Preparation of cultured cells for transmission electron microscope

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Introduction

Researchers working on differentiated cells or cell-substrate interactions *in vitro* must prepare the cultures for transmission electron microscopy to image the cells at high resolution. In practice, workers have found that the procedures used for preparing plant and animal tissues are unreliable when applied to tissue cultures. These procedures often cause the plasma membrane and cytoplasmic membranes to appear as white spaces. We found that the extracted appearance of the membrane is typical of tissues that were never exposed to osmium tetroxide. This artifact is prevented by using hexylene glycol for dehydration, instead of ethanol. Previous workers have used hydroxypropylmethacrylate for dehydration (1). The grade of the epoxy constituent of the resin has been found to be important (2). Previous protocols lead to difficulty in separating the sample from the culture dish. This problem is remedied by making a thin, flexible layer of resin. Here, separate preparation procedures are given for situations in which the tissue culture sample is to be sectioned parallel or transverse to the attachment surface.

Reagents

Commercial quick-setting epoxy glue

Epoxy resin (mixture A and mixture B mixed according to manufacturer with 1.4 % DMP-30):

Mixture A; LX-112® (Ladd) or Embed 812® (EMS) and dodecyl succinic anhydride
Mixture B; LX-112® (Ladd) or Embed 812® (EMS) and nadic methyl anhydride
2,4,6-tri(dimethylaminoethyl)phenol (DMP-30)

Glutaraldehyde, 70% solution

Hexylene glycol

Osmium tetroxide, 1% in deionized or distilled water

Phosphate-buffered saline tablets, make up to PBS, pH 7.5

Polystyrene dishes with cultured cells or tissues

Sodium chloride

Equipment

Embedding oven

Mechanical rotor

Procedure

1. Pre-warm fixative solution (2.5% glutaraldehyde in 0.05 M cacodylate buffer with 0.1M NaCl, pH 7.5) to 37 °C.
2. Remove no more than two dishes at a time from incubator and pour off media.
3. Add glutaraldehyde fixative solution. Leave 20 minutes at room temperature.
4. Rinse 2 times with PBS.
5. Add osmium tetroxide solution and incubate 30 minutes.
6. Rinse once with PBS.
7. Put on 30%, 50%, 70% and 90% hexylene glycol with swirling, 15 minutes each.
8. Wash with 100% hexylene glycol with swirling, 3 times, 10 minutes each.
9. Combine resin and hexylene glycol at 1:2 ratio, add to dish, mix on rotor for 2 hours.
10. Combine resin and hexylene glycol at 2:1 ratio, add to dish, mix on rotor for 2 hours.
11. Replace with 100% resin and place on rotor to mix for 4-6 hours.
12. Replace with fresh 100% resin and mix as above.
13. Replace with 100% resin, adjust level to ~1-2 mm depth, and bake 8-12 hours.
14. Break dish with hammer, peal dish pieces away from cast containing sample.
15. Bake epoxy cast for an additional 16-24 hours.
16. Cut small pieces from the epoxy cast making note of the side with the cells.
17. Glue pieces to blocks for sectioning, using commercial 5-minute epoxy glue.

Timing

48 hours

Critical Steps

Steps 1-2. Take precautions to avoid samples cooling to room temperature before fixation. The use of pre-warmed fixative prevents the disappearance of certain cellular protrusions (see protocol http://www.natureprotocols.com/2007/11/13/preparation_of_cultured_cells.php).

Steps 7-8. The use of hexylene glycol prevents membrane extraction, which appears to be caused by ethanol. The reason for this is not clear, but two possibilities are that: 1) ethanol may contain an

oxidizing compound that re-solubilizes osmium that has been reduced and deposited on membranes, or 2) the sample dries out due to rapid ethanol evaporation, leading to denaturation of membrane structure.

Steps 2-11. Never allow the sample or surface of the dish to dry out.

Steps 9-13. Turning dishes on a mechanical rotor for several hours ensures that hexylene glycol in the sample is displaced by the epoxy resin. The angle of the rotor can be adjusted to ensure that the dish surface remains wet.

Step 13. Use only a thin layer (2 mm) of resin to facilitate separation from the dish.

Troubleshooting

1. If the sample dries out at any time, the ultrastructure will appear abnormal (steps 2-13).
2. If water is not completely replaced by solvent, the resin infiltration will be poor, and the resin will not section well due to residual water (steps 7-8).
3. The same occurs if hexylene glycol is not completely replaced (steps 9-13).
4. The biggest problem in previous protocols has been separating the epoxy layer from the polystyrene dish. Here, separation takes advantage of the flexibility of the epoxy layer when it is warm. The epoxy flexes as the dish is broken away, and the result is a perfectly smooth, shiny cast containing the cell culture at its surface.
5. If the cast appears rough in places, it is because of inadequate dehydration or infiltration with resin (steps 7-13) or because the final layer of epoxy was too thick.

Anticipated Results

The method allows both internal membranes and cell-cell adhesions to be imaged, as illustrated in Figure 1. The membrane is seen in high contrast, and both the internal components of the cell and cell-cell adhesions are obvious. An area of interest in the culture dish can be designated at step 4. This can be done by inspecting the culture in a tissue culture microscope and etching a mark on the dish surface with a diamond or carbide marker.

The above protocol is not suitable for imaging the cell-substrate interface, as the thin sections fold up

at the free edge making the cell contact area invisible, but it can be modified to view the interface. For this, we use the Spurr resin which allows the polystyrene dish to be infiltrated. When transverse sections are made through the tightly bonded resin layer at the level of the dish, the cell-substrate interface is visible. Except for the substitution of the resin and the impossibility of separating the sample from the dish, the steps used are the same as in the main protocol. The infiltration times can be shortened due to the low viscosity of the Spurr resin. The cell layer, sectioned transversely, is illustrated in Figure 2.

References

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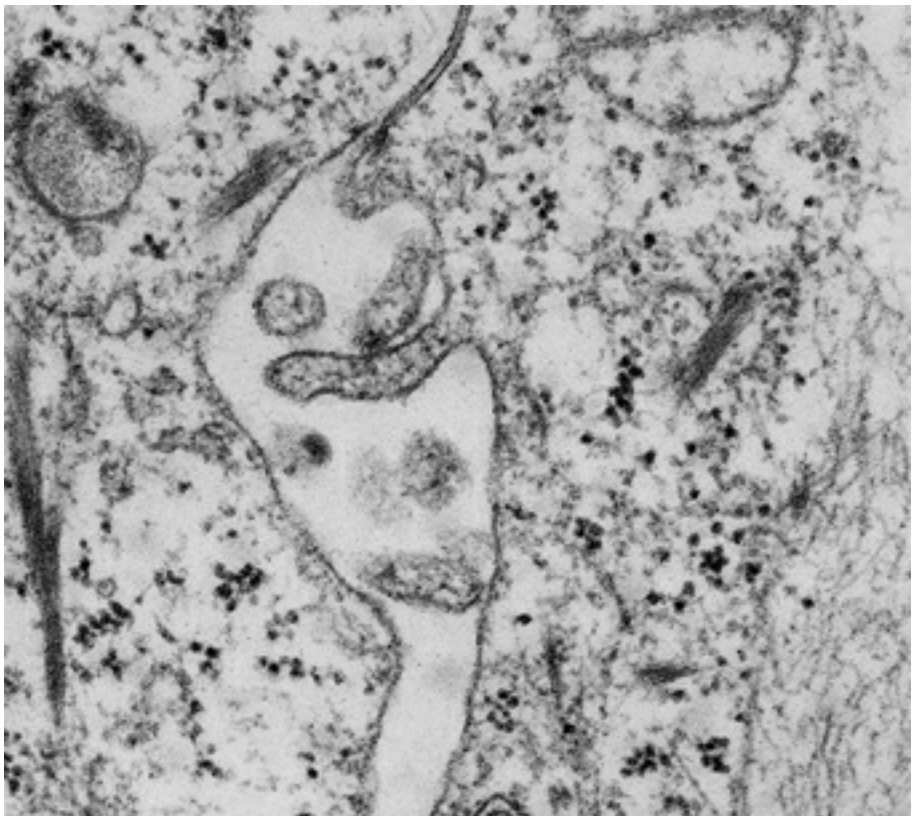
2. Murray, A. B., Helga Schulze, H., and Blauw, E. *In situ* embedding of cell monolayers cultured on plastic surfaces for electron microscopy. *Biotechnic and Histochemistry* **66**, 269 - 272 (1991).

Figures



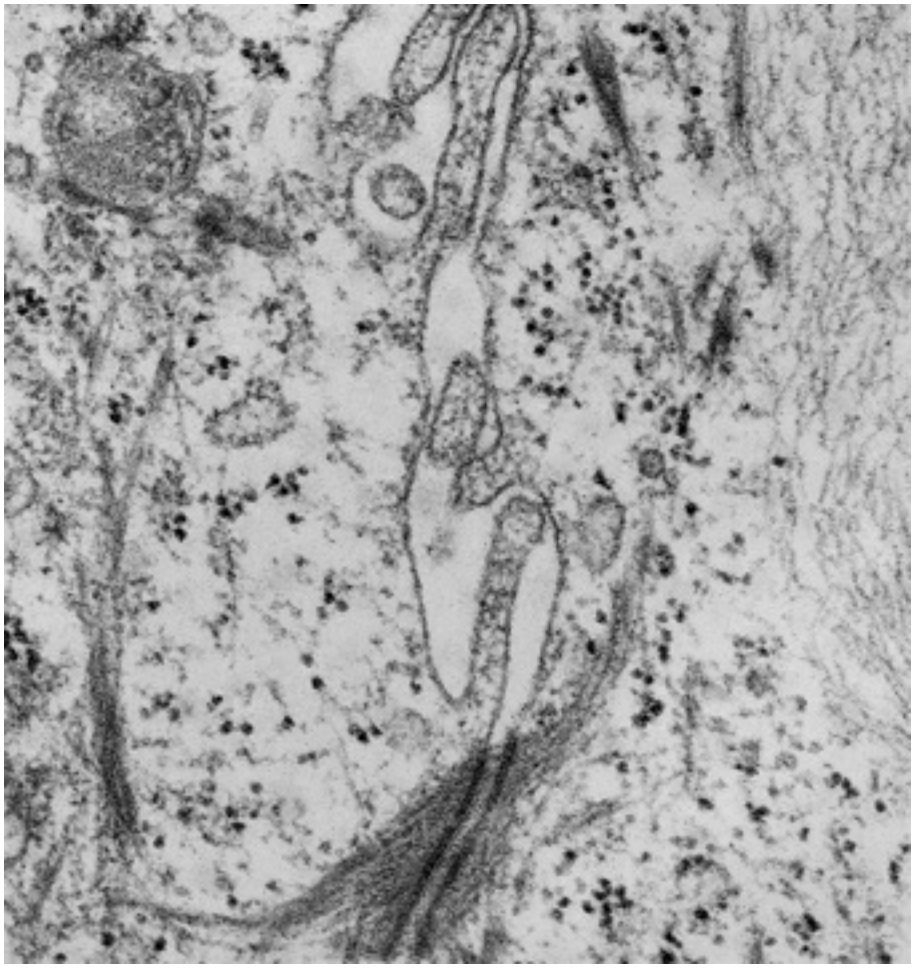
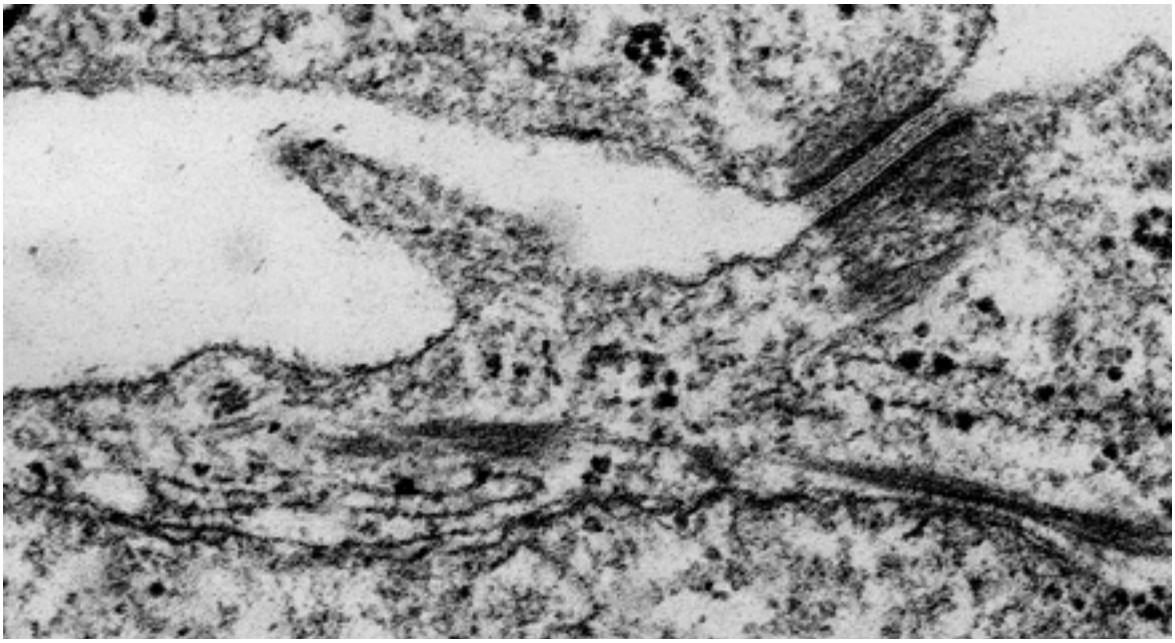


Figure 1

Cell-cell junction between primary rat tracheal epithelial cells _in vitro_ The membranes appear in high contrast, and the cell-cell interface is characterized by gap junctions and desmosomes.



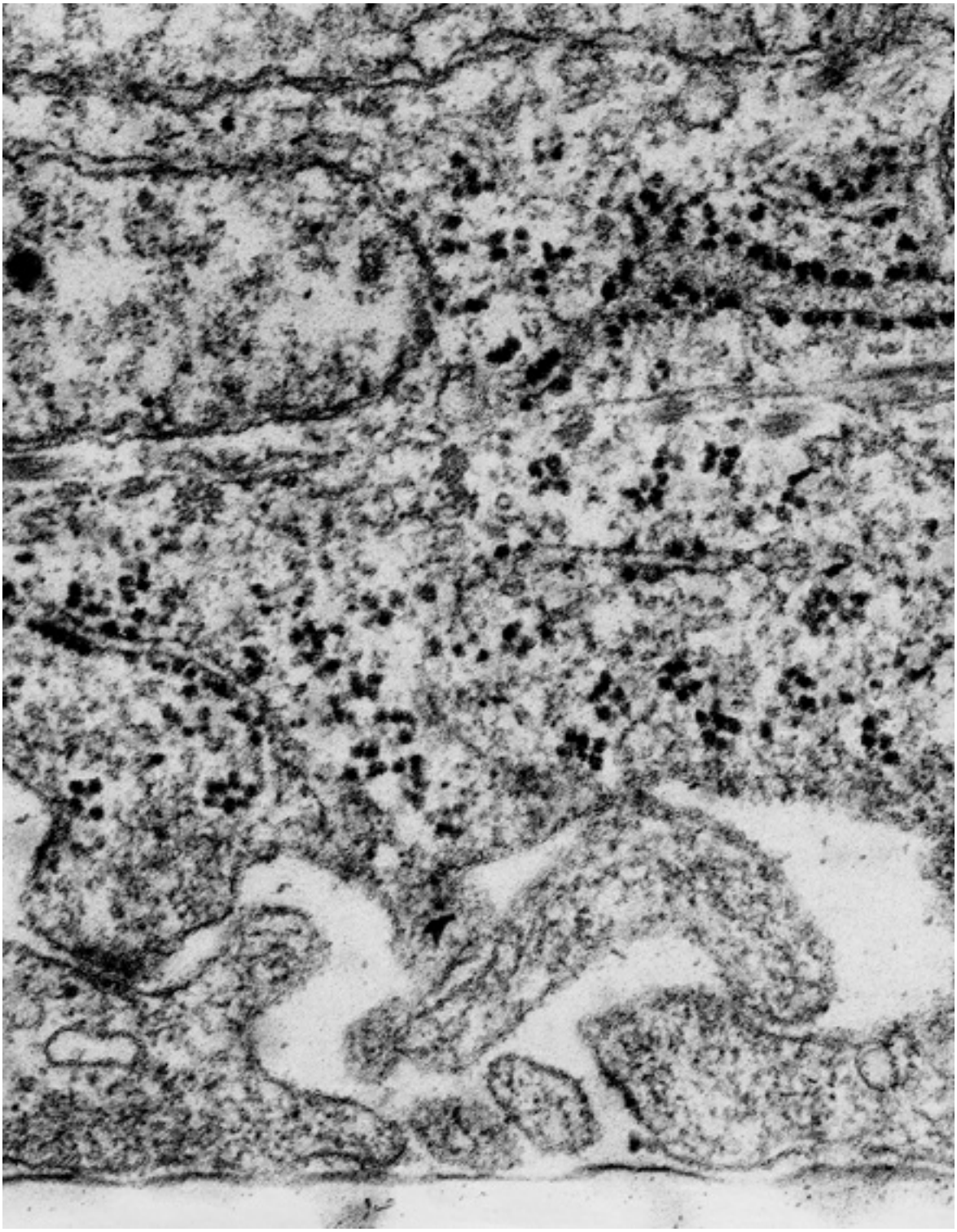


Figure 2

Interface between rat tracheal epithelial cell and plastic dish The membranes appear in high contrast, and the cell-substrate interface is visible, as are the interfaces between adjacent cells.