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Pre- and Post-embedding Immunogold Labeling of Tissue Sections

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Abstract

Despite the improved resolution capacities of fluorescence microscopy over the last 20 years, localization of specific proteins at the ultrastructural level with gold-conjugated antibodies remains a valuable technique in the cell biological tool chest. Ultrastructural immunolocalization of specific proteins in tissues rather than in cultured cells is often advantageous because, in tissues, the interactions between different cell types and with the extracellular matrix are maintained. Here, we describe two immunogold labeling procedures to localize at the ultrastructural level one or more proteins. In the first procedure (pre-embedding), micrometer-thick tissue cryostat sections are immunostained prior to embedding for obtaining ultrathin sections suitable for TEM, while in the second procedure (post-embedding), tissues are embedded in a hydrophobic resin such as Lowicryl K4M and ultrathin sections are first obtained and then immunolabeled. While the former method is better at generating strong immunolabeling, the latter is better at preserving ultrastructure.

Keywords

Tissue; Cryostat; Colloidal gold; Pre-embedding; Post-embedding; Lowicryl

1. Introduction

The use of gold-conjugated antibodies as a method of localizing proteins in cells and tissues at the transmission electron microscopy (TEM) level gained favor in the 1980s. This resulted from the development of relatively simple procedures to generate colloidal gold particles of uniform size and conjugate them to antibodies [1] as well as from the fact that gold particles of 5–25 nm in diameter can be clearly visualized in ultrathin sections prepared for transmission electron microscopy [2]. Moreover, the precision of particle size generation allows the production of antibodies conjugated with gold particles of different diameter for use in double or even triple labeling. The use of nanogold secondary antibodies will not be discussed here but is covered in another chapter of this volume.

The localization of specific proteins in tissues rather than in cultured cells is advantageous because tissue culture procedures often do not maintain or distort interactions between different cell types and/or between cells and the extracellular matrix. There are several ways to prepare tissues for immunogold procedures for TEM. In many of our own studies, we have adapted tissue preparation and fixation protocols from successful light microscopic

localization of antigens for visualization of the same proteins at the electron microscopic level [3].

Here, we describe two of these protocols. The first one is a pre-embedding immunogold labeling procedure in which the labeling is carried out on micrometer-thick cryostat sections of frozen tissues. The labeled cryostat sections, which are too thick for TEM observations, are then embedded in a hydrophobic resin such as Epon and ultrathin sections suitable for TEM are generated. While this procedure is the best in preserving antigenicity and therefore in yielding robust immunogold labeling, it is sometimes poor in preserving ultrastructural details. The second method we describe here is a post-embedding immunogold labeling procedure. Briefly, the fixed tissue piece is embedded in a hydrophilic medium such as Lowicryl K4M and ultrathin sections suitable for TEM are placed on an electron microscope grid and incubated with the antibodies. It has been well established that sections of Lowicryl-embedded fixed tissue exhibit superior ultrastructure detail when compared to pre-embedding labeling of cryostat sections and with antigenicity better preserved than in methods in which tissues are embedded in a hydrophobic resin such as Epon prior to ultrasectioning and immunolabeling [4].

2. Materials

1. 10× Phosphate buffered saline (PBS): 1.3 M NaCl, 27 mM KCl, 100 mM Na₂HPO₄, 18 mM KH₂PO₄. Adjust to pH 7.4 with either 10 M NaOH or concentrated (12 N) HCl, if necessary (*see Note 1*).
2. 1× PBS: add 100 mL of 10× PBS to 900 mL of distilled water and mix well.
3. 4% Paraformaldehyde fixative (*see Note 2*): mix 1 g of paraformaldehyde with 20 mL of water, add two drops of 1 N NaOH, heat to 60 °C until powder dissolves (do not allow to boil!), cool, add 2.5 mL of 10× PBS, adjust pH to 7.4 with 1 N NaOH, and add water to a final volume of 25 mL. Make up fresh for each procedure.
4. Tissue-Tek CRYO OCT compound (ThermoFisher Scientific, Pittsburgh, PA).
5. Acetone.
6. Antibody diluent: 1 % bovine serum albumin (BSA) in 1× PBS.
7. Primary antibodies (*see Note 3*).
8. Gold-conjugated secondary antibodies (Electron Microscopy Sciences, Hatfield, PA).
9. Glutaraldehyde fix: 2.5% glutaraldehyde in sodium cacodylate buffer (Tousimis, Rockville, MD) (*see Note 2*).

¹All solutions should be prepared with distilled water and stored at 4 °C unless otherwise stated.

²Paraformaldehyde, glutaraldehyde, and osmium tetroxide are toxic and volatile. All work with these chemicals should be performed in a fume hood using gloves and protective clothing. Handling and waste disposal should be done according to the guidelines of the local authorities.

³Only use primary antibodies which specificity has been well characterized by Western blotting and which stain by immunofluorescence specimens fixed with 4% paraformaldehyde.

10. EM wash buffer (0.1 M sodium cacodylate): dissolve 21.4 g of sodium cacodylate in 900 mL of water, adjust the pH to 7.2 with 0.2 M HCl, and make up to a final volume of 1 L with water.
11. 0.2 M sodium cacodylate buffer: mix 21.4 g of sodium cacodylate trihydrate ($\text{AsO}_2\text{Na}(\text{CH}_3)_2 \cdot 3\text{H}_2\text{O}$) to 450 mL of water, adjust the pH to 7.2 with 0.2 M HCl, and make up to a final volume of 0.5 L with water.
12. Osmium post-fix solution (*see* Notes 2 and 4): immediately prior to use, mix equal volumes of 4% aqueous osmium tetroxide (OsO_4) solution (Electron Microscopy Sciences, Hatfield, PA) and 0.2 M sodium cacodylate buffer (*see* Note 5).
13. Uranyl acetate staining solution: mix 6.25 g of uranyl acetate powder (Ted Pella, Inc., Redding, CA) (*see* Note 6) with 100 mL of distilled water in an amber bottle and tightly seal. Sonicate for up to 1 h. Centrifuge at $15,000 \times g$ for 5 min prior to use.
14. Sonicator.
15. Table top centrifuge.
16. Centrifuge tubes.
17. Ethanol 100 % (200 proof) and a graded series of ethanol dilutions (30, 50, 70, 90, and 95 %).
18. Reynold's lead citrate staining solution: add 1.33 g of lead nitrate (Electron Microscopy Sciences, Hatfield, PA), 1.76 g of sodium citrate dihydrate (Electron Microscopy Sciences, Hatfield, PA) to 30 mL of distilled water. Mix for 1 h (it is normal for the solution to become cloudy when sodium citrate is added). Then add 8 mL of 1 N NaOH (solution becomes clear when NaOH is added). Add 12 mL of distilled water to a final volume of 50 mL. Stir for 10 min. The solution may be filtered through a Millipore filter to remove any undissolved material. Do not use solution if it is cloudy.
19. Millipore filters 0.2 mm pore size.
20. Propylene oxide (Electron Microscopy Sciences, Hatfield, PA).
21. Embedding resin: EMbed 812 Resin kit (Electron Microscopy Sciences, Hatfield, PA) prepared according to the manufacturer's instructions (*see* Note 7).
22. Liquid nitrogen.

⁴Alternatively, prepare the solution with osmium tetroxide (OsO_4) crystals (Electron Microscopy Sciences, Hatfield, PA). These are available in 1 g quantities in sealed ampoules. The ampoules should be opened in a fume hood. Generally, one can use a diamond tip engraving tool to abrade the ampoule to facilitate its fracture directly into water. The 2% OsO_4 solution can be stored at 4 °C for several months provided it is kept dark. Discard the solution if a black precipitate forms. OsO_4 flames are harmful and all procedures involving OsO_4 should be done in a fume hood and OsO_4 wastes should be disposed of in closed containers (*see* Note 2).

⁵To enhance membrane visualization, a 1.5% aqueous solution of potassium ferricyanide ($\text{K}_3\text{Fe}(\text{CN})_6$) can be added to the 1% OsO_4 fixation solution [6]. The mixing of these two solutions should be performed immediately prior to use and the fixation should be undertaken at 4 °C for 4 h in the dark.

⁶UA is toxic and radioactive. Due caution should be taken with its handling. Long-term exposure, skin contact, and inhalation should be avoided.

23. 1–2 L Dewar container for liquid nitrogen.
24. Epoxy Tissue Stain (Electron Microscopy Sciences, Hatfield, PA).
25. Lowicryl K4M kit (Electron Microscopy Sciences, Hatfield PA)(*see* Note 8).
26. Pelco UVC3 cryochamber (Electron Microscopy Sciences Hatfield, PA).
27. 100 mL Beakers.
28. Cryostat.
29. Cryostat chucks.
30. Scalpel and scalpel blades.
31. Single edge razor blades (Electron Microscopy Science Hatfield, PA).
32. Microscope glass slides (75 × 25 mm, 1 mm thick).
33. Poly-lysine coated microscope glass slides (75 × 25 mm, 1 mm thick).
34. Petri dishes (100 mm in diameter).
35. Whatman #1 filter paper.
36. Liquid blocker PAP pen (Ted Pella, Redding, CA).
37. Staining jars (also called Coplin jars) for microscope glass slides.
38. Parafilm.
39. Tinfoil.
40. Kimwipes.
41. And-capillary tweezers.
42. Tongs.
43. Vacuum oven able to reach 60 °C or higher.
44. Fume hood.
45. BEEM capsules, size 00 (BEEM, West Chester, PA).
46. Flat plastic embedding molds (Electron Microscopy Sciences, Hatfield, PA).
47. Ultramicrotome (Leica Microsystems, Buffalo Grove, IL) with diamond knife (Electron Microscopy Sciences, Hatfield, PA).
48. Binocular dissecting microscope.
49. Copper TEM grids (Electron Microscopy Sciences, Hatfield, PA) (*see* Note 9).

⁷Embedding medium based on Epon 812 was routinely used until the 1970s. However, in 1978 Epon 812 was discontinued. Nonetheless, a number of companies subsequently developed Epon 812 equivalents which are now used in embedding medium formulations. We indicate one such example.

⁸Freshly prepared Lowicryl embedding media may be frozen in small batches (25–50 mL) in tightly covered plastic beakers and stored at –20 °C for several weeks. Allow the aliquots to thaw and reach room temperature for a few hours before use.

⁹TEM grids are available in different mesh sizes. The smaller the mesh size, the better the sections will be supported by the grids and able to resist tearing when exposed to the electron beam. However, too small a mesh size can result in parts of the section being

50. Gold-coated (*see* Notes 9 and 10) TEM grids (Electron Microscopy Sciences, Hatfield, PA).
51. Storage box for EM grids (Electron Microscopy Sciences, Hatfield, PA).
52. Transmission electron microscope.

3 Methods

3.1 Pre-embedding Immunogold Labeling of Tissue Cryosections

1. Human (*see* Note 11) tissues can be obtained either after biopsy or surgical procedure while animal tissues are obtained after euthanasia (*see* Notes 12 and 13).
2. Trim the tissue obtained into small pieces (~5 mm³) (*see* Note 14) with a scalpel or a razor blade. If needed, excess blood should be washed off by rinsing the tissue pieces in 1× PBS until the rinses run clear (*see* Notes 15 and 16).
3. Immediately (*see* Note 15) place tissue pieces in 4% paraformaldehyde fixative (*see* Note 2) and fix for at least 2 h.
4. Place fixed tissue pieces in a 100 mL beaker filled with 50 mL 1× PBS and wash for 10 min. Repeat three times.
5. Place a drop of OCT compound on a cryostat chuck. Place a tissue piece on top of it and surround this tissue piece with more OCT compound (*see* Note 17).
6. Freeze the cryostat chuck (also called holder) supporting the tissue piece included in OCT by placing the chuck on the specimen freezing stage (also called plate) of the cryostat for 1 h (*see* Notes 17 and 18).
7. Set temperature of the cryostat chamber to −20 °C (*see* Note 19).

obscured and, as we all know, the obscured area is always the one containing the best staining. With larger mesh sizes, such as the commonly used 200, the sections are more likely to tear under the electron beam. To help preventing this shortcoming, grids coated with a formvar/carbon film can be used. However the formvar/carbon film will sometimes reduce image contrast. Therefore, decision on what mesh size to use and whether or not to use grids coated with a formvar/carbon film is mostly a matter of personal preference.

¹⁰Do not use copper grids since copper will react with the salt in the antibody solution. Gold, in contrast, will not react with the salt in the antibody solution.

¹¹Human tissues should be obtained only after proper informed consent protocols are put in place and approved by the Institutional Review Board authorization. In addition, human tissue must be handled following Biosafety Level 2 guidelines.

¹²Animal tissues should be obtained only after proper Animal Care and Use Committee authorizations have been secured and by strictly adhering to the experimental protocol approved by that committee.

¹³Ideal tissue preservation is obtained when animals are perfused with 4% paraformaldehyde fixative through the circulatory system prior to tissue sampling [7].

¹⁴It is critical for the tissue pieces to be small enough because this ensures rapid diffusion of the fixative throughout the volume of these pieces and therefore even preservation of biological structures.

¹⁵It is critical to submerge the tissue pieces in the 4% paraformaldehyde fixative as soon as possible after tissue sampling in order to minimize autolysis (i.e., loss of biological structures and protein degradation). Autolysis may also destroy antigenic sites and cause false negative immunogold reactions.

¹⁶Procedures are carried out at room temperature unless otherwise noted.

¹⁷Alternatively, tissue samples can be included in 2% agar prior to placement on the cryostat chuck and freezing.

¹⁸Specimens for cryo-sectioning can be frozen in a variety of other ways, for instance using a cryogen mixture of isopentane cooled by dry ice [8, 9].

¹⁹The optimal chamber and tissue temperature may vary for different tissues [8, 9].

8. After ensuring that the hand wheel is in the locked position, clamp the chuck on the sample holder and allow it to stabilize at the temperature of the cryostat chamber.
9. Unlock the hand wheel and turn it to advance the sample close to the knife.
10. Adjust the micrometer setting to 15 μm and, turning the hand wheel trim the block until achieving a full-face section of the specimen.
11. Adjust the micrometer setting to 5 μm (*see* Note 20), brush trimmings off the knife edge, and lower the antiroll plate onto the knife.
12. Turn the hand wheel to obtain a section.
13. Carefully lift the antiroll plate and with a cold brush smooth out the cryosection.
14. Touch a clean slide against the section on the blade to make the OCT melt and the section adhere to the slide (*see* Note 21).
15. Remove OCT medium surrounding the tissue section by placing the glass slide holding the tissue section for 5 min in $-20\text{ }^{\circ}\text{C}$ acetone contained in a cold Coplin jar placed in the $-20\text{ }^{\circ}\text{C}$ freezer.
16. Dry the tissue section for at least 2 h under a tissue hood.
17. Using a PAP pen, draw a hydrophobic ink circle barrier around the tissue section.
18. Prepare a moist chamber by lying flat a Whatman filter paper moistened with water at the bottom of a 100 mm Petri dish.
19. Lay glass slide flat in the moist chamber with the tissue section facing up. Cover tissue section with a 100 μL drop (or more if necessary, depending on the size of the section) of primary antibody diluted at appropriate dilution (*see* Notes 3, 22, and 23) in antibody diluent. Incubate for 4–18 h at room temperature or at $4\text{ }^{\circ}\text{C}$ if incubating for longer than 4 h.
20. Remove the slide from the moist chamber and place it for 10 min in a PBS-filled Coplin jar. Repeat this wash step three times.
21. Using Kimwipes, remove excess PBS around tissue section.
22. Place slide in a moist chamber as described above for the primary antibody. Cover each section with a 100 μL drop (or more if necessary, depending on the size of the section) of secondary antibody (*see* Notes 24 and 25) diluted 1:20 in

²⁰The thinner the tissue sections, the better the antibody penetration. In addition, for steric reasons, smaller size gold particles may penetrate tissue sections more readily than larger size ones.

²¹Sections of fixed tissues sometimes adhere poorly to glass slides. If this occurs, use poly-l-lysine coated glass slide to enhance adherence of the tissue sections.

²²It is useful to start with the dilution of primary antibody that gives good consistent staining in sections prepared for immunofluorescence microscopy. To reduce nonspecific binding of the secondary antibody to the specimen, serum of the animal species in which the secondary antibody is made should be added to the primary antibody solution at a dilution of 1:20–1:100. To determine the extent of nonspecific immunogold labeling, experiments should be performed by omitting the primary antibody. Additional controls may also involve using a primary antibody known not to recognize proteins of the species from which the specimen was taken.

²³For double immunogold labeling, the primaries should have been raised in different species. Sections can then be incubated at one time with the two antibodies diluted together at their respective dilution in the diluent.

- antibody diluent. Incubate 4–12 h at room temperature or at 4 °C if incubating for longer than 4 h.
23. Remove slide from moist chamber and place it for 10 min in a 1 × PBS-filled Coplin jar. Repeat this wash step three times.
 24. Place slide in a Coplin jar filled with EM wash buffer (*see* Note 26). Wash for 5 min.
 25. Place slide in a Coplin jar filled with glutaraldehyde fix for 30 min.
 26. Wash slides in a Coplin jar filled with EM wash buffer for 15 min. Repeat three times.
 27. Section up, lay slide flat in a fresh Petri dish containing sufficient osmium postfix to fully submerge the tissue section and incubate for 2 h in the dark and under a fume hood (*see* Notes 2, 4, and 5).
 28. Wash slide in a Coplin jar filled with EM wash buffer for 15 min. Repeat three times.
 29. Wash slide for 5 min in a Coplin jar filled with distilled water. Repeat twice.
 30. Tissue section facing up, lay slide flat in a fresh Petri dish and cover section with uranyl acetate stain (*see* Note 6). Stain in the dark for 30 min.
 31. Wash slide for 5 min in a Coplin jar filled with distilled water. Repeat twice.
 32. Dehydrate the section by sequentially incubating the slide in Coplin jars filled with a graded series of ethanol: 30% (3×), 50% (3×), 70% (3×), 90% (twice), 95% (twice), and 100% (3×). Incubate the cells in each bath for 10 min.
 33. Tissue section facing up, lay slide flat in a glass Petri dish (*see* Note 27). Cover the section with ~0.5 mL of a 1:1 mixture of 100% ethanol and propylene oxide. Ensure that the section is covered with the mixture. Infiltrate for 30 min.
 34. Drain excess ethanol and propylene oxide mixture off the section by placing slide at an angle over a piece of filter paper. Using Kimwipes, remove excess mixture around section.
 35. Tissue section facing up, lay slide flat in a glass Petri dish (*see* Note 27). Cover the section with ~0.5 mL of a 1:1 mixture of embedding resin and propylene oxide. Infiltrate overnight, leaving the culture dish uncovered.

²⁴Direct labeling using primary antibodies conjugated with gold can be used in place of the indirect labeling procedure (i.e., using gold-conjugated secondary antibodies for detection), outlined here. One advantage in doing so is that gold particles attached to the primary antibody are closer to the site of antigen than in the indirect procedure where gold particles are separated from the antigen by both primary and secondary antibody molecules. Nonetheless, our preference is the indirect protocol, since multiple secondary antibodies binding to a single primary antibody result in amplification of the gold label signal output.

²⁵For double immunogold labeling, secondary antibodies against each of the primaries should be used. These should have been raised in a species different from that of the two primaries and should be conjugated to gold particles of two different sizes. Sections can then be incubated at one time with the two antibodies diluted together at their respective dilution in the secondary antibody diluent.

²⁶PBS and TBS are optimal for antibody binding, but may form precipitates with uranyl acetate, osmium, and/or lead citrate and thus must be replaced by EM wash buffer at this stage. EM wash buffer is not used to dilute antibodies in previous steps because it is not optimal for antibody binding to antigen. An alternative to EM wash buffer is 0.1 M phosphate buffer, pH 7.2.

²⁷Propylene oxide will dissolve most plastic dishes.

36. Immediately prior to use, prepare fresh embedding resin (*see* Note 28) and remove air bubbles by placing in a vacuum oven set at 20 °C and with the vacuum turned on. Stop process when resin stops bubbling (about 1 h).
37. Drain excess propylene oxide and embedding resin mixture off the section by placing slide at an angle over a piece of filter paper. Using Kimwipes, remove excess mixture around section.
38. Tissue section facing up, lay slide flat in a glass Petri dish. Cover the section with ~0.5 mL of embedding resin. Leaving the Petri dish uncovered, infiltrate 2 h to overnight in a vacuum oven set at 20 °C and with the vacuum turned on.
39. Drain excess embedding resin off the section by placing slide at an angle over a piece of filter paper for a minimum of 1 h. Using Kimwipes, remove excess embedding resin mixture around section. Place slide in a tray fashioned out of tinfoil (Fig. 1a).
40. Cut off the lid of a BEEM capsule and fill it about halfway with fresh embedding resin. Invert the BEEM capsule on top of the section on the slide. Use pressure to ensure that the BEEM capsule stays in place. The viscous nature of the resin ensures that there should be minimal leakage (Fig. 1a).
41. Carefully transfer the tinfoil slide tray containing the slide with the BEEM capsule on top of the slide to an oven set up at 60 °C. Cure the resin for 48 h (vacuum should be turned off).
42. Allow cooling and then plunge the slide with adherent BEEM capsule into a Dewar container filled half-way with liquid nitrogen. Leave immersed for up to 3 min.
43. Use a pair of tongs to lightly tap the slide and then grasp the BEEM capsule to induce its removal from the slide surface. This should be done while the slide is still immersed in nitrogen. The section should now be embedded in the resin (Fig. 1a, b). Discard the slide.
44. Remove the resin “block” from the BEEM capsule by slicing the sides of the BEEM capsule with a razor blade to loosen the block (Fig. 1b) (*see* Note 29). Be careful not to damage the block surface holding the tissue section.
45. Mount the block in a block trimmer and under a binocular microscope trim the block edges using a single edge razor blade to create a four-sided pyramid with walls at a 45° angle and a 0.5–0.75 mm square top surface (Fig. 1c).
46. Mount the trimmed block on an ultramicrotome making sure that the block face is parallel to the knife edge.

²⁸Unused epoxy resin embedding mix can be stored for up to 6 months at –20 °C but it is preferable to use a freshly prepared mix. Epoxy resin waste should be collected and allowed to polymerize before disposal.

²⁹Alternatively, use a BEEM capsule press to remove the epoxy resin block from the BEEM capsule (Electron Microscopy Sciences, Hatfield, PA).

47. Completely fill in the diamond knife water chamber with distilled water so it is leveled with the cutting edge of the diamond blade.
48. Generate alternating ultrathin sections (50–70 nm thickness) (*see* Note 30) and semi-thin sections (approximately 0.5 μm thickness).
49. Collect semi-thin sections on glass slides and stain with Epoxy Tissue Stain according to the manufacturer's instructions.
50. Collect the ultrathin sections by holding a formvar/carbon coated copper grid (*see* Note 9) with a pair of anti-capillary tweezers in the water under the sections and position the grid such that its center is under the sections.
51. Blot the jaws of the tweezers and the bottom of the grid with Whatman #1 filter paper to absorb all of the water and transfer the grid, tissue section side up, onto a dry piece of filter paper in a Petri dish covered with a lid. Allow to dry overnight.
52. Air-dry the grids under the lid of a clean petri dish to protect the sections from dust.
53. Place consecutive grids with consecutive sections in a grid storage box in the order of collection.
54. Examine the thick sections by light microscopy to identify sections generated at the outermost region of the original cryosection.
55. Using the transmission electron microscope, evaluate the labeling of the ultrathin sections generated at the outermost region of the original cryosection and then progressing backwards through the grid collection. Figure 2 shows the immunogold localization of collagen VII in a cryosection of bovine tongue. Gold particles are localized at the junction between the epithelium and the connective tissue, mostly beneath the basement membrane (BM) of the hemidesmosomes (HD) at the point of interaction of so-called anchoring fibrils with the BM. Hemidesmosomes are easily identified by the dense plaque facing the connective tissue. In addition, the distribution of the gold particles indicates that collagen VII is also present in plaque-like aggregates in the dermis (arrows). Altogether, this distribution of collagen VII suggests that this protein plays a role in anchoring epithelial cells to the underlying connective tissue at the level of hemidesmosomes.

3.2 Post-embedding Immunogold Labeling of Tissues Embedded in Lowicryl K4M

1. Human (*see* Note 11) tissues can be obtained either after biopsy or surgical procedure while animal tissues are obtained after euthanasia (*see* Notes 12 and 13).

³⁰50–70 nm ultrathin sections will appear to have a silvery shine when floating on the surface of the water and viewed with the binocular mounted on the ultramicrotome.

2. Trim the tissue obtained into small pieces (~5 mm³) (*see* Note 14) with a scalpel or a razor blade (*see* Note 16). If needed excess blood should be washed off by rinsing the tissue pieces in 1× PBS until the rinses run clear.
3. Immediately (*see* Note 15) place tissue pieces in 4% paraformaldehyde fixative (*see* Note 2) and fix for at least 2 h.
4. Place fixed tissue pieces in a 100 mL beaker filled with 50 mL 1× PBS and wash for 10 min. Repeat three times.
5. Dehydrate tissue pieces in a series of baths containing increasing ethanol concentration as follows: 30 min in 30% ethanol on ice, 1 h in 50% ethanol at –20 °C, 1 h in 70% ethanol at –35 °C, 1 h in 95 % ethanol at –35 °C, and twice in absolute ethanol for 1 h at –35 °C.
6. Infiltrate tissue pieces at –35 °C for 1 h in a 1:1 mix of Lowicryl K4M:100% ethanol, followed by an additional 1 h in a 2:1 mix of Lowicryl K4M:100% ethanol at –35 °C.
7. Incubate tissue pieces overnight in Lowicryl K4M at –35 °C in the Pelco UVC3 cryochamber (*see* Note 31).
8. Place tissue pieces in a BEEM capsule and fill with fresh Lowicryl K4M. Polymerize at –35 °C by UV irradiation in the Pelco UVC3 cryochamber (*see* Note 32).
9. Remove the resin “block” from the BEEM capsule by slicing the sides of the BEEM capsule with a razor blade to loosen the block (Fig. 1a, b). Be careful not to damage the block surface holding the tissue section.
10. Mount the block in a block trimmer and under a binocular dissecting microscope trim the block edges using a single edge razor blade to create a four-sided pyramid with walls at a 45° angle and a 0.5–0.75 mm square top surface (Fig 1c).
11. Mount the trimmed block on an ultramicrotome making sure that the block face is parallel to the Knife edge.
12. Completely fill in the diamond knife water chamber with distilled water so it is leveled with the cutting edge of the diamond blade.
13. Generate ultrathin sections (50–70 nm thickness) (*see* Note 33).
14. Collect ultrathin sections by holding a gold-coated grid (*see* Note 10) with a pair of anti-capillary tweezers in the water under the sections and position grid such that its center is under the sections.

³¹A chest freezer can replace the dedicated apparatus such as the Pelco UVC3 cryochamber for these incubations.

³²UV polymerization is performed at low temperature to prevent excessive heating of the specimen during curing. Rather than using specialized apparatus, one can easily create a UV device that fits in a freezer for this purpose by lining a Styrofoam box with aluminum foil. A handheld UV type lamp is attached to the lid of the box and placed in the freezer. The BEEM capsules should be supported in the box such that they are subject to UV irradiation from all sides.

³³Because Lowicryl K4M is a hydrophilic resin, the block face may become wet during sectioning and this will hinder the sectioning. If this is a recurring issue, reduce the water level a little in the water chamber of the diamond knife without allowing the knife edge to dry.

15. Blot the jaws of the tweezers and the bottom of the grid with Whatman #1 filter paper to absorb all of the water.
16. Place on a piece of Parafilm a 50 μ L drop of primary antibody diluted at the appropriate dilution in antibody diluent (*see* Notes 3, 22, and 23).
17. Gently place the grid, tissue side down, on the drop and incubate for 2 h.
18. Wash off unbound antibody by gently placing the grid, tissue side down, on top of a 50 μ L drop of 1 $\times$ PBS laid on a piece of Parafilm and wash for 5 min. Repeat this wash step three times.
19. Place on a piece of Parafilm a 50 μ L drop of secondary antibody diluted 1:20 in antibody diluent (*see* Notes 24 and 25).
20. Gently place the grid, tissue side down, on the drop and incubate for 2 h.
21. Wash off unbound antibody by gently placing the grid, tissue side down, on top of a 50 μ L drop of 1 $\times$ PBS laid on a piece of Parafilm and wash for 5 min. Repeat this wash step three times.
22. Gently place the grid, tissue side down, on top of a 50 μ L drop of distilled water laid on a piece of Parafilm and wash for 5 min. Repeat three times (*see* Note 34).
23. Proceed to **step 30** if opting to not enhance the contrast of biological structures in tissue sections with uranyl acetateX and Reynold's lead citrate staining solution (**steps 24–29**) (*see* Note 35).
24. Place a 50 μ L drop of uranyl acetate staining solution on a piece of Parafilm.
25. Gently place the grid, tissue side down, on top of the drop and stain for 30 min, protected from light (*see* Note 36).
26. Wash off excess stain by gently placing the grid, tissue side down, on top of a 50 μ L drop of distilled water laid on a piece of Parafilm and wash for 5 min. Repeat this wash step three times.
27. Place a 50 μ L drop of freshly prepared Reynolds lead citrate staining solution on a piece of Parafilm and arrange a few dry sodium hydroxide pellets around the drop (*see* Note 37).
28. Gently place the grid, tissue side down, on top of the drop and cover with a small Petri dish lid or beaker and stain for 2 min.

³⁴This step removes residual PBS to prevent the formation of salt crystals when drying the grids or the formation of precipitates when staining the tissue sections with uranyl acetate.

³⁵Sections can be dried and viewed with the transmission electron microscope prior to staining with heavy metal salts (uranyl acetate and lead citrate) to determine whether the immunolabeling was efficient. Gold particles, especially small ones (5 nm), may be more readily visualized in the absence of heavy metal staining. However, biological structures may be difficult to identify in the absence of heavy metal staining.

³⁶Uranyl acetate staining enhances the electron contrast of biological structures by interacting with lipids and proteins. However, needle-like crystal precipitates may be present in uranyl acetate solutions that have not been cleared by filtration or centrifugation or may form in the presence of residual PBS on the section.

³⁷Lead citrate staining enhances the electron contrast of biological structures by interacting with proteins and glycogens. Lead citrate, however, forms a precipitate of lead carbonate in the presence of CO₂, hence the utility of sodium hydroxide pellets since they adsorb the CO₂. In some cases, the contrast generated by uranyl acetate stain solution may be sufficient to discern biological structures of interest (Fig. 3) and the lead citrate staining step can be omitted.

29. Wash off excess stain by gently placing the grid, tissue side down, on top of a 50 μ L drop of distilled water laid on a piece of Parafilm and wash for 5 min. Repeat this wash step three times.
30. Air-dry the grid by placing it flat, tissue side up, at the bottom of a clean Petri dish covered with a lid to protect the tissue sections from dust. Dried grids can be stored indefinitely in a grid storage box kept in a dust-free, dry place.
31. View grids with a transmission electron microscope. Figure 3 shows results of an experiment in which the respective distribution of the desmosomal proteins desmocollin and desmoplakin was investigated by immunogold labeling in Lowicryl K4M embedded bovine tongue epithelium. The micrographs show good ultrastructural preservation of the different structural domain composing these intercellular junctions, including the dense plaques with attached intermediate filaments and the extracellular space between those two plates. The distribution of the gold particles demonstrates that desmocollin (A) localizes in the intercellular space of the desmosome (arrow) whereas desmoplakin (B) is present in the cytoplasmic desmosomal plaque and in the region where the intermediate filaments attach to the dense plaque (arrow). These distributions suggest a role for desmocollin in cell-cell adhesion and a role for desmoplakin in anchoring intermediate filaments to the desmosomal dense plaque, functions which are also indicated by biochemical data and genetic analysis of the roles of desmocollin and desmoplakin [5].

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Fig. 1.

(a) Picture of a BEEM capsule adhered to a tissue section on a glass slide. The BEEM capsule is about half full of cured gold-colored resin. The BEEM capsule is detached from the slide by immersion in liquid nitrogen (LN₂), (b) The block is removed from the BEEM capsule by slicing the sides of the capsule as indicated, (c) Resin block trimmed into a trapezoidal/pyramidal shape with the tissue section being positioned at the center of the pyramid. The trimming is performed with a razor blade using a binocular dissecting microscope to monitor the operation

Fig. 2. Immunogold labeling of collagen VII in a cryosection of bovine tongue. The primary antibody was a mouse monoclonal against collagen VII prepared in the Jones lab. The secondary antibody was an AuroProbe goat anti-mouse IgG conjugated with 5 nm gold (GE Healthcare UK, Ltd.). Note the gold particles localizing collagen VII to the region beneath the hemidesmosomes (HD) and the basement membrane (BM) at the point of interaction of so-called anchoring fibrils with the BM and also in plaque-like aggregates in the dermis (arrows). Bar: 200 nm

Fig. 3.

Ultrathin sections of Lowicryl K4M embedded bovine tongue epithelium immunogold labeled for (a) desmocollin (primary: rabbit polyclonal antibody against desmocollin, kind gift of Dr. Robert Goldman, Northwestern University, Chicago, IL; secondary: AuroProbe 10 nm gold conjugated goat anti-rabbit IgG) or (b) desmoplakin (primary: mouse monoclonal antibody against desmoplakin from Boehringer Mannheim Biochemical; secondary: AuroProbe 5 nm gold conjugated goat anti-mouse IgG). In (a) and (b) uranyl acetate staining was performed after the immunolabelling. (a) and (b) demonstrate that the desmosome ultrastructure is well preserved. The distribution of the gold particles relative to the ultrastructural features of the desmosome demonstrates that desmocollin (a) localizes in the intercellular space of the desmosome (*arrow*) whereas desmoplakin (b) is present in the cytoplasmic desmosomal plaque (*arrow*) consistent with the known distribution of these two proteins [5]. Note that there is some minor heterogeneity in the size of the colloidal gold particles used in these experiments. Bars: 100 nm