2

Separation of Cell–Cell Adhesion Complexes by Differential Centrifugation

Roger Vogelmann and W. James Nelson

Summary

The number of proteins found associated with cell–cell adhesion substructures is growing rapidly. Based on potential protein–protein interactions, complex protein networks at cell–cell contacts can be modeled. Traditional studies to examine protein–protein interactions include co-immunoprecipitation or pull-down experiments of tagged proteins. These studies provide valuable information that proteins can associate directly or indirectly through other proteins in a complex. However, they do not clarify if a given protein is part of other protein complexes or inform about the specificity of those interactions in the context of adhesion substructures. Thus, it is not clear if models compiled from these types of studies reflect the combination of protein interactions in the adhesion complex in vivo for a specific cell type. Therefore, we present here a method to separate cell–cell contact membrane substructures with their associated protein complexes based on their buoyant behavior in iodixanol density gradients. Analysis of 16 proteins of the apical junctional complex (AJC) in epithelial Madin–Darby canine kidney cells revealed a more simple organization of the AJC adhesion complex than that predicted from the combination of all possible protein–protein interactions defined from co-immunoprecipitation and pull-down experiments.

Key Words: Cell polarity; tight junction; adherens junction; protein complex; Ig superfamily receptors; apical junctional complex; density gradient.

1. Introduction

Cell–cell adhesion complexes are important regulators of cellular functions. The apical junctional complex (AJC) in epithelia regulates cell–cell adhesion between neighboring cells, structural and functional integrity of the epithelial barrier, contractile forces during morphogenesis and wound healing, cell proliferation, cell differentiation, and cell polarity ***(1,2)***. In order to understand

From: *Methods in Molecular Biology, vol. 370: Adhesion Protein Protocols, Second Edition*
Edited by: A. S. Coutts © Humana Press Inc., Totowa, NJ

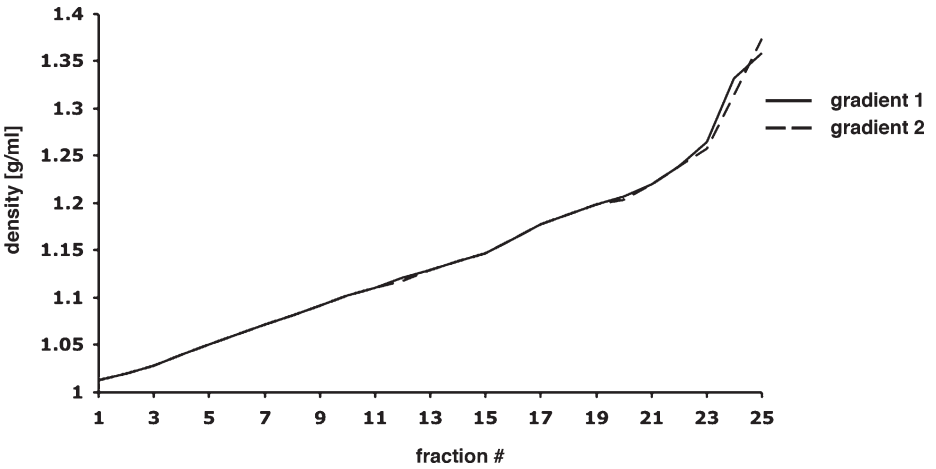

Fig. 1. Density of 10–20–30% iodixanol density gradients. Density δ was determined for each fraction by measuring the refractive index (η) of each fraction [δ = (η * 3.443) - 3.599]. Densities for two different density gradients are shown (gradient 1 and gradient 2).

how adhesion complexes carry out their functions, it is important to know the identity of proteins that localize to the AJC and the nature of the protein–protein interactions as a basis for understanding these different functions. In vitro studies have revealed a large number of proteins and their potential binding partners at the AJC, which has led to models of complex protein networks ***(3–5)***. However, these studies do not clarify if all of these protein–protein interactions occur at the same time in a given cell type, or at a particular stage of assembly of the AJC, or if proteins are separated into different AJC substructures. Whether or not two proteins are in the same complex is critical for the understanding of how adhesion complexes mechanistically regulate multiple functions of the AJC.

Membrane-associated protein complexes can be separated using iodixanol density gradients ***(6,7)***. Iodixanol is an aqueous solution that is iso-osmotic up to a density of 1.32 g/mL and forms self-generating gradients in 1–3 h. It has a very low toxicity toward biological material, and enzyme assays can be carried out in its presence.

Yeaman et al. developed a 10–20–30% iodixanol density gradient to study protein trafficking of the Sec 6/8 protein complex in epithelial cells ***(8)***. This type of gradient exhibits a linear increase in density over almost the entire length of the gradient, and membrane particles are separated in the gradient based on their buoyant characteristics (**Fig. 1**). Cells are broken mechanically in a buffer lacking detergent to preserve membranes in the lysate. After loading the cell lysate at the bottom of the gradient, membranes float up as the iodixanol gradi-

ent self-generates; the separation of different membranes depends on their lipid content and the concentration of associated proteins. After centrifugation, fractions can be sampled from the top of the gradient and analyzed using standard sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immuoblot staining for proteins of interest. Proteins separating in different fractions of the gradient are in neither the same protein complex nor the same AJC substructure. However, proteins that separate in the same fractions may interact with each other in the same complex, which can be further tested by immunoprecipitiation of proteins from the separated membranes.

We utilized the 10–20–30% iodixanol density gradient method to analyze 16 proteins of the AJC in epithelial Madin–Darby canine kidney (MDCK) cells ***(9)***. Our study revealed that the organization of the AJC is simpler than the predicted model based on protein–protein interaction studies. Many of the analyzed proteins are separated into distinct subcomplexes or may interact only transiently during AJC assembly, whereas others may be irrelevant to the formation and maintenance of the AJC in MDCK cells. Iodixanol density gradients are, therefore, a useful biochemical strategy to isolate cell–cell adhesion complexes as a starting point to further study the composition of protein complexes in these structures.

2. Materials

2.1. Cell Culture and Calcium-Switch Experiment

1. Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS; Atlas Biologicals, Fort Collins, CO) and 5 mL of 100X penicillin, streptomycin, and kanamycin (PSK) antibiotics mix. To prepare PSK antibiotics mix, dissolve 6.1 g kanamycin sulfate (100 mg/mL; Invitrogen-Gibco), 1.5 g penicillin "G" sodium (50 u/mL, 1650 u/mg; Invitrogen-Gibco), and 2.5 g streptomycin sulfate (50 mg/mL; Sigma-Aldrich, St. Louis, MO) in 500 mL of phosphate-buffered saline (PBS). Filter through a 0.22-µm filter unit to sterilize, dispense 50 mL per 100-mL bottle, and store at −20°C for up to 6 mo.
2. Washing buffer: dissolve 48 g NaCl, 2.4 g KCl, 6 g glucose (dextrose, monohydrate), 2.1 g $NaHCO_3$ in 5.95 L dH_2O and bring to a final volume of 6 L. Add 1.2 g ethylene diamine tetraacetic acid (will take a while to dissolve), and filter-sterilize using a 0.22-µm filter unit. Aliquot 500 mL each into 500-mL glass tissue culture bottles, and store at 4°C.
3. Washing buffer + trypsin: for trypsin stock solution, dissolve 6.25 g trypsin (Difco, BD, Franklin Lakes, NJ) in 250 mL of washing buffer. Stir 20 min at room temperature (solution will remain cloudy). Centrifuge for 30 min at 10,000 rpm (12,064*g*) 4°C in JA-20 rotor (Beckman Instruments Inc., Palo Alto, CA). Filter-sterilize supernatant using 0.22-µm filter unit, and discard pellet. Aliquot 12.5 mL each of sterile trypsin solution into 20 sterile 50-mL blue cap tubes, store at −20°C. For trypsin working solution (0.0625%), add one tube of sterile trypsin stock solution to one bottle (500 mL) of sterile washing buffer, and mix well.

4. Low-calcium medium (for 6 L): 2.4 g KCl, 1.1989 g $MgSO_4 \cdot 7H_2O$, 35.777 g NaCl, 6.0 g D-glucose (dextrose, monohydratous), 0.06 g phenol red, 0.756 g L-arginine HCl, 0.18774 g L-cystine 2HCl, 1.7752 g L-glutamine, 0.252 g L-histidine $HCl \cdot 2H_2O$, 0.312 g L-isoleucine, 0.312 g L-leucine, 0.435 g L-lysine HCl, 0.192 g L-phenylalanine, 0.288 g L-threonine, 0.06 g L-tryptophan, 0.31188 g L-tyrosine, 0.275 g L-valine (*see* **Note 1**), 60 mL 100X minimum essential medium (MEM) vitamin solution (Invitrogen-Gibco), 6 g $NaHCO_3$, 15.618 g hydroxyethylpiperazine ethanesulfonic acid (HEPES) NaCl, 0.168 mL 100X $CaCl_2$ (dissolve 2.65 g $CaCl_2 \cdot 2H_2O$ in 90 mL of dH_2O and bring up to final volume of 100 mL; filter-sterilize with 0.22-µm filter unit, aliquot 20 mL each into 50-mL blue cap tubes, store at –20°C), 0.84 g $NaH_2PO_4 \cdot H_2O$. Adjust pH to 7.0 using concentrated HCl, adjust volume with dH_2O to 6 L, filter-sterilize, and store media (500 mL/bottle) at 4°C. Add 10% dialyzed FBS, 5 mL of 100X methionine, and 5 mL of 100X PSK for the working solution.
5. Dialyzed FBS: entire procedure is done at 4°C. Prepare 4 L of Tris-saline (10 m*M* Tris-HCL pH 7.5, 120 m*M* NaCl; dialysis solution) and cool to 4°C. Divide 500 mL of FBS into five or six sections of 3/4-in.-wide dialysis tubing that has been rinsed with dH_2O. Put FBS-containing dialysis tubes into one 4-L beaker of Tris-saline and stir gently. Change Tris-saline solution after 24 h and repeat three times. Filter-sterilize FBS using a 500-mL 0.22-µm filter unit, aliquot into 50-mL sterile blue cap tubes, and store at –20°C.
6. 100X Methionine: dissolve 0.15 g of methionine in 90 mL of dH_2O and bring to a final volume of 100 mL. Filter-sterilize in 0.22-µm filter unit, and store at –20°C.
7. Collagen solution: commercial collagen type I solutions can be used. For instructions on how to prepare your own collagen solution, please visit our web page at http://nelsonlab.stanford.edu/lab/labbible.html#_Toc50366772.
8. 75-mm-Well, 0.4-µm polycarbonate membranes (Transwell® filters; Costar Corp., Cambridge, MA).

2.2. Cell Surface Biotinylation

1. Sulfo-NHS-Biotin (Pierce Biotechnology Inc., Rockford, IL) at a final concentration of 0.5 mg/mL in ice-cold Ringers solution. Prepare fresh before each experiment and use immediately.
2. Ringers solution: 10 m*M* HEPES NaCl, pH 7.4, 154 m*M* NaCl, 7.2 m*M* KCl, 1.8 m*M* $CaCl_2$.

2.3. Cell Fractionation in Iodixanol Gradients

1. Dithiobis(succinimidylpropionate) (DSP; Pierce Biotechnology Inc.) stock: 20 mg/mL in dimethylsulfoxide (DMSO) (always make fresh). Dilute DSP stock 1:100 in Ringers solution at room temperature immediately prior to use.
2. Quenching buffer: 120 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, 50 m*M* NaH_4Cl.
3. Homogenization buffer 10X stock: 200 m*M* HEPES·KOH, pH 7.2 (*see* **Note 2**), 900 m*M* K-acetate, 20 m*M* Mg-acetate. Filter-sterilize in 0.22-µm filter unit, and store at 4°C.

4. Homogenization buffer I (make fresh for each experiment): prepare 1 mL for each 75-mm filter. For one gradient (8- × 75-mm filters): 7.97 mL dH_2O, 900 µL homogenization buffer 10X, 90 µL sucrose (2.5 *M* stock), 30 µL Pefabloc (0.1 *M* stock; Hoffmann-La Roche Inc., Nutley, NJ), 8 µL L/A/P (5 mg/mL leupeptin, 5 mg/mL antipain, 5 mg/mL pepstatin in DMSO; Hoffmann-La Roche) (*see* **Note 3**).
5. 15-mL Conical polypropylene tubes (*see* **Note 4**).
6. Homogenization buffer II (make fresh for each experiment): prepare 3 mL for one gradient. 2.63 mL dH_2O, 300 µL homogenization buffer 10X, 30 µL sucrose (2.5 *M* stock), 30 µL Pefabloc (0.1 *M* stock), 8 µL aprotinin (Sigma-Aldrich), 8 µL L/A/P (*see* **Notes 3** and **5**).
7. Homogenization buffer III (make fresh for each experiment): prepare 13 mL for two gradients. 11.57 mL dH_2O, 1.3 mL homogenization buffer 10X, 130 µL sucrose (2.5 M stock).
8. Ball-bearing homogenizer, fitted with 0.3747-in. stainless steel ball (**Fig. 2**) (*see* **Note 6**).
9. 5-mL syringes with Luer-Lok™ tip (BD, Franklin Lakes, NJ), 23G1 needles.
10. Iodixanol: Opti-prep® (Nycomed, Oslo, Norway).
11. Quick-Seal® ultracentrifuge tube, 5/8 × 3 in. (16 × 76 mm) (Beckman Instruments Inc.).

2.4. SDS-PAGE and Immunoblotting

1. 4X SDS sample buffer: for 20 mL solution: 1.6 g SDS, 3.2 mL 1 *M* Tris-HCl pH 6.8, 6 mL glycerol (100%), bromophenol blue, add dH_2O up to 20 mL. Store at room temperature. Before use, add 1 mL of 1 *M* dithiothreitol (store at −20°C) to 4 mL 4X SDS-sample buffer.
2. 10% SDS stock solution, 1 *M* Tris-HCl, pH 8.7 (for separating gel), 1 *M* Tris-HCl, pH 6.8 (for stacking gel). Store at room temperature.
3. 30% Acrylamide/0.8% bisacrylamide solution (National Diagnostics, Atlanta, GA) and *N*,*N*,*N*,*N*'-tetramethyl-ethylenediamine (TEMED; Bio-Rad, Hercules, CA). Acrylamide is a neurotoxin when unpolymerized; see manufacturer's safety instructions before use. 10% Ammonium persulfate (APS; Bio-Rad) in dH_2O. Store all solutions at 4°C.
4. SDS running buffer (10X): 250 m*M* Tris, 1.92 *M* glycine, 1% (w/v) SDS. Store at room temperature. Dilute 1:10 in dH_2O before use.
5. Prestained molecular-weight markers.
6. Western blot transfer buffer: 25 m*M* Tris (do not adjust pH), 192 m*M* glycine, 20% (v/v) methanol.
7. Protran® 0.45-µm nitrocellulose membranes (Schleicher & Schuell, Keene, NH), Immobilon-FL polyvinylidene fluoride (PVDF) Odyssey membrane (Millipore, Billerica, MA) (*see* **Note 7**).
8. Washing buffer: Tris-buffered saline with Tween: 120 m*M* NaCl, 10 m*M* Tris-HCl, pH 7.4, + 0.1% Tween-20.
9. Blocking buffer: LI-COR blocking buffer (LI-COR, Lincoln, NE) in PBS (1:1).

10. Primary antibodies and fluorescently labeled secondary antibodies (1:30,000) are diluted in washing buffer.
11. Secondary antibodies: anti-mouse Alexa Fluor® 680, Alexa® Fluor 680 coupled streptavidin (Molecular Probes, Eugene, OR), and anti-rabbit IRDye® 800 (Rockland Immunochemicals, Gilbertsville, PA).
12. Odyssey® Infrared Imaging System (LI-COR).

3. Methods

3.1. Cell Culture and Calcium-Switch Experiment

The number of cells required for iodixanol gradient separation of the AJC will vary depending on the detection limit of proteins in membrane complexes following centrifugation. Cell–cell adhesion is synchronized by a calcium-switch experiment, which allows adhesion complexes of the AJC to be isolated at different stages of assembly.

1. MDCK II cells are cultured in DMEM + 10% FBS + 1% PSK at 37°C/5% CO_2 in air.
2. For cell passaging, a 150-mm TC culture dish is washed with 8 mL washing buffer and incubated with 8 mL washing buffer + trypsin for 15–30 min at 37°C/5% CO_2 in air. Add 3 mL of complete DMEM media after cells have lifted off the plate to inactivate trypsin; centrifuge at 750*g* for 5 min and resuspend pellet in appropriate amount of media. All work is performed under sterile conditions.
3. Calcium-switch experiment: pass cells at low density (2×10^6 cells/150 mm TC culture dish) on 2 consecutive days. For one gradient use 10 150-mm TC culture dishes, trypsinize cells as described above on day 1 and day 2, and divide all cells (~2×10^8 cells) among 10 new 150-mm TC culture dishes.
4. Collagen coat Transwell filters: add 4 mL of collagen working solution to a 75-mm filter and incubate for 1–2 min at room temperature. Remove collagen solution and dry filters under UV light for 30–60 min at room temperature to sterilize filters (*see* **Note 8**).
5. After trypsinization at day 3, plate 2×10^7 cells on collagen-coated 75 mm 0.4-µm polycarbonate membranes in low-calcium media (5 µ*M* Ca^{2+}) for 30 min to allow attachment. Plate eight 75-mm Transwell filters per density gradient.
6. Transfer cells into regular DMEM (1.8 m*M* Ca^{2+}) for 3 h, 6 h, 9 h, 12 h, 3 d, and 10 d. For time-points 3 and 10 d, replace media every 24 h with fresh DMEM.

3.2. Cell Surface Biotinylation

For some experiments it can be helpful to identify transmembrane proteins of the plasma membrane ***(7,9)***. Cell surface biotinylation allows the identification of transmembrane proteins in different plasma membrane domains. Because of the barrier function of tight junctions, apical and basal–lateral membranes can be biotinylated separately using the Transwell filter system. This step can be omitted if identification of transmembrane proteins is not necessary.

1. Grow MDCK II cells as a confluent monolayer for 3 d on 75-mm Transwell filters.
2. Perform **steps 2–5** in the cold room or on ice. Wash three times in ice-cold Ringers solution.
3. Add Sulfo-NHS-Biotin (0.5 mg/mL) in ice-cold Ringers solution to apical or basal or both compartments of the filter to study proteins in the apical or basal or both membrane domains, respectively (4 mL in apical and 7 mL in basal compartment).
4. Incubate cells twice for 20 min at 4°C with gentle rocking. In between, remove solutions after first 20 min and add fresh Ringers solution with or without Biotin.
5. Wash three times with Ringers solution and proceed with cell fractionation protocol.

3.3. Cell Fractionation in Iodixanol Gradients

3.3.1. Cell Fractionation

During breaking of cells and fractionating of membranes, it is possible that protein complexes can dissociate. In order to preserve existing adhesion structures, we suggest chemically crosslinking proteins prior to mechanical breakage of cells.

1. Wash cells three times with ice-cold Ringers solution in a cold room or on ice.
2. Add 6 mL of freshly prepared 200 µg/mL DSP in Ringers solution to the apical compartment and incubate for 20 min at room temperature with gentle rocking.
3. Perform the following steps in a cold room or on ice: quench the crosslinking reaction with five washes in quenching buffer.
4. Wash cells three times in ice-cold Ringers solution and add 1 mL of homogenization buffer I to the apical compartment. Scrape cells from filter surface and pool cells from eight Transwell filters in 15 mL conical polypropylene tube.
5. Pellet cells by centrifugation at 228*g* for 10 min at 4°C.
6. Resuspend pellet in 2 mL of homogenization buffer II using a 5-mL syringe with a screw cap, 23G1 needle.
7. Homogenize cells in ball-bearing homogenizer fitted with a stainless steel ball bearing (**Fig. 2**); pass cell homogenate back and forth 20 times; flush homogenizer with remaining 1 mL of homogenization buffer II (*see* **Note 9**).
8. Spin homogenate at 930*g* for 10 min at 4°C to pellet nuclei and unbroken cells.

3.3.2. Iodixanol Gradient

After mechanical breakage of cells, membranes are separated by centrifugation in a three-phase 10–20–30% iodixanol density gradient.

1. Prepare 10, 20, and 30% iodixanol solutions according to **Table 1** (*see* **Note 10**).
2. Measure density of each solution with a refractometer. Adjust solutions accordingly to a refractive index (η) of 1.359 for 10%, 1.373 for 20%, and 1.387 for 30% solution (all at room temperature).
3. Add all of the 30% solution containing the cell homogenate to the bottom of the centrifuge tube with a Pasteur glass pipet. Overlay with 4.5 mL of the 20% solution, and fill the tube completely with an overlay of 10% solution (*see* **Note 11**).

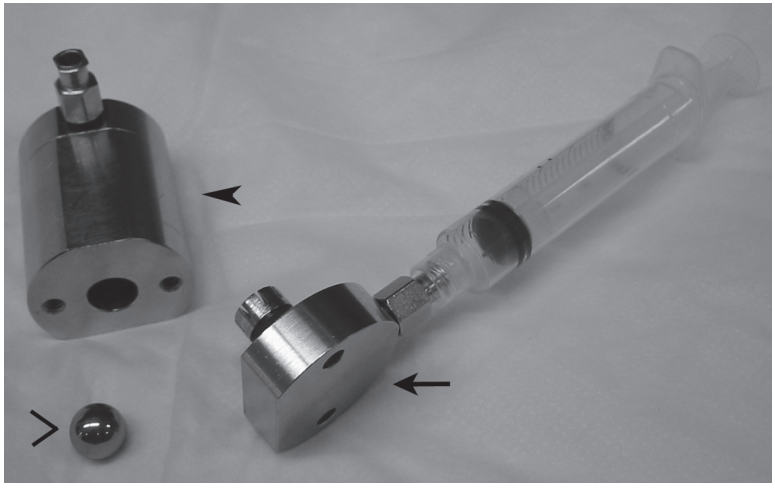

Fig. 2. Ball-bearing homogenizer, designed by Varian Physics Department, Stanford University, CA. The front part (←) is detached to demonstrate a ball bearing (>) and steel cylinder (◀). For proper function, the ball bearing is placed inside the cylinder and the front and back assembled with a screw. Syringes are attached to both syringe adaptors as shown for the front part.

Table 1
Solutions for 10–20–30% Iodixanol Gradient

Solution (%)	Iodixanol, 60% w/v (mL)	Homogenization buffer III (mL)	Postnuclear supernatant (mL)
10	1.25	3.25	—
20	2	2.7	—
30	2.95	—	2.25

4. After the tubes are balanced within 0.1 g of each other, seal the tube neck using standard Beckman equipment for tube sealing.
5. Centrifuge gradients in a VTI 65.1 rotor at 61,000 rpm (350,000*g*) for 3 h 10 min (decel = 5) in L8-80M Beckman Ultracentrifuge (Beckman Instruments Inc.).
6. After centrifugation, open tubes and collect 0.5-mL fractions from the top of the gradient (*see* **Note 12**).
7. Measure the refractive index (η) of each fraction using a refractometer and determine density δ in g/mL [$\delta = (\eta * 3.443) - 3.599$]. Plot the density δ against the fraction number; the density should increase linearly almost over the entire gradient (**Fig. 1**).

Some membranes associated with larger protein complexes remain at the bottom of the gradient and do not float up in the 10–20–30% floating iodixanol

gradient. However, these membranes can be separated in a second iodixanol gradient ***(9)***:

1. Separate membranes in a 10–20–30% floating iodixanol gradient as described above.
2. Combine fractions 19–22 (or other fractions of interest) and dilute to a 10% iodixanol solution.
3. Form a 10–20–30% iodixanol gradient as described above using homogenization buffer III for the 30% solution and the 10% iodixanol solution containing fractions 19–22 from the previous gradient.
4. Proceed as described in **steps 3–7** above.

3.4. SDS-PAGE and Immunoblotting

For the analysis of proteins in the iodixanol gradient, any standard SDS-PAGE and immunoblotting technique can be used. We describe here the method that works well for this laboratory.

1. Add 170 µL of SDS sample buffer to 500 µL of fraction sample, boil for 10 min, and store at −80°C (*see* **Note 13**).
2. Most proteins can be analyzed in linear 14, 10, or 7.5% SDS-polyacrylamide gels. Choose the appropriate separating gel for the molecular mass of your protein of interest. Mix 2.98 mL 1 *M* Tris-HCl pH 8.7, 80 µL 10% SDS, 4 µL TEMED, and 27 µL APS. Add 1.06 mL dH_2O and 3.7 mL acrylamide/*bis* for 14%, 2.2 mL dH_2O and 2.7 mL acrylamide/*bis* for 10%, or 2.8 mL dH_2O and 2 mL acrylamide/*bis* for 7.5% separating gel, respectively. After polymerization of the separating gel, add the stacking gel (3.8 mL dH_2O, 0.64 mL 1 *M* Tris-HCl pH 6.8, 50 µL 10% SDS, 0.5 mL acrylamide/*bis*, 5 µL TEMED, and 25 µL 10% APS). This gel recipe is for two minigels; adjust accordingly for different styles of slab gels.
3. After loading gels with molecular-weight marker and samples, separate proteins in SDS running buffer. Transfer proteins onto nitrocellulose or PVDF membranes using a standard Western blot transfer protocol (*see* **Note 14**).

The quantification of the protein signal after immunoblotting is important to compare sedimentation profiles of different proteins (**Fig. 3**). We use an Odyssey Infrared Imagining System to quantify protein signals, but other quantification methods can be used equally well.

1. Block proteins with LI-COR blocking buffer in PBS (1:1) for 1 h at room temperature.
2. Immunostain proteins with appropriate primary antibodies diluted in washing buffer for 1 h at room temperature.
3. Rinse membranes twice followed by 15-min and three 5-min washes in washing buffer.
4. Incubate membranes with appropriate fluorescently labeled secondary antibody diluted at 1:30,000 in washing buffer for 30 min at room temperature. *It is important to light-protect your membranes in this and the following steps!*

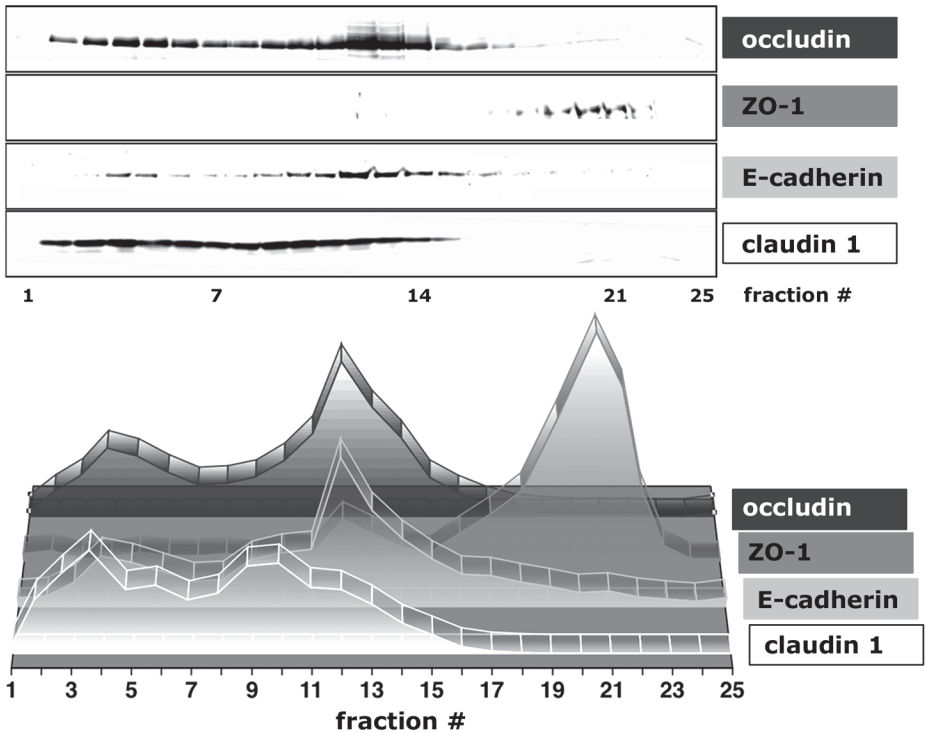

Fig. 3. Proteins of the apical junctional complex (AJC) in polarized epithelia (3 d after cell–cell adhesion) separated in iodixanol gradients. Madin–Darby canine kidney (MDCK) cells were grown as confluent monolayers on Transwell® filters for 3 d after cell–cell adhesion and membranes separated in 10–20–30% iodixanol gradients. The top panel shows immunoblot analysis of indicated proteins. Signal intensity for each protein band was determined as integrated intensity (counts/mm^2) and expressed as a percentage of the sum of integrated intensities in fractions 1–25 (*see* text for details). In the three-dimensional graph, the *y*-axis (arbitrary units) is omitted to increase the clarity of the graphic display.

5. Rinse membranes twice followed by three 5-min washes in washing buffer in the dark. Rinse membranes twice with PBS, and store in PBS at 4°C (protect from light).
6. Scan membranes with Odyssey Infrared Imaging System at 680 and 800 nm.
7. Determine the amount of protein per fraction with Odyssey software 1.2. Express data as integrated intensity of a specified area and correct for background using the appropriate background subtraction method (for details, please refer to user guide, version 1.2 for Odyssey Infrared Imaging System, Chapters 8 and 12). For each protein, the integrated intensities of fractions 1–25 in one gradient can be summed up and the amount in one fraction can be expressed as percent of the sum of fractions 1–25 (*see* **Fig. 3**).

4. Notes

1. Amino acids need to mix at least 1 h to dissolve.
2. Use HEPES·KOH instead of the more common HEPES·NaCl because the salt content is critical for density gradient characteristics. Make 1 *M* HEPES·KOH stock and adjust pH to 7.2 using KCl. Sterile filter (0.22-µm filter unit) and store at 4°C.
3. Dilute 5 mg leupeptin, 5 mg antipain, and 5 mg pepstatin in 1 mL of DMSO, aliquot 50 µL per vial and store at −20°C. Note that any commercial available proteinase inhibitor cocktail can be used according to the manufacturer's instructions.
4. Cell fragments stick less to polypropylene tubes.
5. Use precooled solutions (including dH_2O) or make sure that homogenization buffers are at 4°C before use.
6. The method for breaking cells is critical. We use a ball-bearing homogenizer designed by Varian Physics Department, Stanford University.
7. Immobilon-FL PVDF membranes are preferred for less abundant proteins (higher recovery) and if stripping of blots is desired. In contrast to Immobilon-FL, other PVDF membranes from Millipore have a high background in Odyssey Infrared Imaging System.
8. Collagen coating is critical for rapid cell attachment. Without coating, cells require more than 3 h to attach to polycarbonate membranes.
9. Have about 2 mL of air in the second empty syringe, which is already attached to the ball-bearing homogenizer. Add about 2 mL of air to the syringe containing the cell suspension before attaching to the homogenizer. Move cells completely from one syringe to the other. Frequent small back-and-forth movements while directing the net flow in one direction are very helpful. Clean the ball-bearing homogenizer between different cell lysates by flushing the homogenizer with 4 mL dH_2O; remove excessive fluid in the homogenizer by passing air through it.
10. Prepare solutions for an even number of gradients (balance for centrifuge). Table 1 gives information for one gradient. Prepare 10 and 20% solutions as one pool, respectively, for the number of gradients you are running (multiply numbers in Table 1). After adjustment for refractive index, divide 20% solution in 4.5-mL aliquots.
11. Balance tubes after each step. Tubes should be balanced within 0.1 g of each other after all solutions have been added. Add 20 or 10% solutions very slowly to avoid mixing with 30 or 20% solution, respectively. Sometimes it can be difficult to get the last air bubble out of the tube to fill it completely including the neck. It can be helpful to use a 1-mL syringe and a 26G1/2 needle instead of the Pasteur pipet for the last drops.
12. To open up the ultracentrifuge tubes, use a fresh razor blade and a hemostat. Fractions can be collected by different techniques. Use a small peristaltic pump and an automatic collector while keeping the tip of the collecting tube at the surface of the gradient slowly moving downward. Alternatively, collect initially 100-µL fractions with a P100 or P200 pipet aid and combine them to 500 µL. After the third 500-µL fraction, open the tube completely using a hot razor blade and collect 500-µL fractions with a P1000 pipet aid, moving slowly downward at the surface of the gradient.

13. SDS and K^+ form a precipitate at room temperature. Use samples right after boiling, or when using stored samples warm them up before loading. Avoid prolonged exposure to warm temperatures before loading.
14. Most proteins transfer well in the Western blot transfer buffer described in **Subheading 2.4.6.** Transfer conditions may need to be adjusted for your protein of interest. PVDF membranes are better for less abundant proteins and for stripping of blots. Please note that some PVDF membranes have high autofluorescence when used with the Odyssey Imagine System. Use Immobilon-FL from Milipore to avoid problems with autofluorescence with the Odyssey Imagine System.

Acknowledgments

This work was supported by a Walter V. and Idun Y. Berry Fellowship (R.V.), Deutsche Forschungsgemeinschaft Fellowship VO 864/1-1 (R.V.), a junior investigator award from the Stanford Digestive Disease Center (DDC DK56339; R.V., W.J.N.), and NIH RO1GM35227 (W.J.N.).

References

1. Knust, E. and Bossinger, O. (2002) Composition and formation of intercellular junctions in epithelial cells. *Science* **298,** 1955–1959.
2. Vogelmann, R., Amieva, M. R., Falkow, S., and Nelson, W. J. (2004) Breaking into the epithelial apical-junctional complex—news from pathogen hackers. *Curr. Opin. Cell Biol.* **16,** 86–93.
3. Nelson, W. J. (2003) Adaptation of core mechanisms to generate cell polarity. *Nature* **422,** 766–774.
4. Matter, K. and Balda, M. S. (2003) Signalling to and from tight junctions. *Nat. Rev. Mol. Cell Biol*. **4,** 225–236.
5. Ebnet, K., Suzuki, A., Ohno, S., and Vestweber, D. (2004) Junctional adhesion molecules (JAMs): more molecules with dual functions? *J. Cell Sci.* **117,** 19–29.
6. Grindstaff, K. K., Yeaman, C., Anandasabapathy, N., et al. (1998) Sec6/8 complex is recruited to cell–cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell* **93,** 731–740.
7. Yeaman, C. (2003) Ultracentrifugation-based approaches to study regulation of Sec6/8 (exocyst) complex function during development of epithelial cell polarity. *Methods* **30,** 198–206.
8. Yeaman, C., Grindstaff, K. K., Wright, J. R., and Nelson, W. J. (2001) Sec6/8 complexes on trans-Golgi network and plasma membrane regulate late stages of exocytosis in mammalian cells. *J. Cell Biol.* **55,** 593–604.
9. Vogelmann, R. and Nelson, W. J. (2005) Fractionation of the epithelial apical junctional complex: reassessment of protein distributions in different substructures. *Mol. Biol. Cell* **16,** 701–716.

http://www.springer.com/978-1-58829-533-0

Adhesion Protein Protocols
Coutts, A.S. (Ed.)
2007, X, 234 p. 54 illus., 2 illus. in color., Hardcover
ISBN: 978-1-58829-533-0
A product of Humana Press