 treated with ASA converts DHA to 17R-HDHA (see **Fig. 3**).

#### 3.2.1.3. INCUBATION OF 17-HDHA WITH HUMAN PMNS

1. Human PMNs are freshly isolated from venous blood of healthy volunteers (who declined taking medication for approx 2 wk before donation; BWH protocol no. 88-02642) by Ficoll gradient and enumerated.
2. Cells are divided into  $50 \times 10^6$  cells/1 mL DPBS with  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (DPBS<sup>+/+</sup>), and incubations (40 min,  $37^\circ\text{C}$ ) performed with either 17R/S-HDHA or 17R-HDHA with zymosan A ( $100\ \text{ng/mL}$ ).
3. Incubations are terminated and extracted with deuterium-labeled internal standards (15-HETE and C20:4) for LC-MS-MS analysis.
4. Selected ion monitoring at  $[\text{M-H}] = m/z\ 359$  and  $375$  revealed that human PMN converts 17R-HDHA to di- and tri-HDHA (AT-RvDs), respectively (see **Fig. 3**).

#### 3.2.1.4. INCUBATION WITH MURINE WHOLE BRAIN

1. Mouse whole brains are rapidly isolated from sacrificed mice immediately before incubations.
2. Mouse brains are washed with cold DPBS<sup>+/+</sup> and incubated with ASA (45 min,  $37^\circ\text{C}$ ,  $500\ \mu\text{M}$ ).
3. Incubations are terminated with 5 mL of cold methanol; brain is homogenized and extracted for LC-MS-MS analyses.

#### 3.2.1.5. FORMATION OF 17R-CONTAINING ASPIRIN-TRIGGERED RvDs IN INFLAMMATORY EXUDATES

1. Inflammatory exudates are initiated with intrapouch injection of recombinant mouse TNF- $\alpha$  into dorsal air pouches of 6- to 8-wk-old male FVB mice.
2. ASA ( $500\ \mu\text{g}$ ) is injected locally at 3.5 h followed by injection of  $300\ \mu\text{g}$  DHA/pouch at 4 h after the injection of TNF- $\alpha$ .
3. At 6 h (within the resolution phase), pouches were lavaged (using 3 mL of saline) and exudate cells were enumerated.
4. Cell-free supernatants are collected and extracted for LC-MS-MS analyses.

### 3.2.2. Biological Activity In Vitro

#### 3.2.2.1. REGULATION OF IL-1 $\beta$ IN HUMAN GLIOMA CELLS

1. Human glioma cells (DBTRG-05MG cells) are cultured as recommended by ATCC.
2.  $1 \times 10^6$  cells per well in six-well plates are stimulated for 16 h with  $50\ \text{ng/mL}$  of human recombinant TNF- $\alpha$  in the presence of test compounds (i.e., 17R-HDHA and di-/tri-HDHA,  $10^{-12}$  to  $10^{-7}\ \text{M}$ ) or vehicle (0.04% ethanol).

3. Cells are washed in DPBS<sup>+/+</sup> and harvested in 1 mL of Trizol reagent.
4. RNA is purified and reverse transcription polymerase chain reaction is performed. Primers used in amplifications are: 5'-GGAAGATGCTGGTTCCCTGC-3' and 5'-CAACACGCAGGACAGGTACA-3' for IL-1 $\beta$ ; 5'-TCCACCACCGTGTTCGTTAG-3' and 5'-GACCACAGTCCATGACA TCACT-3' for glyceraldehyde phosphate dehydrogenase (GAPDH).
5. PCR products obtained with these primers are confirmed by sequencing. Analyses are performed for both genes (i.e., GAPDH and IL-1 $\beta$ ) in the linear range of the reaction. Results were analyzed using the NIH Image program (<http://rsb.info.nih.gov/nih-image>).

#### 3.2.2.2. PMN TRANSMIGRATION

Protocols in this subheading are essentially identical to **Subheading 3.1.2.1**.

#### 3.2.3. Bioactions In Vivo

##### 3.2.3.1. MURINE DORSAL AIR POUCH AND PERITONITIS

Protocols in this subheading are essentially identical to **Subheadings 3.1.3.1** and **3.1.3.2**.

### 3.3. 17S-Series RvDs

DHA (C22: 6) is highly enriched in brain, synapses, and retina and is a major  $\omega$ -3 fatty acid. Deficiencies in this essential fatty acid are reportedly associated with neuronal function, cancer, and inflammation. Using new lipidomic analyses using tandem LC-PDA-MS-MS, a novel series of endogenous mediators was identified in blood, leukocytes, brain, and glial cells as 17S hydroxy-containing docosanoids, which were denoted protectins (PD) that also contain docosatrienes (the main bioactive member of the series was 10,17S-docosatriene; the complete stereochemistry was recently established [*see* **ref. 53**]) and 17S series resolvins. These novel mediators were biosynthesized via epoxide-containing intermediates and proved potent (pico- to nanomolar range) regulators of both leukocytes, reducing infiltration in vivo, and glial cells, blocking their cytokine production. These results indicate that DHA is precursor to potent protective mediators generated via enzymatic oxygenations to novel docosatrienes and 17S series resolvins that each regulates events of interest in inflammation and resolution.

#### 3.3.1. Biosynthesis

##### 3.3.1.1. INCUBATION OF DHA WITH HUMAN WHOLE BLOOD

1. Human whole (venous) blood is collected from healthy volunteers (who declined taking medication for approx 2 wk before donation; BWH protocol no. 88-02642).

2. After collection, whole blood was immediately incubated with DHA (10  $\mu$ g) in a covered water bath for 40 min (37°C).
3. Incubations are terminated and extracted for lipidomic and lipid mediator profile analyses by LC-MS-MS.
4. Selected ion monitoring at  $[M-H] = m/z$  343, 359 and 375 revealed that human whole blood converts DHA to mono-, di- and tri-HDHA, respectively (see Fig. 4).

### 3.3.1.2. INCUBATION OF DHA WITH HUMAN PMN

1. Human PMN are freshly isolated from the whole blood by Ficoll gradient. PMN (30–50  $\times 10^6$  cells/mL) are exposed to zymosan A (100  $\mu$ g/mL) followed by addition of either 17S-HDHA (3  $\mu$ g/mL) or DHA (3  $\mu$ g/mL).
2. Cell suspensions are incubated for 40 min at 37°C in a covered water bath.
3. Incubations are terminated and lipidomic analyses carried out by LC-MS-MS.
4. Selected ion monitoring at  $m/z$  359 and 375 revealed that human whole blood converts 17S-HDHA to di- and tri-HDHA (see Fig. 4).

### 3.3.2. Biological Activity In Vitro

#### 3.3.2.1. CYTOSENSOR ANALYSES

1. Changes in extracellular acidification rate are evaluated using a Cytosensor<sup>®</sup> microphysiometer and computer workstation.
2. Glial cells are equilibrated for 30 min in Dulbecco's Modified Eagle's Medium.
3. Extracellular acidification rates are determined by 30-s potentiometric rate measurements ( $\mu$ V/s; pump off cycle) after an 80-s pump cycle with a 10-s delay (120 s total cycle time).
4. Cells are perfused (100  $\mu$ L/60 s) with agonist (DHA or 17S RvD series compounds, 100 nM) or vehicle (0.1% vol/vol ethanol) alone before the first rate measurements.
5. Perfusion duration depends on the ligand being evaluated and ranged from 5 to 15 min. Acidification rates ( $\mu$ V/s) are normalized to basal rates (100%) three cycles before agonist addition.

#### 3.3.2.2. TNF- $\alpha$ -INDUCED IL-1 $\beta$

Protocols in the subheading are identical to **Subheading 3.2.2.1.**

### 3.3.3. Bioactions In Vivo

#### 3.3.3.1. ACUTE INFLAMMATION: MURINE DORSAL AIR POUCH AND PERITONITIS

Protocols in the subheading are identical to **Subheadings 3.1.3.1.** and **3.1.3.2.** (see Fig. 5 for the bioaction of 17S- and 17R-series RvDs).

## 3.4. Summary

Rvs are comprised of five separate chemical series of lipid-derived mediators, each with unique structures and apparent complementary anti-inflammatory properties and actions. Rvs are also generated in their respective epimeric forms

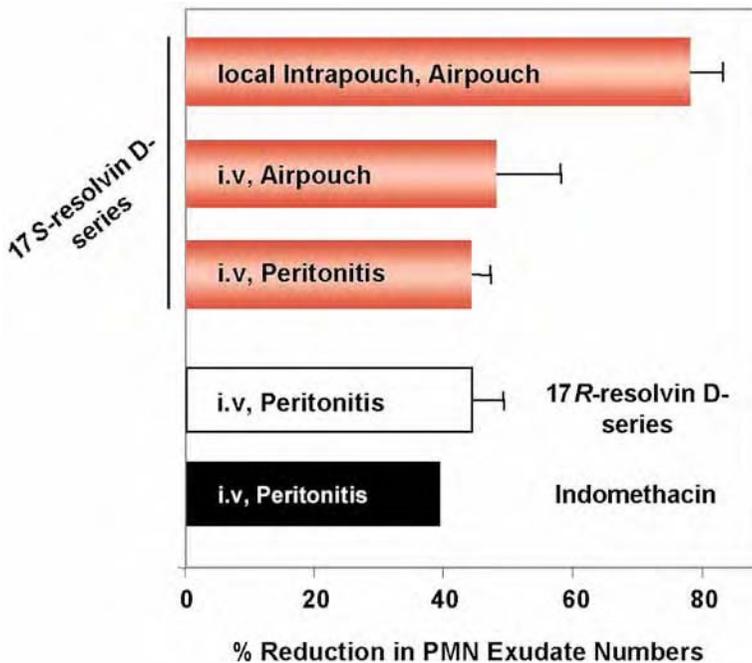


Fig. 5. Anti-inflammatory properties of 17*R*- and 17*S*-containing RvD series. Murine peritonitis and dorsal skin pouch after systemic and topical application. Peritonitis: RvDs (100 ng) or indomethacin (100 ng) was injected intravenously into mouse tail followed by zymosan A into the peritoneum. For direct comparison, mice were treated with 17*R*-RvDs via intravenous injection followed by zymosan A. Mice were sacrificed and peritoneal lavages were collected (2 h) and cells enumerated. Air pouch: RvDs (100 ng) were injected intrapouch or intravenously followed by intrapouch injection of TNF- $\alpha$ . At 4 h, air pouch lavages were collected and enumerated. Values represent mean  $\pm$  SEM from three to four different mice; all gave  $p < 0.05$  when infiltrated PMN was compared with vehicle control.

when aspirin is given in mammalian systems (11,12). The Rvs each dampen inflammation and PMN-mediated injury from within, key culprits in many human diseases. The results of these initial studies underscore a role in resolution as well as catabasis and give a possible therapeutic potential for this new arena of immunomodulation and host protection. Hence it is likely that the Rv and their AT-related forms may play roles in other tissues and organs involved in physiological and pathological processes as host-protective mediators. In view of the important roles of DHA and EPA in human biology and medicine uncovered to date (7-11), the physiological relevance of the Rvs is likely to extend well beyond our current appreciation (11-15).

#### 4. Notes

1. Handling cytokines: It usually is advisable to aliquot cytokines because they are in general dissolved in water and kept at  $-20^{\circ}\text{C}$ . Keep and dissolve cytokine stocks according to the manufacturer's instructions. Repeated freezing and thawing can lead to degradation of these protein mediators.
2. COX-2 expression: expression of COX-2 should be verified in the current experimental conditions. COX-2-specific antibodies as well as COX-2 nucleotide primers are available from most vendors that supply eicosanoids. Thus, verification of cytokine COX-2 induction by either Western blot or reverse transcription polymerase chain reaction is recommended.
3. Handling aspirin: aspirin hydrolyses rapidly in an aqueous environment. Therefore, prepare aspirin stocks in ethanol immediately before each experiment and store solid aspirin in a desiccator to prevent decomposition. **Note** that aspirin is the only known non-steroidal anti-inflammatory drug that acetylates COX-2.
4. Extraction protocol optimized for LX and RV: Terminate the incubation by adding two volumes of ice-cold methanol and place the mixture at  $-20^{\circ}\text{C}$  for at least 20 min for protein precipitation. Centrifuge at 800g for 20 min at  $4^{\circ}\text{C}$ . Collect the supernatant in a 50-mL tube and dilute the sample with at least five volumes of pure water. Rapidly adjust the pH of this aqueous suspension to pH 3.5 with HCL (1 N). Load the acidified samples into solid-phase extraction cartridges ( $\text{C}_{18}$  Sep-Pak), wash the cartridge with 10 mL of water, and elute compounds with 8 mL of hexane, followed by 8 mL of methyl formate, and a final elution with 8 mL of methanol. LX, RV, HETEs, and most leukotrienes elute in the methyl formate fraction, whereas peptide leukotrienes ( $\text{LTC}_4$ ,  $\text{LTD}_4$ ,  $\text{LTE}_4$ , etc.) elute in the methanol fraction. Take the methyl formate fraction to dryness under a gentle stream of nitrogen and then resuspend compounds in a small volume of methanol (i.e., 100  $\mu\text{L}$ ).
5. LC-MS-MS analyses: LC/MS/MS analysis is conducted on an LCQ (Finnigan MAT, San Jose, CA) quadrupole ion trap mass spectrometer system equipped with an electrospray atmospheric pressure ionization probe. Compounds were dissolved in methanol and injected into the high-performance liquid chromatography (HPLC) component, which consisted of a Spectra SYSTEM P4000 quaternary gradient pump (Thermo Separation Products, San Jose, CA), LUNA C18-2 (150  $\times$  2 mm, 5  $\mu\text{m}$ ) or C18-2 (100  $\times$  2 mm, 5  $\mu\text{m}$ ) columns (Phenomenex, Torrance, CA), and a rapid spectra scanning SpectraSYSTEM UV2000 UV/VIS absorbance detector (Thermo Separation Products). The column is eluted at 0.2 mL/min isocratically for 20 min with methanol/water/acetic acid (65/35/0.01, v/v/v) followed by a 20 min linear gradient to 99.99/0.01 methanol/acetic acid (v/v). The spray voltage is set to 8 kV and the heated capillary to  $250^{\circ}\text{C}$ , and a maximum ion injection time of 350 ms. Over a 2.4 s scan cycle, full-scan MS are acquired by scanning between  $m/z$  95-450 in the negative ion mode, followed by the acquisition of product ion MS/MS of the most intense molecular anions (e.g.,  $[\text{M}-\text{H}] = m/z$  349.5 for RvE1,  $[\text{M}-\text{H}] = m/z$  343, 359 and 375 for mono-, di- and tri-HDHA, respectively). Also,

a Chiralcel OB-H column (J. T. Baker) was used to determine R and S alcohol configurations of monohydroxy-PUFA using isocratic (hexane:isopropanol; 96:4 vol:vol).

6. Human PMNs: PMNs should be used immediately after isolation (i.e., ~3 h after venipuncture, accounting for isolation) or placed in an ice bath (4°C). This will slow the decrease of enzymatic activity that starts once the cells are removed from blood. Note that careful records should be kept on the blood donors. Unlike cultured cells, peripheral blood cells are exposed to a diverse environment that can include cytokines and mono-HETEs that “prime” leukocytes. Such diversity results in noticeable donor variations in the data. For example, in endogenously “primed” PMN such as from asthmatic patients, 5S,15S-diHETE and LX, which normally are associated with transcellular biosynthesis, are generated endogenously from a single cell type (PMN).
7. TNF- $\alpha$ -initiated murine air pouches. Male FVB mice (6 to 8 wk) fed with laboratory rodent diet 5001 (Lab Diet, Purina Mills; containing less than 0.25% arachidonic acid, 1.49% EPA and 1.86% DHA) were anesthetized with isoflurane. Dorsal air pouches were raised by injecting 3 mL of sterile air subcutaneously on day 0 and 3. On day 6, inflammation in the air pouch was induced by local injection of recombinant murine TNF- $\alpha$  (20 ng) dissolved in 100  $\mu$ L of sterile PBS. At indicated times, air pouches were lavaged twice with 3 mL of PBS. Lavage cells were collected by centrifugation and enumerated by light microscopy. Cell-free lavage fluids were used for determination of lipid mediators by lipidomic analysis.
8. Handling lipid mediators: RVs are sensitive to light, oxygen, and heat and should be stored at 20 to -80°C. This is especially critical for native EPA and DHA, which upon exposure to oxidative conditions will degrade to a variety of compounds. Thus, prepare a concentrated stock and a “working or daily stock” that you will use as reference materials for the experiments. As a general rule, each time a stock is used, pass a gentle stream of nitrogen into the vial before closing. This will slow the oxidative degradation. Determine the concentration of RV stocks by measuring the ultraviolet absorbance and using the specific extinction coefficient. This is especially important if stocks are used on a regular basis. If possible, HPLC analysis of your stocks is an invaluable tool to determine the purity and, for example, percent isomerization.
9. Vehicle controls: the total amount of vehicle (ethanol) added to your cell incubation or coinubations should not exceed 0.1%. Higher concentrations of alcohol can affect cell integrity (i.e., enzyme activity and membrane integrity). It is therefore desirable to prepare stocks of compounds at 1000 to 5000 times the final concentration so that only small aliquots are added directly to cell suspensions.
10. Zymosan A-initiated murine peritonitis: Male FVB mice (6–8 wk old) were anesthetized with isoflurane. Compounds were delivered as a bolus injection either into the tail vein in 100  $\mu$ L of sterile saline or intraperitoneally in 200  $\mu$ L of sterile saline and followed by intraperitoneal injection of zymosan A (1 mg/mL). Vehicle controls were performed in parallel. At indicated time points, mice were

ethanized with an overdose of isoflurane, peritoneal lavages collected and centrifuged. Aliquots of the cells were stained with Trypan blue and numerated by light microscopy.

11. Internal standards: it is essential to terminate incubations with solutions containing an internal standard since this will serve as an invaluable tool for correction of product profiles (e.g., HPLC retention times) and for calculating recoveries of LX and Rv after extraction and sample workup. The internal standard should be a stable measurable compound that is not generated during the cell incubations and does not interfere with the product analysis. PGB<sub>2</sub> usually meets those criteria for analysis of LX and Rv. Depending on the sensitivity of the analytical systems to be used (i.e., LC-MS, GC-MS, or PDA-UV-HPLC), 50 pg to 200 ng are added to the incubations.

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## Isolation and Analysis of Lipid Rafts in Cell–Cell Interactions

Aimee Landry and Ramnik Xavier

### Summary

Lipid rafts are dynamic structures made up of proteins and lipids that float freely within the liquid-disordered bilayer of cellular membranes and have the ability to cluster to form larger, more-ordered platforms. These clustered structures have been identified in all cell types and have been shown to play critical roles in signal transduction, cellular transport, and cell–cell communication. Lipid rafts also have been implicated in facilitating bacterial/viral entry into host cells and in human disease, highlighting the significance of understanding the role lipid rafts play in physiological and pathological signaling outcomes. In this chapter, we provide protocols to isolate lipid rafts from polarized and nonpolarized cells and outline novel technologies to analyze signal transduction cascades in vivo.

**Key Words:** Lipid rafts; detergent-insoluble membranes; cholesterol; signal transduction; biochemical fractionation; microscopy; proteomics; FRET; FRAP; flow cytometry; lymphocytes; polarized epithelial cells.

### 1. Introduction

Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids, and are associated with specific microanatomic features of cells from diverse lineages. They float freely in the resting state, carrying a few passenger proteins, but aggregate to form functional platforms mediating diverse signaling cascades when activated (*1–4*). Lipid rafts have been described in all cell types to date. Cholesterol content and sphingolipids play a central role in the genesis of these of these microdomains. Analysis of the consequences of directed mutations in mice have established a role for lipid rafts in signal transduction (*5,6*). Caveolae, characteristic flask-shaped invaginations of endodermal cells are one class of lipid raft structure; another is the luminal surface of polarized

epithelium, which is highly enriched in raft-like structures. In other cells, such as lymphocytes and neurons, rafts are isolated structures 50 nm in diameter and do not appear to be associated with any specific ultrastructural features. In lymphocytes, rafts are estimated to represent 40% of total membrane as determined by fluorescence anisotropy measurements. For the purposes of this overview we will refer to lipid rafts as detergent-insoluble membranes (DIMs). Both phosphatidylinositol-linked proteins and palmitoylated transmembrane proteins are concentrated in rafts, as well as nearly all lipidated intracellular proteins, including myristoylated, palmitoylated, and prenylated molecules.

A protein's affinity to lipid rafts can be modified by enzymatic reactions, which alter the biochemical properties of the given protein, allowing the cell to dynamically regulate molecular mechanics which govern cellular function. Glycosylphosphatidylinositol (GPI)-linked proteins often are found located within lipid rafts and can be cleaved by phospholipase. Rab GTPases, key regulators of membrane targeting and fusion, require the covalent attachment of geranyl-geranyl isoprenes near their C terminus in a multi-step process, which can subsequently increase lipid affinity (7). Src family kinases, which are responsible for plasma membrane targeting, contain a unique domain in the N-terminus that is modified by dual acylation and palmitoylation (8).

The ability of lipid rafts to isolate protein receptors from the majority of the plasma membrane enables the compartmentalization of signaling components, concentrating certain elements within lipid rafts while excluding others (9,10). HaRas, which is palmitoylated and farnesylated, is localized to lipid rich microdomains. In contrast, KiRas, which is farnesylated but not palmitoylated, associates with the inner leaflet of the plasma membrane and is excluded from lipid rafts. Thus, a posttranslational addition of palmitate effectively segregates these two GTPases in the plasma membrane (11). Recent studies have suggested that an equivalent domain organization is present in the cytoplasmic leaflet as well. In polarized epithelial cells, lipid rafts are distributed asymmetrically at the apical surface and play a role in protein localization, trafficking, internalization, and proteosomal degradation (12,13).

Proteins are thought to target lipid-rich domains resulting from the fact that the number of lipid sites exceeds the number of binding sites; this system allows lipid rafts to recruit many different proteins to a membrane without saturating protein-binding sites. Lipids also can be produced and degraded rapidly by enzymes in a matter of seconds, allowing the characteristics of a membrane to be rapidly remodeled based on the physiological need of the cell at a given time point. In addition, lipid-binding interactions often occur at a low affinity, enabling rapid protein equilibration and movement inside the cell.

Microdomains also play key roles in the life cycle of microbes by facilitating access to cellular receptors, influencing trafficking to appropriate subcellular sites, and play a critical role in the assembly of enveloped viruses during replication (14). Lipid rafts also have an important function in the organization of signal transduction during lymphocyte activation (15,16). It appears that differential partitioning of signaling proteins within the lipid rafts is an important mechanism by which integration of signal transduction pathways is achieved. In T- and B-cells, lipid rafts appear to play an important role in development, signaling and differentiation.

Understanding raft-mediated membrane trafficking is important to cell communication. Lipid rafts have been shown to be involved in fundamental processes of all eukaryotic cells, and 30% of all cellular proteins are membrane proteins. Lipid rafts have drawn the attention of a variety of investigators with broad range of research interests. Neurobiologists have identified lipid rafts as the basis of synaptic transmission (17). Lipid rafts have also been shown to be essential for antigen presentation and processing and have been implicated in several immunological disorders such as systemic lupus erythematosus (18). An expanding list of monogenic diseases can be traced to altered lipid raft function (19,20). In addition, lipid rafts play a central role in the pathogenesis of numerous common and complex diseases, such as diabetes, atherosclerosis, cancer, and neurodegenerational disorders such as Alzheimer's disease (21). Lipid rafts are thought to serve as specific targets for entry of immunomodulators (e.g., toxins or lipopolysaccharides) of bacterial origin into host cells or viruses (e.g., human immunodeficiency and influenza viruses).

Persuasive evidence for the existence of lipid rafts comes from analysis of model membranes, liposomes, and biophysical methods such as fluorescence energy transfer and single particle tracking experiments (22). Considerable progress has been made in studying and understanding the role lipid rafts play in receptor-mediated signal transduction. Here, we briefly review the role of rafts in a model system familiar to our laboratory and provide protocols to isolate and analyze rafts in nonpolarized and polarized cell signaling.

## 2. Materials

### 2.1. Cell Stimulation

1. Purified antibodies of interest.

### 2.2. Cholesterol Depletion and Enrichment

1. Methyl- $\beta$ -cyclodextrin (M $\beta$ CD; Sigma, St. Louis, MO, cat. no. C-4555). Stock solutions made in dH<sub>2</sub>O and stored at  $-20^{\circ}\text{C}$ .
2. Cholesterol complexed to M $\beta$ CD (Sigma, cat. no. C-4951). Stock solutions made in dH<sub>2</sub>O and stored at  $-20^{\circ}\text{C}$ .

### 2.2.1. Sucrose Gradient

1. Refrigerated ultracentrifuge capable of reaching speeds of 200,000g and appropriate rotor.
2. Glass Dounce homogenizer (Bellco Glass Inc.; cat. no. 1984-10007).
3. SW-40 polyalomer tubes (Beckman, cat. no. 331372).
4. Mes-buffered saline (MBS): 25 mM Mes (pH 6.5), 150 mM NaCl. Store at 4°C.
5. MBS lysis buffer: 22 mM MES (pH 6.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM EDTA, 1 mM phenylmethyl sulfonyl fluoride, and 1 µg/µL aprotinin. Store at 4°C.
6. Sucrose solutions: 80% (w/v) sucrose (80 g of ultra pure sucrose into a final volume of 100 mL of MBS). Heat approx 50 mL of MBS while stirring and gradually add sucrose until dissolved. Do not boil. Increase volume to 100 mL with additional MBS. 35% (w/v) and 5% (w/v) sucrose solutions can be made by diluting 85% stock. Store at 4°C.
7. Apparatus for sodium dodecyl sulfate polyacrylamide gel electrophoresis and Western transfer (Bio-Rad, cat. no.165-3318).
8. Polyvinylidene difluoride membrane (Millipore, cat. no. IPVH20200).

### 2.2.2. Linear Gradient

1. Buffer A: 0.25 M sucrose, 1 mM EDTA, 20 mM Tris-HCl, pH 7.8.
2. Buffer A+: Buffer A+ 0.2 mM AEBSF, 1 µg/mL aprotinin, 10 µM bestatin, 3 µM E-64, 10 µg/mL leupeptin, 2 µM pepstatin, and 50 µg/mL calpain inhibitor I.
3. Percoll (Sigma, cat. no. P-1644).

### 2.2.3. Sodium Carbonate Gradient

1. SCB: 50 mM sodium carbonate, pH 11.0.
2. SCB+: SCB + 0.2 mM AEBSF, 1 µg/mL aprotinin, 10 µM bestatin, 3 µM E-64, 10 µg/mL leupeptin, 2 µM pepstatin, and 50 µg/mL calpain inhibitor I.
3. Branson Sonifier 250, or similar.

### 2.2.4. OptiPrep Gradient

1. Base buffer: 20 mM Tris-HCl, pH 7.8, 250 mM sucrose, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>.
2. Base buffer+: Base buffer + 0.2 mM AEBSF, 1 µg/mL aprotinin, 10 µM bestatin, 3 µM E-64, 10 µg/mL leupeptin, 2 µM pepstatin, and 50 µg/mL calpain inhibitor I.
3. OptiPrep (Granier BioOne, cat. no. No. 1030061; www.gbo.com).

## 2.3. Protein Localization and Aggregation of Lipid Rafts

1. Glass slides and cover slips.
2. Clear nail polish.
3. PAP Pen (Sigma, cat. no. Z377821).
4. 0.01% poly-L-lysine (Sigma, cat. no. P-4707).

5. 4% (w/v) paraformaldehyde (PFA): add 4 g of PFA powder to approx 80 mL of dH<sub>2</sub>O. Stir and heat solution until solution reaches 60 to 70°C (but do not allow to boil). Add 10 N NaOH drop-wise until solution clears. Remove from heat and allow to cool. Add 10 mL of 10X phosphate-buffered saline (PBS) and adjust final volume to 100 mL. Adjust pH to 7.4, filter, and store at 4°C for up to 1 mo. Note: PFA powder is toxic if inhaled. Work in a fume hood or wear a mask while measuring out the powder to avoid inhalation.
6. Tween-20 (Sigma, cat. no. P-9416).
7. Bovine serum albumin (BSA; Sigma, cat. no. A-3059).
8. Primary antibodies of interest.
9. Fluorescent secondary antibodies.
10. VectaMount (Vector Laboratories, cat. no. H-5000, [www.vectorlabs.com](http://www.vectorlabs.com)).

## 2.4. Molecular Probes and Fluorescent Microscopy

1. Vybrant Lipid Raft Labeling Kit (Molecular Probes; [www.probes.com](http://www.probes.com)).
2. Bodipy sphingomyelin analog (C5 DMB-SM; Molecular Probes; [www.probes.com](http://www.probes.com)).

## 2.5. Immunolocalization

1. Goat-anti-mouse IgG Dynabeads, M-450 4 × 10<sup>8</sup> beads/mL (Dynal, Oslo, Norway; [www.dynalbiotech.com](http://www.dynalbiotech.com)).
2. MPC-E magnet for 1.5-mL microcentrifuge tubes (Dynal).
3. Dynal MPC-1 magnetic partial concentrators for 15- or 50-mL centrifuge tubes (Dynal).
4. Rotating wheel.
5. 100 mM sodium vanadate: dissolve 9.5 g of sodium orthovanadate in approx 400 mL of dH<sub>2</sub>O. Adjust pH to 10.2 with HCl. Heat in microwave until solution starts to boil. Adjust pH to 10.2 with 1 M NaOH. Heat in microwave until solution starts to boil. Check pH is between 9.9 and 10.1. Repeat with new reagents if pH is not correct.
6. 10 mM pervanadate: 10 μL of 100 mM sodium vanadate stock solution, 10 μL of 30% H<sub>2</sub>O<sub>2</sub>, and 80 μL of dH<sub>2</sub>O. Incubate solution at room temperature (RT) for 10 min before preparing HB+ buffer.
7. 1000X CLAP: 5 mg of chymostatin, 5 mg of leupeptin, 5 mg of antipain, and 5 mg of pepstatin. Dissolve in 500 μL of dimethyl sulfoxide. Store in 50-μL aliquots at -20°C.
8. HB buffer: 250 mM sucrose, 10 mM Na-HEPES, pH 7.2, 1 mM MgCl<sub>2</sub>, 10 mM NaF, and 1 mM sodium vanadate. Adjust to a final volume of 500 mL with dH<sub>2</sub>O.
9. HB+ buffer: add 1 : 1500 of 10 mM pervanadate and 1 : 1000 of 1000X CLAP to HB.
10. Laemmli sample buffer (BioRad; cat. no. 161-0737).

## 2.6. Electron Microscopy

1. 5-mm copper wire loops.
2. Mesh copper grids (Agar Scientific, UK).

**Table 1**  
**Volumes for Preparing Reducing Solution for Various-Sized Gold Particles**

Gold size (nm)	2–3	4	4.5	5.5	6	7.5
1% tannic acid (mL)	2.5	1.25	0.75	0.5	0.25	0.13
25 mM potassium carbonate (mL)	2.5	1.25	0.75	0.5	0.25	0.13

3. Filter paper (Whatman).
4. Fish skin gelatin, 45% aqueous solution (Sigma, cat. no. G-7765).
5. Glutaraldehyde, EM grade, 25% stock (Agar Scientific, UK, cat. no. R1020).
6. Methyl cellulose (Sigma, cat. no. M-6385).
7. Parafilm.
8. Paraformaldehyde, EM grade, 16% stock (Agar Scientific, cat. no. R1026).
9. Adobe Photoshop (San Jose, CA).
10. Image J 1.26 (<http://rsb.info.nih.gov/ij/>) or NIH Image 1.82 (<http://rsb.info.nih.gov/nih-image>).
11. Affinity-purified antibodies of interest.
12. Commercially available K-function analysis program.
13. Transmission electron microscope (JEOL 1010 or similar).
14. 1% trisodium citrate: dissolve 0.5 g of sodium citrate in 50 mL of dH<sub>2</sub>O. Store at RT.
15. 1% tannic acid: dissolve 0.5 g of tannic acid in 50 mL of dH<sub>2</sub>O. Store at RT.
16. 25 mM potassium carbonate: dissolve 0.173 g of potassium carbonate in 50 mL of dH<sub>2</sub>O. Store at RT.
17. Reducing solution: 2 mL of 1% trisodium citrate, X mL of 1% tannic acid, and X mL of 25 mM potassium carbonate (*see* **Table 1** for quantities). Add dH<sub>2</sub>O for a final volume of 10 mL. Make fresh for each use.
18. 1% gold chloride: dissolve 1 g of gold chloride in 100 mL of dH<sub>2</sub>O. Store at 4°C. Stable for up to 2 yr.
19. Gold solution: add 0.5 mL of 1% gold chloride to 39.5 mL of dH<sub>2</sub>O. Make fresh for each use.
20. 10% BSA: dissolve 2 g of BSA in 20 mL of PBS. (For use in electron microscopy [EM], centrifuge at 100,000g for 1 h at 4°C to remove any remaining particles. Store at 4°C for 5 mo.)
21. 0.1% BSA: add 0.5 mL of 10% BSA to 50 mL of PBS. Make fresh for each use.
22. 2% methyl cellulose: dissolve 2 g of methyl cellulose in 98 mL of dH<sub>2</sub>O heated to 90°C. Cool on ice while continuing to stir. Store in a sealed container for 72 h at 4°C.
23. Centrifuge at 100,000g for 1 h. Decant supernatant and store at 4°C.
24. 10X potassium acetate (KOA) buffer: add 11.92 g of HEPES, 22.58 g of potassium acetate, and 1.02 g of magnesium chloride to 180 mL of dH<sub>2</sub>O. Add six pellets of KOH and continue to adjust pH using additional KOH (1 M) to 7.4. Add additional water for a final volume of 200 mL. Aliquot and store at –20°C.

**Table 2**  
**Donor/Acceptor Antibodies for FRET**

FRET Pair	Donor Imaging Path			Acceptor Imaging Path		
	Excitation Filter	Dichroic	Emission Filter	Excitation Filter	Dichroic	Emission Filter
FITC-TRITC	480/40	505 LP	535/40	545/30	565 LP	610/60
FITC-Cy3	480/40	505 LP	535/40	540/25	565 LP	605/55
Cy3-Cy5	540/25	565 LP	605/55	640/20	660 LP	680/30
BFP-GFP	390/20	420 LP	460/50	475/40	460 LP	535/50
GFP-Cy3	475/40	460 LP	505/40	540/25	565 LP	605/55
CFP-YFP	436/20	455 LP	480/40	500/20	515 LP	535/30
CFP-DsRed	436/20	455 LP	480/40	546/12	560 LP	620/20

All values are expressed as center wavelengths (in nm) with the spread on either side of the peak value. Donor- and acceptor-only images are acquired separately with donor and acceptor filter sets, respectively. FRET images are acquired with donor excitation filter, donor dichroic and acceptor emission filters. These filter combinations were adapted from the catalog of filter sets published by Chroma Technology Corporation. FRET, fluorescence resonance energy transfer; TRITC, tetramethylrhodamine isothiocyanate; BFP, blue fluorescent protein; GFP, green fluorescent protein; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein.

25. 1X KOAc buffer: thaw and add 2 mL of 10X KOAc buffer (above) to 18 mL of PBS. Make fresh for each use.
26. Fixative: add 2 mL paraformaldehyde (16% stock), 32  $\mu$ L of glutaraldehyde (25% stock), and 0.8 mL of 10X KOAc buffer to 5.17 mL of dH<sub>2</sub>O in a fume hood. Store at 4°C for up to 2 wk.
27. 25 mM glycine: dissolve 93.8 mg of glycine in 50 mL of PBS. Aliquot (1 mL each) and store at –20°C.
28. Fish skin gelatin: add 3 mL of fish skin gelatin (45% aqueous solution) to 11.5 mL of PBS. Store at 4°C for up to 6 mo.
29. Blocking solution: add 200  $\mu$ L of 10% fish skin gelatin and 200  $\mu$ L of 10% BSA to 10 mL of PBS. Make fresh for each use.
30. 3% uranyl acetate: dissolve 1.5 g of uranyl acetate in 50 mL of dH<sub>2</sub>O. Aliquot and store at 4°C for 1 yr.

## 2.7. Fluorescence Resonance Energy Transfer

1. Lipid-based delivery system, such as TransFection (Bio-Rad, cat. no. 170-3250).
2. Donor/acceptor antibodies for FRET ([Table 2](#)).
3. Normal serum from secondary antibody host diluted in PBS (as recommended by manufacturer).
4. NaBH<sub>4</sub> (1 mg/mL).
5. 100% methanol (–20°C) or 0.1% Triton X-100 (4°C) for fixation (if necessary).

6. Confocal laser scanning imaging system with appropriate lasers for excitation of donor-acceptor fluorescence resonance energy transfer (FRET) pairs.

## **2.8. Fluorescence Recovery After Photobleaching**

1. Polybead polystyrene beads (6.0  $\mu\text{m}$  in diameter; Polyscience, cat. no. 07312).
2. Purified antibodies of interest.
3. Blocking Solution: 10 mg/mL in PBS. Filter-sterilize and store at 4°C for several months.
4. Confocal microscope (Carl Zeiss, LSM 510, or similar) and with a 25 mW Argon laser.

## **2.9. Proteomics**

1. Leucine-deficient media with serum.
2. Leucine-deficient media without serum.
3. L-Leucine (Sigma, cat. no. L-8912).
4. L-Leucine-3,3,3-D<sub>3</sub> (Sigma, cat. no. 48,682-5).
5. Protein concentration determination kit.
6. 6 M urea-2 M thiourea (in 10 mM HEPES, pH 8.0).
7. 2.5 M sodium acetate, pH 5.0.
8. 1,4-dithio-DL-threitol (DTT; Fluka, cat. no. 43817).
9. Iodoacetamide (Sigma, cat. no. I-1149).
10. LysC enzyme (Sigma, cat. no. A-3422).
11. 50 mM ammonium bicarbonate.
12. Trypsin (Promega, cat. no. V5113).
13. Empore disks (3M, Minneapolis, MN, cat. no. 2215).
14. Reverse-phase analytical columns (various manufacturers).
15. 5.6 to 64% acetonitrile (Fluka cat. no. 00682).
16. Mass spectroscopy (MS) (QSTAR-Pulsar quadrupole time-of-flight hybrid; PE-Sciex, Thornhill, Ontario, Canada), or similar.

## **2.10. Analysis Using the Fluorescence-Activated Cell Sorter**

1. Fluorescent antibodies specific against membrane antibodies.
2. Fluorescence-activated cell sorting (FACS) machine (FACSCalibur or similar), corresponding software program (i.e., CellQuest).

## **3. Methods**

### **3.1. Cell Preparation**

#### *3.1.1. Cell Stimulation*

Lipid rafts are most commonly isolated from cells based on their differential solubility in nonionic detergents. Raft size is increased by clustering, leading to a new mixture of molecules. This clustering can be triggered at the

extracellular side by ligands, antibodies, or lectins (23); within the membrane by oligomerization (24); or by cytosolic agents (cytoskeletal elements, adapters, scaffolds [25,26]). Raft clustering occurs at the plasma membrane as well as intracellularly, such as in the endosomal lumen. Ligand binding or oligomerization can alter the partitioning of proteins in and out of rafts. Increased raft affinity of a given protein and its activation within rafts (e.g., phosphorylation by Src-family kinases can initiate a cascade of signaling events, leading to further increase of raft size by clustering (27). In the example presented below, Jurkat cells are stimulated with anti-CD3 and CD28 antibodies to activate the T-cell receptor (TCR) complex. This method can easily be adapted to other cells.

1. Count cells and determine the total number needed for the experiment (*see Note 1*).
2. Collect required cells by centrifugation (800g for 5 min at 4°C) and wash once in 1 mL ice-cold PBS. Resuspend in 200 µL of PBS per sample.
3. Transfer cells to labeled microcentrifuge tubes and add 1 µg of both CD3 and CD28 antibodies and incubate in a 37°C water bath for various time points (*see Note 2*). If the samples will be in the water bath for more than 5 min, agitate intermittently.
4. Stop the reaction by adding 1 mL of ice-cold PBS to each tube and wash twice (800g for 5 min at 4°C).

### 3.1.2. Cholesterol Depletion and Enrichment

Because of the important role cholesterol plays in the stabilization of lipid rafts, reversible depletion or enrichment of plasma membrane cholesterol are two useful techniques that can be used to examine the biological roles of functional and non-functional lipid rafts. If a protein is found to be associated with DIM, treatment with cyclodextrin, saponin, and sphingomyelinase may be applied to test whether the association depends on cholesterol and sphingomyelin. M $\beta$ CD extracts cholesterol from membranes and disrupts domains whose integrity depends on cholesterol. Saponin complexes cholesterol and is thought to sequester it away from other interactions.

#### 3.1.2.1. CHOLESTEROL DEPLETION

1. Collect cells by centrifugation and wash once in MBS. Incubate cells with 10 mM M $\beta$ CD at 37°C for 30 min.
2. Cells can now be used in functional studies, or washed, repelleted, lysed, and subjected to sucrose-gradient fractionation.

#### 3.1.2.2. CHOLESTEROL ENRICHMENT

When precomplexed with cholesterol, cyclodextrins can deliver cholesterol back into the plasma membrane and restore cellular functions previously interrupted by cholesterol depletion.

1. Wash cholesterol-depleted cells. Resuspend cells in MBS containing 3 mM M $\beta$ CD: cholesterol and incubate at 37°C for 30 min.
2. Wash cells once and use in subsequent studies (*see* **Notes 3–5**).

### 3.2. Gradient Isolation of DIM Fractions

#### 3.2.1. Isolation of DIM Fractions

This protocol takes advantage of the differential solubilization of lipid rafts and the plasma membrane to isolate populations of DIMs. In our experience, Triton X-100 is the most effective detergent in membrane solubilization and recovery of proteins enriched in lipid microdomains. Composition of isolated DIMs depends on the temperature and on the nature and concentration of detergent used. An elegant study recently demonstrated that DIMs can be isolated at 37°C in the presence of Brij 98 (28). Commonly used detergents are Triton X-100, Brij 58, Brij 96, LubrolWX, CHAPS, and Brij 98. Composition of lipid rafts depends on the detergent used. Recent studies have suggested that all rafts isolated do not reflect the same aspect of membrane organization (28,29). Keep in mind that proper controls for incomplete solubilization of membrane fractions are important for each experiment. A common problem with this method in to analyze lipid rafts organization in cells is that lipid reorganization occurs during solubilization so that detergent-resistant membrane structures may differ from membranes before detergent extraction. Despite this caveat, however, the isolation of DIMs will remain a valuable tool for the analysis of biological membranes.

##### 3.2.1.1. SUCROSE GRADIENT

1. Precool the ultracentrifuge, rotor, and all solutions to 4°C (*see* **Note 6**).
2. Resuspend the pellet in 2 mL of MBS buffer. Incubate the cells on ice for 30 min. Disrupt cell membranes by homogenizing for ten strokes in a glass Dounce homogenizer at 4°C.
3. Transfer 2 mL of the total cell homogenate to the bottom of a SW-40 centrifuge tube and add 2 mL of 80% sucrose; mix well.
4. Slowly layer 6 mL of the 35% sucrose solution on top of the sample layer; to prevent the disruption of the sucrose gradient, hold the pipet at a 45° angle on the side of the centrifuge tube.
5. Layer 4 mL of the 5% sucrose solution on top of previous two layers. Make sure that the tube is filled to within 2 mm of the top.
6. Balance the tubes to within 0.01 g and place them in the rotor. Centrifuge samples for 16 h at 200,000g at 4°C.
7. Using a pipet, carefully collect 12 fractions of 1 mL each, starting from the top of the gradient. Alternatively, extract the visible band at the 35%: 5% sucrose interface with a syringe. Proteins localized to lipid rafts are usually recovered in the low-density sucrose gradient fractions of two, three, and four (*see* **Notes 7–9**).

8. Prepare aliquots for sodium dodecyl sulfate polyacrylamide gel analysis and separate the proteins by electrophoresis. Transfer to a polyvinylidene difluoride membrane. Identify proteins of interest by immunoblotting with antibodies according to manufacture's directions (*see* **Note 10**).

### 3.2.1.2. DETERGENT-FREE ISOLATION OF LIPID RAFTS

Detergents have routinely been used to isolate lipid rafts over the past several years, although questions have recently been raised surrounding this technique. It is thought by some that the extraction of lipid rafts with detergent may generate artificial clusters of lipid rafts that do not exist *in vivo*. The following is a list of three protocols that avoid the use of detergents, but care should be taken in deciding which protocol will best serve your research needs.

### 3.2.1.3. LINEAR GRADIENT

Originally developed to isolate caveolae from cells or tissues, this protocol uses sonication to disrupt the cell membrane and was used to identify novel proteins associated with lipid rafts based on their unique buoyant density (**28,30**). The main advantage of this protocol is the purified lipid rafts obtained without the use of harsh detergents. This purity, however, also results in low overall yields.

1. Wash four confluent 10-cm plates of cultured adherent cells (~7–8 mg of total protein) with PBS. Remove PBS and scrape cells into Buffer A+. Transfer into a 15-mL centrifuge tube.
2. Centrifuge cells (1000g for 5 min at 4°C) and resuspended in an additional 1 mL of Buffer A+.
3. Lyse cells with 10 strokes of a Dounce homogenizer using a tight-fitting pestle.
4. Follow homogenization by shearing, passing lysates through a 23-gage needle 10 times.
5. Centrifuge cells (1000g for 5 min at 4°C). Collect the supernatant and transfer to a separate tube.
6. Repeat lysing of the remaining pellet with 1 mL of additional Buffer A+. Lyse cells with 10 strokes of a Dounce homogenizer using a tight-fitting pestle.
7. Follow homogenization by shearing by passing lysates through a 23-gage needle 10 times.
8. Centrifuge cells (1000g for 10 min at 4°C). Combine both cleared lysates.
9. Carefully layer the combined lysates on top of 8 mL of 30% Percoll and centrifuge for 30 min at 84,000g.
10. A visible band should be seen one-third of the way down the gradient. Collect band with a needle and syringe.
11. Adjust total volume of the plasma membrane fraction to 2 mL with additional Buffer A+.

12. Sonicate fraction with six pulses at 50% duty cycle using a Branson Sonifier 250. Allow the sample to settle for 1 min. Repeat sonification two more times for a total of 18 pulses.
13. Mix sonicate with 2 mL of Buffer A+ containing 50% OptiPrep.
14. Carefully layer an 8 mL OptiPrep gradient (10–20%) on top of the 25% sonicate and centrifuge for 90 min at 52,000g.
15. Isolate fractions of equal volume as above (*see Subheading 3.2.1.1.*) and determine the distribution of key lipid raft proteins via Western blotting.

#### 3.2.1.4. SODIUM CARBONATE GRADIENT

The following is another detergent-free method of isolating lipid rafts that preserves the interactions between resident prenylated caveolin-associated proteins while generating a purified population of caveolin-rich membrane domains; using this protocol, Song et al. (28,31) were first to identify novel interactors of caveolin, including Ras. The main advantage of this protocol is its simplicity; disadvantages include a long spin time and potentially significant contamination of the isolated raft fraction with other membranes.

1. Wash two 10-cm plates of cultured adherent cells with PBS. Scrape cells and transfer into a 15-mL centrifuge tube.
2. Centrifuge cells (800g for 5 min at 4°C) and resuspended in 2 mL of SCB+.
3. Lyse cells with 20 strokes of a Dounce homogenizer using a tight fitting pestle.
4. Follow homogenization by shearing by passing lysates through a 23-gage needle 10 times.
5. Sonicate lysate three times 15 s using a Branson Sonicator 250.
6. Combine lysate with 2 mL of MES buffer containing 90% sucrose. Transfer mixture to the bottom of a 12-mL centrifuge tube.
7. Carefully layer 4 mL of MES buffer containing 35% sucrose on top of previous layer.
8. Add an additional 4 mL of MES buffer containing 5% sucrose and centrifuge for 16 to 20 h at 175,000g.
9. Take twelve 1-mL fractions and determine the distribution of key lipid raft proteins via Western blotting.

#### 3.2.1.5. OPTIPREP GRADIENT

Here, we outline a recent isolation method for the preparation of detergent-free lipid rafts where the lysis of cells by shearing in an isotonic buffer containing calcium and magnesium yields a highly purified raft fraction and optimal protein yield (28).

1. Wash four 10-cm plates of cultured adherent cells (~7–8 mg of total protein) with PBS. Remove PBS and scrape into 3 mL of Base Buffer+. Transfer into a 15-mL centrifuge tube.
2. Centrifuge cells (1000g for 5 min at 4°C) and resuspended in 1 mL of Base Buffer+.

3. Lyse cells by passing through a 23-gage needle 10 times, being careful to avoid air bubbles.
4. Centrifuge cells (1000g for 10 min at 4°C). Transfer the cleared lysate to a separate tube.
5. Lyse the remaining pellet for a second time with 1 mL of additional Base Buffer+.
6. Lyse cells by passing through a 23-gage needle 10 times, being careful to avoid air bubbles.
7. Centrifuge cells (1000g for 10 min at 4°C). Combine both cleared lysates.
8. Add 2 mL of Base Buffer + 50% OptiPrep to the combined post-nuclear (now 25% OptiPrep) supernatants and place in the bottom of a 12-mL centrifuge tube.
9. Carefully layer an 8 mL of OptiPrep gradient (0–20%) on top of the combined lysates.
10. Centrifuge for 90 min at 52,000g.
11. After centrifugation, a diffuse band will appear one-third of the way down the gradient and a distinct band will be apparent at the 20:25% interface.
12. Isolate 0.67-mL fractions as mentioned previously (*see Subheading 3.2.1.1.*) and determine the distribution of key lipid raft proteins via Western blotting.

### 3.3. Useful Protocols for DIM Analysis

#### 3.3.1. Protein Localization and Aggregation of Lipid Rafts

Lipid-enriched microdomains are principally composed of sphingolipids and cholesterol. The operational definition of rafts is solubility in cold detergent and the functional definition of lipid raft-dependent signaling has been traditionally based on cholesterol depletion studies. However, cholesterol depletion, in addition to affecting lipid rafts structure, can also alter cellular processes such as actin reorganization, receptor mediated signaling and endocytosis. Introducing fluorescent tags to proteins is a valuable way of assessing the sub-cellular distribution of proteins in response to receptor activation (32,33). This permits one to assess both the spatial and temporal aspects of protein expression. Two different approaches have been used to identify lipid rafts in cell membranes. One is to observe the random or directed motion of membrane lipids and lipid-anchored proteins in the plane of a bilayer. This approach is useful to identify the size of DIMs. Further microscopic probes can track molecule mobility on the nanometer scale and following lipid raft association induced by ligand receptor interaction. The second approach is to measure the proximity of molecules in rafts by FRET. In addition, fluorescent lipid analogs have been used to define rafts in other lipid microdomains in cells in culture.

The use of fusion proteins, such as green fluorescent protein (GFP), allows an investigator to examine protein distribution in live cells while avoiding the potential artifacts of cell fixation and permeabilization. However, the concerns include the fact that GFP-tagged proteins are often expressed in levels higher than the endogenous protein, and could have an effect on protein localization.

Furthermore, the GFP tag is large, approx 27 kDa in size, and could interfere with tracking recruitment to microdomains. This is further compounded by GFP-dependent dimerization. Despite these limitations, fluorescent tags are a very useful approach to monitor lipid domain based signaling events. It is essential to ensure that the fluorescent-tagged protein co-localizes to the same compartment as the endogenous protein. The availability of different fluorescent proteins (yellow fluorescent protein and cyan fluorescent protein) allows two proteins to be tagged with distinct spectral variants. In these studies, expressed proteins are visualized before and after activation by digital imaging using confocal and low light charge-coupled device (CCD) camera-based microscopes. In addition to live imaging, cells can be visualized after fixation and staining with Cy3-labeled cholera toxin B-subunit.

Lipid rafts in the plasma membrane outer leaflet are enriched in sphingolipids and cholesterol, but the exact lipid composition of the inner membrane raft is unknown. To determine the segregation and kinetics of recruitment of proteins to the outer and inner layer lipid enriched domains, Zacharias et al. generated fusion of GFP spectral variants linked to consensus sequence of different lipids (34). In an elegant series of experiments, the authors demonstrated that lipid acyl (myristoylated and palmitoylated) proteins were localized to the outer membrane, whereas proteins containing the geranyl-geranyl tail were excluded from the outer lipid enriched caveolae. In follow-up experiments the authors were able to recover the acylated proteins from the DIM fraction. Zacharias found that premylated proteins were insensitive to cholesterol depletion, demonstrating that this approach is a valuable tool to monitor the differential recruitment of proteins to lipid enriched microdomains (35).

The following is a protocol for a conjugation assay, which enables one to identify physiological events that take place at the point of contact between two cells, such as the site of the immune synapse between T-cells and antigen-presenting cells (APCs). It is also helpful to track the migration of various proteins within the cells with the aid of GFP-tagged proteins or proteins stained with fluorescence antibodies. Here, we outline an experiment using Jurkat T-cells and a cultured Raji B-cell line loaded with staphylococcal enterotoxin E (SEE; Toxin Technology, Sarasota, FL) toxin to investigate the activity of proteins before, during, and after stimulation with TCR. It is important to keep in mind that the protocol can be easily adapted to other cells lines to best suit your own specific research needs.

### 3.3.1.1. SLIDE PREPARATION

1. Encircle a dime-sized area on a glass microscope slide with a PAP Pen to define the experimental area. Add approx 100  $\mu$ L of 0.01% poly-L-lysine to center of the

circle and incubate in a moist chamber for 30 min at RT. Wash the slides four times with deionized (d)H<sub>2</sub>O before use.

### 3.3.1.2. CELL PREPARATION

1. Count cells and determine the total number needed for the experiment (*see Note 11*).
2. Resuspend the APC cells ( $\sim 1 \times 10^5$  cells/50  $\mu$ L; *see Note 12*).
3. Resuspend the T-cells ( $\sim 1 \times 10^5$  cells/50  $\mu$ L; *see Note 13*).
4. Mix the T-cells and APCs  $\pm$  SEE in a 1:1 ratio and centrifuge briefly to ensure proper conjugate formation.
5. Incubate cell lines together for various time points at 37°C (*see Note 14*).
6. Resuspend the cell mixture well and spot 100  $\mu$ L of on lysine-coated slides. Incubate at RT for 10 min to allow complexes to adhere to the lysine. Carefully aspirate media.
7. Check for conjugation efficiency under bright light microscopy.
8. Fix the conjugates by adding approx 150  $\mu$ L of 4% paraformaldehyde–0.1% Tween-20 in PBS to the slide.
9. Incubate the slides at RT for an additional 10 min.
10. Wash the slides twice in a large volume of PBS at RT for 10 min.
11. Block the cells by adding approx 150  $\mu$ L of 1% BSA in PBS and incubate for 30 min at RT.
12. Dilute the primary antibody as recommended by manufacturer in a total volume of 100  $\mu$ L of PBS. Add antibody solution to slides and incubate for 1 h at RT or overnight at 4°C.
13. Wash cells twice in a large amount of PBS at RT for 10 min.
14. Dilute the fluorescent-conjugated secondary antibody 1:5000 in 100  $\mu$ L of PBS and incubate for 1 h at RT (*see Note 15*).
15. Wash cells twice in a large amount of PBS at RT for 10 min.
16. Aspirate the majority of the PBS and add a small amount of VectaMount to the cells. Add the cover slip and seal edges with nail polish to prevent the samples from drying out.
17. Wait at least 30 min before viewing lipid raft aggregates and protein localization under the microscope (*see Note 16*).
18. Use confocal laser scanning microscopy to visualize lipid rafts in T-cell membranes before and after cross linking with anti-TCR.

### 3.3.2. Molecular Probes and Fluorescent Microscopy

Commercially available kits allow for convenient labeling and crosslinking of lipid rafts, such as Molecular Probe's Vybrant Lipid Raft Labeling Kit, which uses a fluorescent cholera toxin subunit B conjugate (in one of three colors to accommodate multiple staining parameters) to selectively bind to the GM1 proteins embedded with lipid rafts. An antibody specific for cholera toxin subunit B then crosslinks the labeled lipid rafts into distinct patches that are then easily visualized by fluorescence microscopy. These kits allow an investigator to

identify physiologically significant membrane proteins that associate with lipid rafts. Detailed instructions are included with each kit and can vary from one manufacture to another; it is highly recommended that you familiarize yourself with the directions for the particular kit you will be using before starting your experiments. Another fluorescent microscopic technique uses changes in the emission spectrum of a bodipy sphingomyelin analog (C5 DMB-SM) to identify where lipid probes concentrate within the plasma membrane. When the probe becomes more concentrated, its emissions shifts from green to red; therefore, the existence of lipid rafts would have been expected to lead to small regions of red fluorescence superimposed on the green emission. Unfortunately, some studies have proven the ineffectiveness of this particular probe, where with low concentrations of C5 DMB-SM, the plasma membrane appears entirely green, indicating the absence of highly concentrated sphingolipid domains (36). On the other hand, it is entirely possible that the presence of the bodipy moiety on the end of a shortened acyl chain of this sphingolipids analogue creates a bulky probe that does not pack well into the ordered environment suggested for lipid rafts (37). In an attempt to circumvent such technical limitations, Kuerschner et al. recently has devised a method of labeling lipids for fluorescent microscopy that closely mimics the natural conformation (38). This new reporter lipid is a product of a modified Wittig olefination reaction and consists of five conjugated double bonds in the long chain of the fatty acid and has excitation and emission wavelengths of 350 nm and 470 nm, respectively. This molecule is also able to incorporate into the structure of membrane lipids such as sphingomyelin and have been shown to have no drastic effects on the phase behavior, making it ideal for in vivo studies (39).

### 3.3.3. Immun isolation of Proteins Associated With Lipid Rafts

An elegant approach to isolate raft-associated proteins was first put forward by Harder et al. (9). Here, DIM fractions from unstimulated and stimulated Jurkat cells were coupled with magnetic beads coated in specific antibodies. The proteins are isolated with a magnet and the raft-associated proteins within the resulting lysates were identified via immunoblotting. This protocol is presented as a technique for isolating lipid rafts in lymphocytes and it can be easily modified for other cell–cell interactions. The interested reader is also referred to Harder et al. for more detailed information (9).

#### 3.3.3.1. COATING THE DYNABEADS

1. Determine the number of cells needed. Aliquot half that number of M-450 goat-anti-mouse Dynabeads into a sterile microcentrifuge tube (*see Note 17*).
2. Collect beads on an MPC magnet for 1 min. Remove the supernatant and wash the beads twice by resuspending in 1 mL of serum-containing media.

3. Resuspend in 1 mL serum-containing media + 50 mM HEPES buffer for up to  $4 \times 10^8$  beads.
4. Add anti-CD3 and anti-transferrin receptor antibodies at 2  $\mu\text{g}$  per  $10^7$  beads and incubate for 1 h at RT on a rotating wheel.
5. Collect the beads on the magnet and remove supernatant.
6. Wash the beads twice by resuspending in 1 mL of serum-containing media.
7. Resuspend the beads at a concentration of  $4 \times 10^8$  beads per milliliter in serum-containing media + 50 mM HEPES buffer.

#### 3.3.3.2. ISOLATION OF TCR COMPLEXES

1. Collect the number of cells needed for the experiment by centrifugation ( $\sim 1\text{--}2 \times 10^7$  cells per data point).
2. Resuspend the cells in 1 mL of serum-containing media + 50 mM HEPES buffer per data point and pellet in a microcentrifuge tube. Loosen the cell pellet by gently flicking the tube.
3. Add the antibody-coated Dynalbeads at a ratio of 1:2 (e.g.,  $0.5 \times 10^7$  beads: $1 \times 10^7$  cells), mix well, and rotate on a lab wheel for 5 to 10 min.
4. Transfer samples to a 37°C water bath for the desired time points.
5. Add 1 mL of ice-cold HB buffer to stop reaction, and pellet cells in a refrigerated microcentrifuge tube for 2 min at 800g.

#### 3.3.3.3. DISRUPTION OF CELLS

1. Harder et al. use a nitrogen cavitation procedure to disrupt the cell sample; for more information, the interested reader is referred to Harder and Kuhn. (9).

#### 3.3.3.4. SAMPLE PREPARATION

1. Collect the beads with the magnet for 5 min, on ice.
2. Aspirate supernatant and wash samples twice by resuspending in 1 mL of ice-cold HB buffer.
3. Resuspend beads in 1 mL of HB + buffer and transfer to microcentrifuge tubes.
4. Retrieve beads magnetically and remove supernatant.
5. Add an appropriate amount of 2X Laemmli sample buffer to the beads and heat samples for 5 min at 95°C.
6. Separate proteins by electrophoresis and transfer onto a polyvinylidene difluoride membrane. Identify proteins of interest by immunoblotting.

#### 3.3.4. Electron Microscopy

In contrast with light microscopy, EM can only provide a snap shot of the cell at one particular time. The resolution of EM, however, is much greater than light microscopy, and particulate markers can provide the means to quantitatively study lipid raft organization at the molecular level (40). Recently, Prior et al. (41) developed a novel methodology to examine lipid raft markers on the inner leaflet of the plasma membrane using a combination of EM studies and

statistical analysis to screen proteins associated with, and colocalizing within, lipid rafts. This approach has potential to further examine plasma membrane organization and the spatial dynamics of regulated signaling and membrane trafficking events associated with the cell surface. However, it is important to keep in mind that there are limitations with this method; labeling methods for electron microscopy are often harsh and can damage delicate cellular ultrastructures and disrupt lipid organization.

#### 3.3.4.1. PREPARING ANTIBODY-CONJUGATED COLLOIDAL GOLD

Immunogold electron microscopy allows one to directly visualize membrane-anchored proteins at high resolution. It has been used to determine the size and distribution of lipid rafts, to monitor colocalization, and to observe Ras signaling domains (41). Briefly, plasma membranes are fixed, labeled with gold-conjugated antibodies, and viewed on a transmission electron microscope. Once the x,y coordinates are extracted from the images, Ripley's K function is used to determine whether the observed gold pattern is random or clustered into lipid rafts. A modification of the K-function also can be used to measure the co-localization of two proteins, each labeled with a different-sized gold particle (i.e., 2 nm and 5 nm).

1. Heat 40 mL of the reducing solution and 10 mL of Gold Solution to 60°C and combine, stirring vigorously. Boil for 5 min (the solution will turn a deep red once colloids form).
2. Cool the solution on ice and adjust the pH to 8.5 with 5 N NaOH using pH paper.
3. Identify the optimal antibody concentration by adding 0 to 5 µg of the affinity-purified antibody of interest (diluted in a total volume of 20 µL) to 250 µL of the gold colloid solution made in the previous two steps. Incubate at RT for 5 min.
4. Add 100 µL of 10% NaCl. Titrate for the smallest amount of antibody that stabilizes the gold (where no color change occurs).
5. Incubate this gold-antibody solution for 10 min at RT.
6. Add 10% BSA to a final concentration of 0.1%.
7. To concentrate gold and remove unbound antibody, centrifuge at 120,000g for 2–3 nm gold or 100,000g for 5 nm gold for 1 h at 4°C.
8. Collect the loose portion of the pellet and store at 4°C.

#### 3.3.4.2. PREPARING PLASMA MEMBRANE SHEETS

1. Incubate pioloform-coated grids with 1 mg/mL poly-L-lysine for 10 min. Wash twice in 1 mL of distilled water for 1 min. Air-dry and store in a dust-free container.
2. Culture cells on 10-mm glass cover slips as below (fluorescence recovery after photobleaching (FRAP) assay protocol); wash cells with PBS to remove cellular debris.
3. Place groups of poly-L-lysine-coated grids onto filter paper with the coated side facing up.

4. Remove cover slip from culture dish and remove excess solution by touching the edge of the cover slip to the filter paper. Place the cover slip face down on the grids.
5. Place filter paper on top of the cover slip and press down using a silicone bung for 1 to 2 s.
6. Remove the filter paper and turn the cover slip over so the grids are on top.
7. Carefully add 1X KOAc buffer around the grids until they float up onto the top of the drop.
8. Wash the grids by transferring them to new drops of 1X KOAc buffer on Parafilm.
9. Fix for 15 min with fixative. Wash twice by transferring the grids to fresh drops of PBS for 1 min each.
10. Quench free aldehyde groups with 25 mM glycine for 10 min.
11. Block for 10 min with blocking solution to prevent the antibodies from binding to nonspecific sites.
12. Centrifuge the gold-conjugated antibody at maximum speed in a microcentrifuge for 2 min and dilute in additional blocking solution. Float the grids on top of drops of the diluted antibody for 30 min.
13. Wash the labeled grids with blocking solution five times for 4 min each.
14. Wash the grids with dH<sub>2</sub>O five times for 2 min.
15. Incubate the grids in a mixture of nine parts 2% methyl cellulose and one part 3% uranyl acetate for 10 min on ice.
16. Pick up the grids in 5-mm wire loops, drain the excess solution on filter paper, and leave to dry in the loops for 10 min.

#### 3.3.4.3. IMAGING AND ANALYSIS

1. Using a transmission electron micrograph, scan plasma membrane sheets at low magnification ( $\times 4$ –6000), adjusting field brightness to maximize the contrast between the plasma membrane and background; take micrographs at higher magnification ( $\times 20,000$ ).
2. Scan negatives at 1 in. width, 1500 dpi resolution, and 8-bit depth using the imaging software program, and save files as TIFF formats.
3. Using Adobe® Photoshop® or a similar software program, select an area of the negative approx  $800 \times 800$  pixels and paste into a new file.
4. Remove the background from the image using the curves and brightness/contrast tools. Save as a new TIFF file.

#### 3.3.4.4. CALIBRATION AND ANALYSIS

1. Identify from an area containing 200 to 400 gold particles; open the file using Image J software or other similar program.
2. Set the image's threshold between 0 and 170.
3. Modify "set measurements" to record center of mass to eight decimal places.
4. Modify "analyze particles" to count the number of gold particles at different sizes from a minimum of 2 pixels to a maximum of 75 pixels for 5 nm of gold.
5. Record the number of particles after each step and repeat the threshold adjustments.

6. Modify “analyze particles” to locate the center of mass of all gold particles within the required pixel range to eight decimal places. Save as a text file.
7. Using Microsoft Excel<sup>®</sup>, convert the coordinates from pixels (the units generated in the previous step) into nanometers (the conversion factor is determined by the scale bar included with each micrograph or from images of calibrating spheres).
8. At this point, the x,y data points can be entered into any commercially available K-function analysis program to assess protein colocalization; for a more in-depth analysis protocol, the interested reader is referred to Prior (41).

### 3.4. Biophysical Analysis

Because lipid rafts are less than 50 nm in size, it is impossible to visualize individual rafts on standard fluorescent microscopes. However, various methods exist that can detect lipid raft aggregates and associated proteins, and these will be explained in detail below. Such experiments provide important information regarding the colocalization of lipid rafts with raft proteins (localized and excluded) and should be used to confirm biochemical data based on detergent insolubility. However, it is essential to keep in mind that not all microscopic evidence clearly supports the theory of lipid rafts. Recently, Glebov and Nichols used FRET analysis to measure the proximity of two different GPI-anchored fluorescent proteins in T-cells. The signal they detected was density-dependent, cholesterol-independent, and similar to data from non-raft proteins, suggesting that resting T-cells do not contain lipid rafts (42). These findings strengthen work previously performed by Kenworthy and Edidin, who found diffuse distribution, and hence little evidence of clustering, of overexpressed 5' nucleotidase, a GPI-anchored protein, in polarized MDCK cells; this suggests that this protein is randomly dispersed across the apical plasma membrane and is not found in organized clusters as would be expected with lipid rafts (43). It is possible, however, that these cells do in fact contain lipid rafts but these rafts are smaller than originally thought (<10 nm in diameter), and contain very few GPI-anchored proteins, making it doubtful that any one raft would contain both the FRET donor and acceptor proteins (44). With these points in mind, it is essential that all biophysical experiments performed on lipid rafts contain proper positive and negative controls to best analyze ensuing data.

#### 3.4.1. Fluorescence Resonance Energy Transfer

FRET detects the energy transfer between two molecules only a few nanometers apart and is a powerful technique that can be used to identify intermolecular interactions within living cells. Energy transfer from an excited donor fluorophore molecule to an acceptor fluorophore results in quenching of the donor channel signal and an increase in the acceptor signal; because FRET only occurs between molecules in very close proximity; it is an excellent technique to study clustering

and co-association of fluorescently-tagged proteins (45–47) and, although promising, it must be stressed that the absence of interactions is not always indicative of the lack of interaction. Steric constraints between the donor and acceptor could result in a false-positive result (44). Summarized herein is a simplified outline to FRET assays; for a more in-depth exploration of the theory behind this technique, please refer to the excellent reviews by Gordon (45) and Herman (47).

#### 3.4.1.1. SAMPLE PREPARATION

1. Plate 293 human embryonic kidney cells onto glass cover slips in suitable growing media. Incubate cells at 37°C with 5% CO<sub>2</sub> until the cells are approx 50% confluent.
2. Transfect 1 to 2 µg of plasmid DNA into the cells using a lipid-based delivery system, such as TransFection (Bio-Rad). Overexpressed fluorescent signals can be visualized between 6 and 24 h after transfection.
3. If staining for endogenous or nonfluorescent over-expressed proteins, wash cells in PBS and choose appropriate donor–acceptor antibody concentrations for labeling (i.e., fluorescein isothiocyanate, Cy3, or Cy5). Typical donor–acceptor molar ratios range from 1:1 to 1:5. Normal serum from the secondary antibody host diluted in PBS should be used as a blocking agent.
4. Prepare the primary antibody in blocking reagent and incubate according to manufacture's instructions (*see Note 18*).

#### 3.4.1.2. CELL FIXATION AND MOUNTING

1. Wash cells in a large volume of PBS to remove all traces of serum which could interfere with antibody binding.
2. Fix cells in 4% PFA for 30 min at RT.
3. Wash cells twice with additional PBS after fixation.
4. To decrease autofluorescence, incubate the cells with 1 mg/mL NaBH<sub>4</sub> for 5 min.
5. Wash cells in PBS. Aspirate most of the excess fluid from the cover slip, and add a drop of Vectashield directly onto the cells.
6. Cover and seal the edges with nail polish to prevent the samples from drying out. Store at 4°C if not viewed on a confocal laser scanning microscope immediately.

#### 3.4.1.3. IMAGING PARAMETERS AND DATA ACQUISITION

FRET is detected by exciting labeled specimens with light at a corresponding wavelength to the absorption spectrum of the donor and detecting light emitted at the wavelength corresponding to the absorption spectrum of the acceptor (45). Energy transfer between the two proteins of interest is visible by both the quenching the donor fluorescence signal in the presence of the acceptor and in the sensitized emission spectrum of the acceptor.

1. Three individual samples of each specimen must be prepared to collect FRET measurements. Cells containing only the signal from the donor (d); cells containing

only the signal from the acceptor (a); and cells containing signals from both donor and acceptor (f). Each sample is imaged with a donor filter set (D); an acceptor filter set (A), and a FRET filter set (F). This approach will identify the FRET signal that results from energy transfer that has been transferred from the donor to acceptor molecules while reducing signal bleeding and crosstalk (47).

2. Exposure time, light intensity, and camera settings for each of the filter sets, or imaging pathways, are determined by scanning a representative area of d with D, a with A, and f with F.
3. Once the imaging parameters are determined for each filter set, they must not be altered during data acquisition. Computer macros can be used to automate acquisition once the parameters are set.
4. Collect at least 10 fields for every specimen with each of the three filter sets.

#### 3.4.1.4. QUANTIFICATION AND ANALYSIS

1. Identify regions on the cover slip that are not covered in cells (background) and calculate the average fluorescence signal from this area.
2. Identify signals emitted from the cells (foreground) and calculate average fluorescence signal from these areas.
3. Normalize results by subtracting background signals from foreground signals for each FRET measurement.

#### 3.4.2. FRAP Analysis

FRAP is an important technique used to measure the diffusion mobility of membrane components labeled with fluorophores, fluorescent antibodies, or GFP fusion proteins. In FRAP assays, a short pulse of intense laser light irreversibly destroys (photobleaches) fluorescence in a defined area of the cell. Recovery of fluorescence into the photobleached area occurs as a result of diffusional exchange between bleached and unbleached molecules. This technique is useful in the study of the dynamics of signaling molecules in Jurkat cells after lipid raft activation as described by Tanimura et al. (46).

##### 3.4.2.1. BEAD PREPARATION

1. Wash  $1 \times 10^7$  Polybead polystyrene beads (6.0  $\mu\text{m}$  in diameter) with 1 mL of PBS and resuspend in a final volume of 1 mL of PBS. Add 10  $\mu\text{g}$  of the purified antibody to the beads. Poly-L-lysine-coated beads can be used as a negative control in these experiments.
2. Incubate bead:antibody mixture at RT for 90 min on a rotating wheel.
3. Centrifuge in a microcentrifuge at maximum speed for 30 s.
4. Remove supernatant and discard. Wash the beads three additional times with PBS.
5. Add 1 mL of blocking solution to the beads and incubate at RT for 30 min on a rotating wheel.
6. Centrifuge in a microcentrifuge at maximum speed for 30 s.
7. Remove supernatant and discard. Wash the beads three additional times with PBS.
8. Resuspend the coated beads in 1 mL of PBS and store at 4°C for up to 3 wk.

#### 3.4.2.2. CONJUGATE FORMATION

1. The cells used in these experiments will need to express a GFP fusion protein. Both stable cell lines and transiently transfected cells can be used.
2. Determine the number of cells needed for the experiment. Harvest by centrifugation and wash once in 10 mL of serum-free media.
3. Resuspend the cells in media at a concentration of  $1 \times 10^7$  cells per milliliter and transfer 10  $\mu$ L of the cell suspension ( $1 \times 10^5$  cells) to a microcentrifuge tube.
4. Add 10  $\mu$ L of antibody-coated beads in PBS ( $1 \times 10^5$  beads for a 1:1 cell:bead ratio) and mix.
5. Incubate the cell:bead mixture at 37°C for 20 min.

#### 3.4.2.3. SAMPLE PREPARATION FOR FRAP

1. During the cell:bead conjugate incubation (above), encircle a dime-sized area on a glass microscope slide with a PAP Pen.
2. Add 3  $\mu$ L of the cell:bead mixture into the center of the circle and place a cover slip on top. Seal edges with nail polish to prevent the media from evaporating.

#### 3.4.2.4. FRAP ASSAY

FRAP assays are conducted at RT using a confocal microscope with a  $\times 63$  objective lens and the 488-nm line of a 25-mW Argon laser. Before data collection can begin, it is necessary to determine how often the bleaching process should be performed with full laser power to reduce the fluorescence in the region of interest to background levels. It is also important to perform preliminary experiments to determine how long recovery of fluorescence takes to reach a plateau after bleaching. A whole cell scan at low laser power (75% power, 10% transmission) before the bleach and photobleach the cell with full laser power (100% power, 100% transmission) 40 times (2 s) is a recommended starting point. Recovery is followed after 2 s with low-laser power at 2-s intervals for 30 s, at 10-s intervals for 120 s, and then 60-s intervals for 6 min.

1. Place the sample on the stage and the scan slide to detect a cell of interest at imaging laser intensity.
2. Define the region of interest for the photobleach with an area of approx  $1 \mu\text{m} \times 1 \mu\text{m}$  for bleaching. Program the computer's software to automatically change settings quickly for the photobleaching routine. It should record the image of the cell before photobleaching, execute the bleach, and record an image after the photobleaching and throughout the recovery.
3. Begin the FRAP cycle by executing the program and monitor recovery on the screen.
4. Check the cell during and after completion of the FRAP cycle to ensure that the region of interest has not changed.
5. Save the image as an image database file and calculate fluorescent intensities in the region of interest for the bleached region ( $I_b$ ) and a nonbleached region ( $I_r$ ) for each time point.

6. Calculate the relative intensity (rI) for each time point:  $rI_{(t)} = I_b(t) - I_r(t)$
7. Calculate the percentage recovery as follows: % recovery =  $[rI_{(t)} - rI_{(post)}/rI_{(pre)} - rI_{(post)}]$ , where  $rI_{(pre)}$  = initial (prebleach) relative intensity;  $rI_{(post)}$  = post-bleach relative intensity;  $rI_{(t)}$  = relative intensity at each time point.
8. Draw a FRAP curve using a calculation software, such as Microsoft Excel (% recovery vs time in min (see **Note 19**).

### 3.4.3. Proteomics

Proteomics, the large-scale study of the protein complement of a given cell, has proven to be an invaluable technique in the identification of novel proteins associated with lipid rafts. von Haller et al. were the one of the first to use MS to identify proteins that co-purified with DIM fractions of T-cells and found that the proteins identified in the screen essentially fell into one of two categories: cytoskeletal proteins and proteins involved in signal transduction (48). Since then, proteome analysis has offered evidence that several additional classes of proteins associate with DIM fractions isolated from stimulated T-cells (cell adhesion, heat shock, and GTP-binding proteins [49,50]). Foster et al. (51) have taken this approach one step further by classifying proteins as “raft” proteins or “raft-associated” proteins based on biochemical properties such as their susceptibility to cholesterol depletion and detergent solubilization. This screen uses stable isotope encoding to add a functional dimension that significantly improves specificity of the data, providing strong evidence that their groupings are real and are not statistical artifacts (51). Stable isotope labeling by amino acids in cell culture (SILAC) entails the addition of labeled, essential amino acids to amino acid-deficient cell culture media; these labeled amino acids are incorporated into all newly synthesized proteins, or “encoded into the proteome” (52). The advantages of this technique include the fact that no chemical labeling or affinity purification is required, and the method is convenient and inexpensive to perform on a large scale. This method also allows investigators to identify large numbers of proteins from a single experiment in an unbiased manner.

#### 3.4.3.1. SILAC

1. Culture cells in leucine-deficient media. Supplement one population with normal isotopic abundance L-Leucine (Leu, 105 mg/L) and the other with 99% isotopic abundance L-Leucine-5,5,5-D3 (LeuD3; 107.5 mg/L).
2. Passage cells three times for a minimum of seven population doublings.

#### 3.4.3.2. CHOLESTEROL DEPLETION AND ISOLATION OF DETERGENT-RESISTANT FRACTIONS

1. Serum-starve 10-cm plates of each cell population (Leu, LeuD3) for 18 h before cholesterol depletion.

2. Disrupt membrane cholesterol in one population (Leu) of cells with 10 mM M $\beta$ CD for 30 min at 37°C (see **Subheading 3.1.2.1.**). Treat the second population of cells (LeuD3) with a carrier control only (see **Note 20**).
3. Lyse Leu, LeuD3 cells for 1 h at 4°C in MBS lysis buffer.
4. Clear lysates by centrifuging at 10,000g for 10 min at 4°C.
5. Determine protein concentration of each sample using a commercially available kit.
6. Equal amounts of protein from the Leu (treated) and LeuD3 (untreated) cell populations were combined and mixed with an equal volume of 90% sucrose in MBS for a final concentration of 45%.
7. Transfer the solution to the bottom of an ultracentrifuge tube. Carefully add additional layers of 35 and 5% sucrose on top.
8. Balance the tubes to within 0.01 g and place them in the rotor. Centrifuge samples for 16 h at 200,000g at 4°C.
9. Extract the low-density band (at ~18% sucrose) with a needle and syringe. Dilute the band with four volumes of additional MBS. Centrifuge samples for an additional 2 h at 200,000g at 4°C to pellet the detergent-resistant material.

#### 3.4.3.3. HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND TANDEM MS

1. Solubilize the pellet from the second centrifugation step in a small volume of 6 M urea/2 M thiourea (in 10 mM HEPES, pH 8.0). Precipitate proteins by diluting in five volumes of absolute ethanol. Adjust to 50 mM sodium acetate with a 2.5 M stock solution, pH 5.0.
2. Allow the solution to stand at RT for 2 h. Centrifuge at 12,000g for 10 min at RT.
3. Resolubilize the pellet in additional urea/thiourea. Reduce the solution by adding 1  $\mu$ g of DTT per 50  $\mu$ g of estimated total protein. Incubate for 30 min at RT.
4. Alkylate the solution by adding 5  $\mu$ g of iodoacetamide per 50  $\mu$ g of estimated total protein. Incubate for 30 min at RT.
5. Digest the solution by adding 1  $\mu$ g of LysC per 50  $\mu$ g of estimated total protein. Incubate for 3 h at RT.
6. Dilute the sample in four volumes of 50 mM NH<sub>4</sub>HCO<sub>3</sub>. Digest with 1  $\mu$ g of trypsin per 50  $\mu$ L of estimated total protein. Incubate for 12 h at RT.
7. Prepare the stop and go extraction (STAGE) tips by punching out small disks of C18 Empore filters using a 22-gage flat syringe and plugging the disks into P200 pipet tips.
8. Equilibrate column by forcing approx 100  $\mu$ L of methanol through the Empore disk with a syringe fitted to the end of the pipet tip.
9. Remove any remaining organic solvent in the column by forcing a small volume of 0.5% acetic acid through twice.
10. De-salt peptide mixture with the STAGE tips and bomb-load onto reverse-phase analytical columns for liquid chromatography (LC). Elute peptides from the columns with three-step linear 2.5-h gradients running from 5.6 to 64% acetonitrile and sprayed directly into the orifice of a MS.
11. Identify peptides by LC/MS/MS by information-dependent acquisition of fragmented spectra for multiply charged peptides.

12. Search acquired proteins against a known database, such as the Human International Protein Index database (<http://www.ensembl.org/IPI/>), using MASCOT (Matrix Science, London, UK, <http://www.matrixscience.com>).

#### 3.4.4. Flow Cytometry

Gombos et al. (53) recently outlined a novel flow cytometry based assay that characterizes surface proteins and raft association by utilizing two defining characteristics of lipid rafts, cholesterol dependency, and detergent resistance. Here, the authors stained specific surface protein and compared treated and untreated groups (M $\beta$ CD, nonionic detergent, or both); raft-associated proteins will be significantly affected by cholesterol depletion and/or detergent solubilization show dramatic differences in fluorescence in treated groups versus untreated groups. Raft-independent proteins will not be affected by cholesterol depletion and/or detergent solubilization and will therefore show very little difference between treated and untreated groups. This method is easy to perform and allows for rapid screening of multiple surface proteins.

1. Count and pellet cells by centrifugation (*see Note 21*).
2. Divide the total number of cells into two populations. Treat one half of the cells with M $\beta$ CD to disrupt cholesterol in the plasma membrane (*see Subheading 3.1.2.1*). The remaining (untreated) cells will serve as a control population for cholesterol dependence.
3. Divide both M $\beta$ CD-treated and untreated cell populations into half again. Treat one half of the cells with either 0.5% Triton X-100 or NP-40 for 5 min on ice. Wash with PBS. The remaining cells will serve as a control population for detergent sensitivity.
4. Label all cells with fluorescent antibodies specific against surface markers (*see Notes 22 and 23*).
5. Collect the following data for each sample on a flow cytometer:  
Forward scatter (FSC) and Side scatter (SSC).  
Fluorescence intensity (FL1, FL2, or FL3 histograms, depending on the color of the antibody).
6. Analyze FACS data by using the following histograms:  
Autofluorescence of untreated, unlabeled cells (background) (FL<sub>Bg</sub>).  
Fluorescence of labeled, untreated cells, proportional to total protein expression (FL<sub>Max</sub>).  
Autofluorescence of detergent-treated, unlabeled cells (FL<sub>Bg-Det</sub>).  
Fluorescence of cells treated after detergent for 5 min (FL<sub>Det</sub>).  
Calculate the extent of detergent resistance (FCDR) as follows:  $FCDR = (FL_{Det} - FL_{Bg-Det}) / (FL_{Max} - FL_{Bg})$ .

The closer the FCDR value is to 1, the less soluble a given protein is in the detergent. Likewise, a low FCDR value is indicative of a highly-soluble protein.

A significant decrease in the FCDR value after cholesterol depletion (lipid raft disruption) indicates that the observed resistance is a result mainly due to localization of the protein in lipid rafts.

When a high FCDR value does not change significantly after M $\beta$ CD treatment, it can be assumed that the protein does not bind directly to lipid rafts (e.g. cytoskeletal anchorage only).

#### 4. Notes

1. Both cultured cell lines and primary cells can be used in stimulation experiments. As with all experiments, experimental design is critical, and the inclusion of a negative control (unstimulated cells) is essential. Start with a minimum of  $1 \times 10^6$  cells per sample.
2. Try 0, 1, 2, 5, 10, and 15 min; if the number of cells is limited, select the critical time points.
3. The use of positive and negative controls is recommended strongly. For example, Src family kinases that are palmitoylated do not associate with DIMs after the depletion of cholesterol.
4. Cyclodextrins also can remove other lipid components from membranes; therefore, it is useful to check that any effects can be reversed by using cholesterol-loaded cyclodextrin carrier to deliver cholesterol back to cells.
5. Not all lipid rafts appear to be equally sensitive to the depletion of cholesterol. Recent studies have suggested that the depletion of cholesterol reduces lateral mobility of membrane proteins because it disrupts the highly regulated interactions of PIP<sub>2</sub> with molecules controlling actin cytoskeleton (54).
6. It is essential that while extracting proteins associated with DIM fractions that all reagents and buffers be kept at 4°C and the experiments themselves be carried out on ice or in a cold room. It has been shown that the DIM fractions themselves can be lysed by detergents at higher temperatures.
7. Milder detergents such as the Brij family of detergents produce larger raft fractions, whereas 1% Triton X-100 produces less buoyant fractions containing less than 50% of the typical raft molecules and only a few nonpalmitoylated transmembrane proteins.
8. If two proteins that reside in the same membrane float to different densities during DIM preparation it can be concluded that they had different lipid environments before detergent extraction.
9. Regardless of the conditions for detergent treatment, a completely soluble membrane protein localized to the same membrane as the protein of interest should be included in the analysis.
10. For a comprehensive list of proteins associated with DIM fractions from T-cells, please refer to Razzaq et al. (55); such proteins can be used as markers for cell biological studies.
11. Both cultured cell lines and primary cells can be used in conjugation assays. In the example given here, Jurkat T-cells are conjugated to SEE-loaded B-cells to investigate

the effects of TCR stimulation. This protocol, however, can easily be adapted to other cell systems. An important consideration for these particular experiments is the use of a negative control (Raji B-cells without SEE).

12. To prepare the APC cell population, use  $1 \times 10^6$  cells per sample. Significant cell loss will occur through subsequent washing steps. Aliquot the cells needed for the negative control and set aside. Resuspend the remaining cells in 500  $\mu$ L of media + 5  $\mu$ g/mL (SEE) and incubate at 37°C for 30 min. Resuspend the two cell populations in 50  $\mu$ L per sample and keep cells on ice.
13. Count the cells needed for the T-cell population ( $\sim 5 \times 10^5$  cells/50  $\mu$ L) and pellet. Wash once in additional media and resuspend for a final cell count of  $1 \times 10^5$  cells/50  $\mu$ L. Keep on ice.
14. The time points used in conjugation assays depend on whether the protein of interest migrates early after activation or not. When in doubt, begin with various time points, such as 0, 1, 2, 5, 10, and 15 min.
15. Fluorescence antibodies are sensitive to light. The signal fades as a result (photo-bleaching). Be sure to keep slides covered from this point onward
16. For a detailed discussion on conjugate quantification, *see* Burack et al. (24).
17. Use  $1 \times 10^6$  cells per sample. Before working with Dynalbeads, read the literature provided with the reagents and become familiarized with the technical aspects of the beads.
18. If cytoplasmic labeling is required, permeabilize the plasma membrane to allow antibodies into the cell; 100% methanol at  $-20^\circ\text{C}$  or 0.1% Triton X-100 at  $4^\circ\text{C}$  are both effective techniques.
19. For an excellent review that explains how to interpret FRAP data, *see* Sprague (56).
20. Total cholesterol content can be measured from a 10- $\mu$ g aliquot using Amplex red kit (Molecular Probes), according to the manufacturer's instructions.
21. Approximately  $1 \times 10^6$  cells per sample will be needed.
22. It is important to include proper positive and negative controls in the FACS analysis. In addition to any other proteins of interest, stain cells for GM1 or CD48 as positive controls (both markers are raft residents) and CD2 or CD71 as a negative control (both markers are excluded from lipid rafts).
23. For an excellent protocol on FACS sample preparation and analysis, *see* Vacchio and Shores (57).

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