

Visualizing Clathrin-Mediated IgE Receptor Internalization by Electron and Atomic Force Microscopy

Alan R. Burns, Janet M. Oliver, Janet R. Pfeiffer, and Bridget S. Wilson

1	Introduction.....	235
2	Materials	239
3	Methods.....	241
4	Notes	244
	References.....	244

Summary A significant step in the immunoglobulin E (IgE) receptor signaling pathway in mast cell membranes is receptor internalization by clathrin-coated vesicles. Visualization in native membrane sheets of the emerging clathrin lattice structures containing the IgE receptor and associated signaling partners has been accomplished with high-resolution transmission electron microscopy (TEM). More recently, membrane sheets with labeled clathrin have also been characterized with atomic force microscopy (AFM) in combination with fluorescence imaging. We discuss here the procedure for creating fixed, native cell membrane sheets, labeling with immunogold or fluorescent labels, and utilization for TEM or AFM/fluorescence imaging of clathrin-mediated IgE internalization.

Keywords Atomic force microscopy; clathrin; electron microscopy; IgE receptor; membrane sheets.

1 Introduction

1.1 *Immunoglobulin E Receptor Signaling, Membrane Organization, and Clathrin-Coated Pits*

Receptor-induced endocytosis via clathrin-coated pits is a critical pathway for internalization of external ligands (1). Using high-resolution transmission electron microscopy (TEM) and immunogold labeling of intact membrane sheets, we have shown that the crosslinked immunoglobulin E (IgE) receptor

FcεRI is internalized through pits (2,3). Following activation of IgE-primed receptors by multivalent antigen, FcεRI crosslinking results in clustering and redistribution to “dark” membrane regions that are characteristically more electron dense than bulk membrane and are often bordered by clathrin-coated pits. The tyrosine kinase Syk is also recruited to the dark patches and mixes extensively with the receptors (2). The dark regions are caused in part by osmium staining but also are thought to harbor numerous proteins (3). For example, TEM has identified in the receptor-rich dark patches in activated cells the lipid remodeling enzyme PLCγ2, phosphatidylinositol 3-kinase, the scaffolding adaptor Gab2, the guanosine 5′-triphosphate (GTP) exchange factor Vav, the adaptor protein Grb2, the adaptor/ubiquitin ligase Cbl, and others, leading to the hypothesis that these areas represent primary sites of active signaling (4).

Clathrin-coated vesicles are striking features of membrane sheets. In actively endocytosing cells, clathrin vesicles are often grouped closely together in various stages of vesicle formation prior to the fission process (Fig. 1A). These sites often border dark patches of membrane (arrow, Fig. 1A) or are closely associated with cable-like structures (Fig. 1B) previously shown to contain filamentous actin (4). They can be readily identified by the characteristic honeycomb lattice appearance of the budding vesicle (5). We also identify them through immunogold and immunofluorescence labeling with anticlathrin antibodies (Figs. 1A, 2B), as well as through immunogold labeling of adaptor proteins such as AP2 (5) (labeled with anti-AP2 antibodies; see arrows, Fig. 1B). Other features, such as the specific targeting of the Eps15 endocytic factor (5) to the neck of pre-fission vesicles, can be demonstrated using this technology (arrows, Fig. 1D). Importantly, immunogold-labeled IgE receptors can be visualized as they enter clathrin-coated vesicles (arrow, Fig. 1C). The presence of clathrin-coated pits identifies dark patches not only as primary signaling domains but also as the principal location for receptor internalization. Although Syk is present in the dark patches, it does not accompany the receptor into coated pits (2) thus, internalization very likely represents an important step in signal termination.

1.2 Atomic Force Microscopy of Membrane Sheets

In the preceding subheading, we briefly summarized the significant insights regarding lateral distributions of signaling proteins following FcεRI activation that can be obtained with high-resolution TEM and nanoscale immunogold labeling of membrane sheets. This work is complemented by the use of atomic force microscopy (AFM) to examine the topography of the cytoplasmic face of plasma membrane sheets. AFM has been used extensively to characterize biological samples because it provides true height topography and can be routinely performed in natural fluid environments, a clear advantage over vacuum conditions imposed by TEM. It has also been well established that AFM can provide undistorted images of soft,

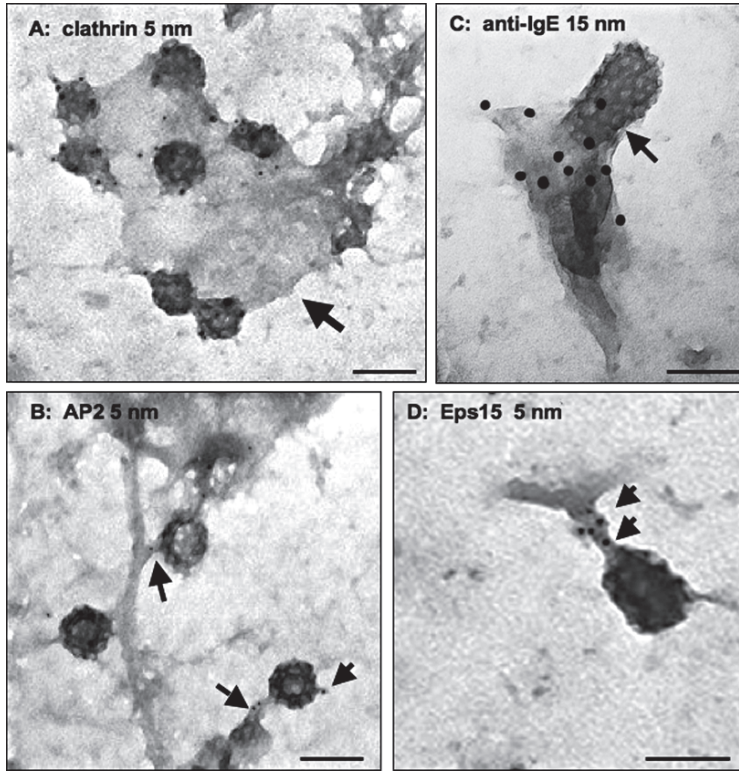


Fig. 1 TEM images of clathrin-coated vesicles on membrane sheets. All scale bars = 0.1 μm. (A) A cluster of budding vesicles labeled with anticlathrin antibodies and 5-nm gold secondary antibodies. The arrow points to the dark patch in the membrane sheet, which is caused in part by osmium staining. (B) Vesicles labeled with antibodies to the clathrin adaptor AP-2. Several 5-nm gold particles are marked with arrows. (C) Vesicle containing gold-labeled IgE receptor (arrow). Cells were primed with IgE and stimulated with 15-nm colloidal gold conjugated to rabbit anti-IgE for 2 min. The dark "signaling" patch immediately surrounding the vesicle contains numerous gold particles marking receptors. (D) The neck of this vesicle is labeled with antibodies to the endocytic protein Eps15 (arrows point to 5-nm gold particles).

compliant membrane structures (6), including membrane sheets (7,8). A key feature of our AFM is the ability to correlate membrane topographic features with the locations of fluorescently tagged proteins and lipids through simultaneous acquisition of AFM and confocal fluorescence images (9,10) (see Fig. 2 A,B).

Examples of AFM imaging of the inner (cytoplasmic) side of plasma membrane sheets are shown in Fig. 2. Simultaneous AFM (Fig. 2A) and fluorescence (Fig. 2B) imaging of mast cell sheets has revealed numerous irregular-shape raised domains that label with both resting and activated FcεRI and clathrin. They are most likely clusters of proteins that show up as the dark patches in TEM discussed above (Fig. 1A) (7). Higher-resolution AFM images indicate that these particular domains of

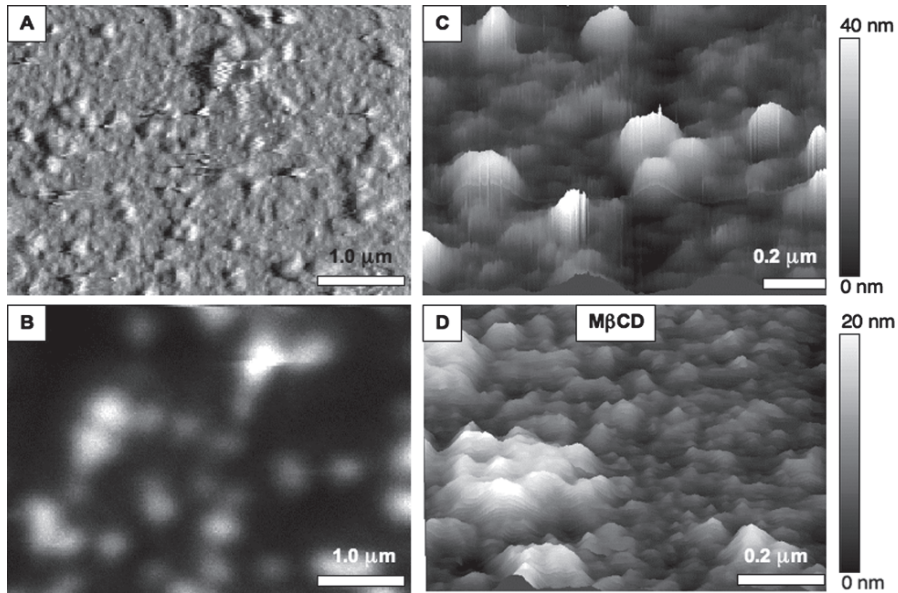


Fig. 2 AFM images of membrane sheets labeled with mouse monoclonal anticlathrin heavy chain and Alexa 488F(ab')₂ goat antimouse IgG. **(A)** AFM deflection image revealing raised domains that label with clathrin. **(B)** Fluorescence image acquired simultaneously with AFM image in **(A)**. Note the strong correlation of fluorescence spots with raised domains. **(C)** AFM topography of clathrin-labeled raised domains in control cells. Note the round appearance and average height of approx 40 nm. **(D)** AFM topography of clathrin-labeled raised domains in membranes harvested from cells that have been subjected to cholesterol depletion with methyl-β-cyclodextrin (MβCD). Note that the domains are now flatter and half the height of the control cells in **(C)**.

clustered proteins are rounded and have a height relative to the surrounding membrane of approx 40 nm. Treatment of cells with methyl-β-cyclodextrin (MβCD), which extracts cholesterol from the membrane, reduces the height and flattens the clathrin-labeled domains. This is consistent with TEM data and reveals that cholesterol is an important component of the clathrin–signaling protein complexes.

1.3 Membrane Sheets for TEM and AFM

TEM analysis procedures are described elsewhere (11), as are the methods of AFM of membranes (12). The focus of this chapter is thus preparation of membrane sheets for TEM and AFM by which components of the IgE signaling network, including clathrin, are labeled. Successful membrane sheets will contain obvious features in TEM such as clathrin-coated pits and, for nonhematopoietic cells, caveolae. The appearance of these endocytic structures varies somewhat among cell types

and preparative techniques, and it is advisable to use specific antibodies (anticlathrin, anticaveolin) to positively identify these features early in a membrane sheet project. This is particularly important for AFM, for which height and width of domains extending into the cytoplasm may be insufficient for full identification. It is important to recognize that membrane sheets seen by TEM often contain a mix of coated pits, as well as flat clathrin arrays with diameters from 80 to 100 nm. As mentioned, the flat arrays are abundant in cells treated with M β CD (11). Flat array “remnants” are also abundant on mast cell membrane sheets within 10 min of antigen stimulation and internalization of over 100,000 IgE receptors.

The overall strategy for sheet preparation is based on the “rip-and-flip” procedure originated by Sanan and Anderson (13) for harvesting and fixing apical membrane surfaces. Similar “unroofing” procedures for ventral surfaces were discussed thoroughly by Heuser (14) and Drees et al. (15).

2 Materials

2.1 Laboratory Supplies

1. 15- and 25-mm round glass coverslips (Electron Microscopy Sciences, Hatfield, PA).
2. TEM grids: Hexagonal, nickel (no. 26431, 200 mesh; Ernest Fullam, Latham, NY), Formvar[®] and carbon coated.
3. Filter paper (Whatman’s ashless, no. 42, 11.0-cm circles).
4. Cellulose acetate disks (Osmonics, Minnetonka, MN, cat. no. A02SP02500).
5. No. 1 black rubber stopper (TEM only).
6. A 37°C slide warmer with lid (Cole-Parmer, Vernon Hills, IL); line sides with damp paper towels to establish moist chamber.
7. Fume hood.
8. Curved forceps (2 pair; Electron Microscopy Sciences, cat. no. 72801-D).
9. Aspirating apparatus.
10. Divided Petri dishes.
11. Parafilm[®].
12. Glow discharge apparatus.
13. Moist chamber for sheet incubation during labeling.
14. Coverslip holder (Warner Instruments) for 25-mm coverslips used in AFM.

2.2 Working Solutions

Prepare on day of use. Sterile, doubly distilled water is used unless otherwise indicated.

1. HEPES buffer: 25 mM HEPES, 25 mM KCl, 2.5 mM Mg (CH₃COO)₂, pH 7.2.

2. Phosphate-buffered saline (PBS) buffer: 0.137 *M* NaCl, 0.003 *M* KCl, 0.007 *M* Na₂HPO₄, pH 7.4.
3. Bovine serum albumin (BSA): 0.1% in PBS buffer.
4. Poly-L-lysine: Prepare 8 mg/mL poly-L-lysine hydrobromide stock and store at −20 °C. Thaw and dilute in water on day of use. For TEM, use 0.48 mg/mL. For AFM, use 0.2 mg/mL.
5. 2% paraformaldehyde (from 16% EM grade stock solution, Electron Microscopy Sciences) in HEPES buffer.
6. 2% glutaraldehyde (from 8% EM grade stock solution, Electron Microscopy Sciences) in PBS.
7. Sodium cacodylate buffer: Prepare stock solution of 0.2 *M* sodium cacodylate, pH 7.2, and store at 4 °C. Use at 0.1 *M* (TEM only).
8. 1% osmium solution (from 4% stock solution of osmium tetroxide OsO₄, Electron Microscopy Sciences). Dilute on day of use in 0.1 *M* sodium cacodylate buffer (TEM only).
9. 1% tannic acid in sodium cacodylate buffer. Filter with a 0.2-μm Millipore filter disk (TEM only).
10. 1% uranyl acetate, prepared in water from 2% stock solution (uranyl acetate powder, a Electron Microscopy Sciences) (*see* [Note 1](#)). Once made, keep in 60-mL syringe wrapped with foil. Use new 0.2-μm Millipore filter and filter small amount for staining; add to equal volume of water (TEM only).

2.3 Immunogold Labels for TEM

Best results are obtained with monoclonal antibodies or affinity-purified polyclonal antibodies. Labeling specificity should be confirmed by Western blotting or other immunochemical techniques. It is often desirable to label two membrane proteins on the same sheet using primary antibodies raised in different species, followed by gold-conjugated secondary reagents of two distinct sizes (e.g., 5-nm antimouse secondary antibodies and 10-nm antirabbit secondary antibodies). In our experience, gold reagents from Amersham (GE Healthcare) are reliable in size and quality. Because primary antibodies are raised in only a limited number of species, it is rarely possible to label more than two different proteins in one experiment.

2.4 Fluorescence Labels for AFM/Fluorescence Imaging

Various fluorophores conjugated to secondary antibodies are used in combination with the primary antibodies noted above. Alexa 488-conjugated secondary reagents from Molecular Probes or Jackson Laboratories are brightly fluorescent and more photostable than fluorescein-conjugated antibodies. For IgE receptor studies, care must be taken to control for crossreactivity with mouse IgE used to prime receptors. Various protein-

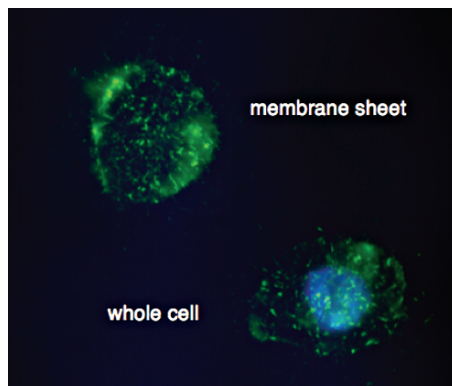


Fig. 3 Demonstration of the combination of fluorescent membrane label (in this case, fluorescein-phalloidin as a marker for the cortical actin cytoskeleton) and Hoechst nuclear stain to identify membrane sheets suitable for AFM imaging. A “good” membrane sheet candidate for AFM imaging is seen in the upper left-hand corner, whereas the cell in the lower right-hand corner is a whole cell, or is incompletely “ripped,” since it contains an intact Hoechst-stained nucleus.

labeling kits are also available from commercial sources (Molecular Probes, Sigma). Finally, it is important to note that Hoechst 33342 nuclear stain (e.g., Molecular Probes) is required to distinguish whole cells from sheets (Subheading 3.3.; Fig. 3).

3 Methods

3.1. *Whole Cell Activation, Labeling, and Fixation Prior to Sheet Preparation for Both TEM and AFM*

1. Prepare cells by plating onto sterile, 15-mm clean glass coverslips. Culture 1–4 d, to 70–80% confluent.
2. Decide if you will “activate” the cells (stimulate immune response) or subject the cells to other pretreatments (cholesterol depletion, pharmacological inhibitors, etc.). Specific procedures will differ with cell lines and signaling systems of interest.
3. Step 3 is specific to activation of the mast cell IgE receptor system. Prime cells with dinitrophenyl (DNP)-specific IgE for 2–24 h, followed by rinsing with PBS or fresh medium. IgE may be labeled with a fluorophore (e.g., Alexa 488 IgE). Remove coverslips with cells from culture dish and wick off excess medium. Gently wipe bottom of coverslip and place on surface of slide warmer set at 37 °C. Quickly (to keep cells from drying out) add 100 µL of DNP-BSA medium (0.1 µg/mL) and incubate the coverslips for a defined incubation period (1–10 min). Close the lid to keep chamber moist.

4. One can stop the reaction at the end of incubation by transferring to cold buffer. However, we recommend a light pre-fix at this stage by adding 100 μ L 0.5% paraformaldehyde to the coverslip (see [Note 2](#)). The coverslip is then held in the moist chamber for 7 min at room temperature, followed by a rinse in HEPES buffer.

3.2 Preparation of Sheets for TEM

1. Line 2-mL Petri dish with Parafilm and apply large drops of thawed, diluted poly-L-lysine solution.
2. Glow discharge EM grids (previously Formvar and carbon coated) for 30 s.
3. Float grids on poly-L-lysine drops for 30 min at room temperature with cell contact side *down*; cover Petri dish.
4. Transfer the grids facedown to distilled water for 10 s.
5. Using a sheet of filter paper, wick off excess water and place onto filter paper, treated side *up*.
6. Prepare cellulose acetate disks on glass or plastic surface. First, place a drop of HEPES buffer on the surface, apply the disk, then aspirate off excess medium so that the disk does not move when tapped with the aspirator. Place two or three grids with the poly-L-lysine side up on the acetate disk.
7. Remove excess buffer from cell coverslip by wicking. Turn the coverslip so that cells face the grids and put the coverslip over grids. Place a cork or other stopper on top and press for 1–20 s depending on cell type and confluence. The time/firmness of this step is established by trial and error.
8. Gently remove the stopper. Turn the coverslip over and remove grids by sliding to the edge of the coverslip. Transfer grids *facedown* onto droplets of 2% paraformaldehyde (grids will float on top of droplet). Hold for 10–30 min at room temperature.
9. Transfer grids to PBS in quadrant Petri dishes. Since cells are now fixed, all further steps are performed at room temperature.
10. To label the cytoplasmic face of the sheet with gold-conjugated probes and the traditional two-step protocol, prepare 100 μ L droplets of primary antibody solutions. Keep things organized by using divided Petri dishes or labeled sheets of Parafilm. Aim for saturating concentrations of the primary antibody (2–10 μ g/mL in 0.1% BSA solution). If feasible, identical grids should be incubated with isotype-matched, irrelevant IgG as a negative control. Multiple grids (two or three) can be placed facedown on each droplet of antibody solution for labeling. Incubate 30–60 min in moist chamber.
11. Rinse three times with PBS buffer for 5 min.
12. Set up 100- μ L droplets of secondary antibody conjugated to 3- to 10-nm colloidal gold particles in 0.1% BSA solution. Use the recommended dilution from manufacturer (usually 1:20). Place grids facedown on droplets and incubate for 30 min in the moist chamber.

13. Rinse three times for 5 min in PBS buffer, then postfix by incubating grids face-down on droplets of 2% glutaraldehyde for 10 min, again in the moist chamber.
14. Transfer to PBS buffer. Grids may be stored overnight at this step.
15. Proceed with osmium staining using the droplet method (see [Note 3](#)). Working in the hood, place small squares of Parafilm on the glass plate and secure to the plate by rubbing gently. Add 100- μ L droplets of 1% osmium to squares, transfer the grids facedown, and incubate for 10 min in the hood.
16. Transfer the grids facedown to Petri dishes containing 0.1 M sodium cacodylate buffer. Rinse once for 5 min.
17. Transfer grids to a Petri dish containing water. Rinse twice for 5 min.
18. Make new Parafilm squares on glass and add droplets of freshly filtered tannic acid to each square. Transfer the grids facedown to these droplets and incubate 10 min.
19. Transfer grids to a Petri dish containing water. Rinse three times for 5 min each.
20. Make new Parafilm squares for filtered uranyl acetate. Add 100- μ L droplets to each square. Transfer the grids facedown to the droplets and incubate 10 min.
21. Transfer the grids to water, rinsing twice for 1 min each. Wick off excess water from the back of the grid (do not touch face) and air dry. Store faceup in a labeled container lined with Whatman paper until ready for TEM.

3.3 Preparation of Sheets for AFM/Fluorescence

Instead of grids, we use 25-mm diameter glass coverslips that are eventually mounted in a Warner coverslip holder. Instead of gold nanoparticles, fluorescent labels are used.

1. Clean 25-mm coverslips in ethanol and glow discharge for 30 s.
2. Immerse in thawed, diluted poly-L-lysine for 30 min.
3. Rinse in water for 10 s and air dry.
4. Fix cells (grown on 15-mm coverslips $\pm$ stimulation at 37 °C) in 0.5% paraformaldehyde in PBS buffer containing Hoechst 33342 nuclear stain for 7 min at room temperature.
5. Rinse 15-mm coverslip containing fixed cells in PBS buffer.
6. Dip these 15-mm coverslips in HEPES buffer, wick off most of the moisture with a Kimwipe®, and place cell side down onto center of the 25-mm poly-L-lysine-coated coverslip, resting on moist filter paper. Do not apply pressure.
7. Leave glass “sandwich” for 10 min at room temperature, making sure filter paper on the bottom stays moist.
8. Transfer the two coverslips, still sandwiched together, into a Petri dish filled with HEPES buffer. Position the sandwich with the 25-mm coverslip on top; while floating, coverslips will separate, and the 25-mm coverslip may be removed with forceps.

9. Fix 25-mm coverslip with 2% paraformaldehyde for 30 min.
10. Rinse coverslips and label sequentially with primary and fluorophore-conjugated secondary antibodies (or other fluorescent probes).
11. Rinse in PBS buffer three times.
12. Mount in coverslip holder and quickly add PBS buffer before sheets dry out.
13. Can be stored covered with Parafilm at 4 °C overnight before AFM/fluorescence imaging.
14. Once at the AFM microscope, it is critical to use both the fluorescent membrane label (to locate sheets on the coverslips) and the Hoechst label (to avoid whole cells and partially fragmented cells that still contain a nucleus). An example is shown in Fig. 3, in which the presence of a Hoechst-stained nucleus rules out the cell in the lower right corner for AFM imaging.

4 Notes

1. The 2% uranyl acetate is a saturated solution and has a tendency to fall out of solution. Gentle heating is needed when making stock solution.
2. This light pre-fix has the distinct advantage that it helps to preserve the cortical cytoskeleton attached to the inner membrane without rendering the cells too “stiff.” If you intend to label the outside of the whole cell with antibody and gold reagents, it is advisable to substitute a stronger pre-fix (such as 2% paraformaldehyde) and to extend the incubation time (up to 30 min) to prevent antibody-induced cluster artifacts (11,16). However, it tends to lower the yield and quality of sheets recovered. Prepare 2% paraformaldehyde solution in the slightly hypotonic HEPES buffer to facilitate a small amount of cell swelling and improved recovery of sheets (14).
3. Osmium staining can obscure gold labels in clathrin-coated pits and can be omitted if desired.

Acknowledgments A.R. Burns was supported in part by the Division of Materials Science, Office of Basic Energy Sciences, U.S. Department of Energy. Sandia is a multiprogram laboratory operated by Sandia Corporation, a Lockheed Martin Company, for the United States Department of Energy under contract DE-AC04-94AL85000. B.S. Wilson was supported in part by National Institutes of Health grants A1051575 and CA119232, and J.M. Oliver was supported through National Institutes of Health grants GM49814 and P20 GM067594 that established the New Mexico Center for the Spatiotemporal Modeling of Cell Signaling. Use of the electron microscopy facility at the University of New Mexico School of Medicine is gratefully acknowledged.

References

1. Maxfield, F., and McGraw, T. (2004) Endocytic recycling. *Nat. Rev. Mol. Cell Biol.* **5**, 121–132.
2. Wilson, B., Pfeiffer, J., and Oliver, J. (2000) Observing FcεRI signaling from the inside of the mast cell membrane. *J. Cell Biol.* **149**, 1131–1142.

3. Wilson, B., Steinberg, S., Liederman, K., et al. (2004) Markers for detergent-resistant lipid rafts occupy distinct and dynamic domains in native membranes. *Mol. Biol. Cell* **15**, 2580–2592.
4. Wilson, B., Pfeiffer, J., Surviladze, Z., Gaudet, E., and Oliver, J. (2001) High resolution mapping reveals distinct FcεRI and LAT domains in activated mast cells. *J. Cell Biol.* **154**, 645–658.
5. Brodsky, F., Chen, C.-Y., Knuehl, C., Towler, M., and Wakeman, D. (2001) Biological basket weaving: formation and function of clathrin-coated vesicles. *Annu. Rev. Cell Dev. Biol.* **17**, 517–568.
6. Müller, D., Heymann, J., Oesterhelt, F., et al. (2000) Atomic force microscopy of native purple membrane. *Biochim. Biophys. Acta* **1460**, 27–38.
7. Frankel, D., Pfeiffer, J., Surviladze, Z., et al. (2006) Revealing the topography of cellular membrane domains by combined AFM/fluorescence imaging. *Biophys. J.* **90**, 2404–2413.
8. Franz, C., and Müller, D. (2005) Analyzing focal adhesion structure by atomic force microscopy. *J. Cell Sci.* **118**, 5315–5323.
9. Burns, A. (2003) Domain structure in model membrane bilayers investigated by simultaneous atomic force microscopy and fluorescence imaging. *Langmuir* **19**, 8358–8363.
10. Burns, A., Frankel, D., and Buranda, T. (2005) Local mobility in lipid domains of supported bilayers characterized by atomic force microscopy and fluorescence correlation spectroscopy. *Biophys. J.* **89**, 1081–1093.
11. Wilson, B., Pfeifer, J., Raymond-Stinz, M., et al. (2007) Exploring membrane domains using native membrane sheets and transmission electron microscopy. In *Lipid Rafts, Methods in Molecular Biology* (T. McIntosh, ed.), Humana Press, Totowa, NJ, v. 398, pp. 245–261.
12. Burns, A. (2007) Atomic force microscopy of lipid domains in supported model systems. In *Lipid Rafts, Methods in Molecular Biology* (T. McIntosh, ed.), Humana Press, Totowa, NJ, v. 398, pp. 263–282.
13. Sanan, D., and Anderson, R. (1991) Simultaneous visualization of LDL receptor distribution and clathrin lattices on membranes torn from the upper surface of cultured cells. *J. Histochem. Cytochem.* **39**, 1017–1024.
14. Heuser, J. (2000) The production of cell cortices for light and electron microscopy. *Traffic* **1**, 545–552.
15. Drees, F., Reilein, A., and Nelson, W. (2005) Cell adhesion assays: fabrication of an ε-cadherin substratum and isolation of lateral and basal membrane patches. In: *Cell Migration: Developmental Methods and Protocols, Methods in Molecular Biology*, Vol. 294 (J.-L. Guan, ed.), Humana Press, Totowa, NJ, pp. 303–320.
16. Kusumi, A., and Suzuki, K. (2005) Toward understanding the dynamics of membrane-raft-based molecular interactions. *Biochim. Biophys. Acta* **1746**, 234–251.