Protocol

Immunogold Staining of Ultrathin Thawed Cryosections for Transmission Electron Microscopy (TEM)

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This protocol was adapted from "Ultrastructural Immunocytochemistry," Chapter 7, in *Immunohistochemistry: Methods Express* (ed. Renshaw), from the *Methods Express* series. Scion Publishing Ltd., Oxfordshire, UK, 2006.

INTRODUCTION

A pre-embedding method of immunochemical staining is used if antigens are damaged by resin embedding, or if the best preservation of membranes is required. Applying immunogold reagents to sections of lightly fixed tissue, free of embedding medium, can be a very sensitive method of immunochemical staining. Cells or tissues are fixed as strongly as possible and then treated with a cryoprotectant, which is usually a mixture of sucrose and polyvinylpyrrolidone (PVP). They are frozen onto pins in liquid nitrogen and sectioned at approximately -100°C . The frozen sections are thaw-mounted on to Formvar/nickel film grids and the cryoprotectant is removed by floating the grids on drops of phosphate-buffered saline (PBS). The immunogold staining is performed on the unembedded sections, which are subsequently contrast counterstained and infiltrated with a mixture of methylcellulose and uranyl acetate. In this protocol, samples are sectioned at low temperature, thaw-mounted onto film grids, immunochemically stained, contrast counterstained, and embedded/encapsulated in situ on the grid before viewing by transmission electron microscopy (TEM).

RELATED INFORMATION

Ultrastructural Immunocytochemistry (Skepper and Powell 2008a) describes methods and considerations for the use of immunogold staining, including fixation, controls, resolution, and quantification. The following protocols provide detailed procedures for immunogold staining of various sections for TEM:

Immunogold Staining of Epoxy Resin Sections for Transmission Electron Microscopy (TEM) (Skepper and Powell 2008b)

Immunogold Staining of London Resin (LR) White Sections for Transmission Electron Microscopy (TEM) (Skepper and Powell 2008c)

Immunogold Staining Following Freeze Substitution and Low Temperature Embedding after Chemical Fixation or after Cryoimmobilization for Transmission Electron Microscopy (TEM) (Skepper and Powell 2008d)

For more comprehensive descriptions of the range of techniques available, see Griffiths et al. (1993) and Skepper (2000).

MATERIALS

CAUTIONS AND RECIPES: Please see Appendices for appropriate handling of materials marked with <I>, and recipes for reagents marked with <R>.

Reagents

<R>Antibodies, primary (optimally diluted in PBSG)

<R>Antibodies, secondary (optimally diluted in PBSG)

Use a secondary antibody raised against the species of the primary antibody and conjugated to 10- or 15-nm colloidal gold particles.

<R><R>Formaldehyde (2% w/v) (made from freshly depolymerized paraformaldehyde)

<R>Formaldehyde (8%) in PIPES

Methylcellulose (2%, w/v)

Methylcellulose is used in embedding media to reduce shrinkage, thus preventing the sections from collapsing totally during air-drying before viewing by TEM.

<R>PBSG

<R>PBS (1X) containing 10% (w/v) gelatin

<R>Phosphate-buffered saline (PBS) (pH 7.6)

PIPES buffer (0.1 M) (pH 7.4)

Sodium chloride (0.9%, w/v)

Sucrose (1.9 M) and 10% (w/v) polyvinylpyrrolidone (PVP-10)

Sucrose and PVP-10 are used as cryoprotectants.

Sucrose (2.3 M)

A 50:50 mixture of 2.3 M sucrose and 2% methylcellulose is used as retrieval fluid; see Step 7.

Tissue (small pieces) or cells of interest

<R>Uranyl acetate (3%, w/v; aqueous)

2% methylcellulose and 3% aqueous uranyl acetate are mixed in ratios varying from 9:1 to 5:1; see Step 13.

Equipment

Cell scraper

Diamond trim tool and 45° ultradiamond knife (Diatome AG)

Eyelash (to maneuver sections)

Filter paper (hardened)

Frozen cryosection module (FCS) and cryo-prep center (CPC) freezing station (Leica Microsystems)

<R>Liquid nitrogen

Loop (copper, 1-mm diameter)

Micropipettor with tips

Microscope (transmission electron) (FEI Tecnai 120)

Nickel/Formvar grids (400 mesh)

Sectioning pins

Tubes (1.5-mL microcentrifuge)

Ultramicrotome (EM UCT; Leica Microsystems)

METHOD

Tissue Preparation and Sectioning

If it is possible to use strong fixation during tissue preparation (4%-8% formaldehyde for 2-4 h), the preservation of cell membranes is excellent (Liou et al. 1996) (see Fig. 1). These sections can be stored on buffer at 4°C for several hours or even overnight, if more blocks are to be sectioned. However, caution should be exercised if the tissue has been fixed very lightly, because the ultrastructure will deteriorate as a function of the time that the section is floated on the buffer.

1. Rinse cells or small pieces of tissue twice in 0.9% sodium chloride.
2. Incubate cells or tissue pieces in formaldehyde (8%) in PIPES for 1 h at 4°C. If the cells are adherent, scrape them free from the substrate with a cell scraper and transfer to 1.5-mL tubes.
3. Rinse four times in 0.1 M PIPES buffer at room temperature over a period of 20 min and twice in H₂O.

FIGURE 1. Ultrathin thawed cryosection of placental syncytium. Sections were fixed in 6% formaldehyde, cryoprotected in sucrose and PVP, and retrieved from the microtome on methylcellulose and sucrose. Cells were immunolabeled for copper/zinc superoxide dismutase. Gold particles can be seen over both the cytoplasm (C) and the nucleoplasm (N). Bar, 200 nm. (Reprinted with permission from Scion Publishing Ltd. © 2006.)

4. Incubate in 1X PBS containing 10% gelatin for 2 h and centrifuge to form a pellet. Cool to 4°C and fix in 2% formaldehyde for 2 h.
Small pieces of fixed tissue can be trimmed to 0.5 mm in one dimension.
5. Trim to 0.5-mm³ cubes and incubate in 1.9 M sucrose and 10% PVP-10 overnight at 4°C.
6. Freeze the cubes onto sectioning pins in liquid nitrogen. Transfer to the frozen cryosection module (ultramicrotome with a cryochamber) at a temperature of between -90°C and -130°C and cut thin sections of 90-140 nm.
See Troubleshooting.
7. Maneuver the sections away from the cutting edge of the knife with an eyelash. Place a drop of a 50:50 mixture of 2.3 M sucrose and 2% methylcellulose in a 1-mm diameter copper loop and retrieve the sections from the frozen cryosection module. Allow the sucrose to thaw at room temperature and touch the sections onto the Formvar surface of a nickel/Formvar grid.
The droplet of retrieval fluid should be moved rapidly toward the sections, which will jump toward it and "disappear." The sucrose must remain liquid while the sections are retrieved or they will not fully decompress, so speed is critical.

Tissue Staining

Immunogold staining is carried out essentially the same as for resin sections with several modifications. The absence of an embedding medium means that many primary antibodies will bind strongly after 0.5-2 h exposure, so it is often convenient to use more dilute primary antibody solutions and stain immunochemically overnight. This conserves antibodies and tends to produce less nonspecific background staining.

8. Transfer sections to drops of PBSG.
9. Incubate sections on drops of optimally diluted primary antibody in PBSG at room temperature overnight.
10. Rinse sections on ten 100-μL drops of 1X PBS, dispensed with a micropipettor, for 2 min on each drop.
11. Incubate sections on drops of optimally diluted species-specific secondary antibodies in PBSG (conjugated to 10- or 15-nm gold particles) at room temperature for 2 h.
12. Rinse sections on ten 100-μL drops of 1X PBS for 2 min on each drop.
13. Rinse briefly in H₂O and incubate sections on drops of 2% methylcellulose and 3% aqueous uranyl acetate in ratios varying from 9:1 to 5:1, until the desired contrast is achieved. Blot away excess stain using hardened filter paper and air-dry sections before viewing at 80 kV in a transmission electron microscope.
See Discussion.

TROUBLESHOOTING

Problem: The sucrose tends to crumble.

[Step 6]

Solution: In tissues with voids such as blood vessels, the sucrose tends to crumble rather than section. This effect is particularly noticeable in fragile embryos. It can be prevented by filling the lumen of blood vessels with gelatin (Russell et al. 1998) or by infiltration of embryos with polyacrylamide gel (Tokuyasu 1983).

DISCUSSION

This technique for immunogold staining of lightly fixed, unembedded tissue is frequently referred to as the Tokuyasu technique (Tokuyasu 1973; 1983; 1986; 1989) after its pioneer. It is one of the few methods that is consistently used for immunochemical staining of sparse and labile membrane-bound proteins such as receptor molecules, and has been used to great advantage in the study of receptor internalization and endocytosis (Liou et al. 1997; Klumperman et al. 1998).

Small cubes of fixed tissue (<0.25 mm³) impregnated with either 2.3 M sucrose (Tokuyasu 1986) or a mixture of 1.9–2.1 M sucrose and 10% PVP as described here, to act as cryoprotectants (Tokuyasu 1989), are mounted on pins and frozen in liquid nitrogen. The high concentrations of cryoprotectant make rapid freezing unnecessary. Frozen sections can be retrieved on a drop of cold 2.3 M sucrose, a mixture of sucrose and PVP, or more recently introduced alternative retrieval fluids, e.g., a 50:50 mixture of 2% methylcellulose and 2.3 M sucrose (described here), or 1.5%–2% methylcellulose and 0.3%–3% uranyl acetate. Because the tissue has been fixed and sectioned directly after cryoprotection, residual reactive aldehyde groups may remain in the sections. These can be quenched by exposure to 0.1%–1% (w/v) lysine or glycine in phosphate- or Tris-buffered saline for 10 min.

After immunochemical staining, the sections are contrast counterstained and encapsulated in a matrix to prevent gross collapse of the sections caused by surface tension effects during subsequent air-drying. The most commonly used stain is uranyl acetate, which produces a negative contrast. Many variations and alternatives to uranyl acetate have been proposed, and are discussed in detail by Griffiths et al. (1993). Sections prepared as described in this protocol are stable for a considerable time. The thickness of the section and that of the final embedding layer influence the contrast in the transmission electron microscope. The thinnest, flattest sections produced with diamond knives produce the best contrast between the section and antibodies conjugated to small colloidal gold particles (5–20 nm) (Peters et al. 2003).

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