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DISCLAIMER

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A VIEW ON TECHNIQUES OF PREPARATION OF CELL AND TISSUE CULTURE SPECIMENS FOR ELECTRON MICROSCOPY

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ABSTRACT

Many investigators fix and embed cell cultures *in situ* for electron microscopy. Maximum structural integrity is maintained, and cell-to-cell contacts are easily studied. Coating coverslips with evaporated carbon is a popular method for allowing easy separation of polymerized blocks from the coverslips. Because carbon coatings are hydrophobic, we have developed a new method whereby the carbon coating is overlaid with evaporated silicon monoxide, providing a surface that wets easily and still allows separation of polymerized blocks from the coverslip. Our complete technique for coating coverslips, fixation, dehydration, and embedding is described.

During the past several years, numerous techniques have been published concerning electron microscopy of cells and tissues grown *in vitro*. Perhaps the greatest variability in these methods lies in the manner in which specimens are embedded. This variability may occur because the specimen is often too thin to be manipulated with forceps and the embedding epoxy sticks to the substrate (glass or plastic).

Many experimenters (Brinkley *et al.*, 1967; Lipetz and Cristofalo, 1972; Ross *et al.*, 1975; Codling and Mitchell, 1976) utilize *in situ* fixation and embedding; some use carbon-coated coverslips to allow the plastic-embedded specimen to snap free when polymerized (Lipetz and Cristofalo, 1972). Indeed, in trying several methods for allowing embedded blocks to be easily separated from the substrate, the carbon-coated coverslip method works best. Unfortunately, carbon-coated slips are hydrophobic, and for roller-tube applications, this hydrophobia can cause a problem because specimens are continuously dipped in and out of the incubation medium.

This paper outlines, in detail, an *in situ* culture fixation and embedding technique developed in our laboratory. A new method of coating the substrate is described.

MATERIALS AND METHODS

Coverslip Preparation

Glass coverslips are cleaned by dipping them in absolute alcohol followed by distilled water and allowing them to dry. The slips are placed on similarly cleaned glass slides in a vacuum evaporation chamber, and a carbon rod is evaporated (a total of about 10 mg of carbon is sufficient to begin with) until the slips are medium gray in color (0.20 to 0.30 on a Kodak Gray Scale or 0.25 optical density as measured on a densitometer). The slips are then coated with silicon monoxide by evaporation of approximately 10 mg in a tungsten wire basket. The silicon coating appears slightly yellow. Adding the silicon monoxide on the carbon undercoat allows greater wetting of the coverslip surface while still preventing the epoxy from adhering too tightly to the glass. Care must be exercised to shield other electrodes in the chamber when evaporating the silicon monoxide because the silicon coats other electrodes as well. Subsequent use of the silicon-coated electrodes to evaporate carbon will result in both carbon and silicon monoxide evaporating together, which will interfere with conduction if specimens are used for scanning electron microscopy. It may be necessary, therefore, to evaporate the carbon, open the chamber, cover the carbon electrodes with aluminun foil, close the chamber, and evaporate the silicon monoxide from the wire basket on the second pair of electrodes. The coverslips are then boiled in distilled water for several minutes for further cleaning. They can be stored between sheets of soft tissue and sterilized before use.

Coated coverslips are placed, coated surface up, in petri dishes, and cells with medium are innoculated into the dish. For roller-tube use, explants are placed on the coated surface of the coverslip in the usual manner except that care must be taken not to scratch the surface of the coverslip to damage the coating.

Fixation and Dehydration

The fixative employed is of personal choice. All types that we have tried seem to work well (see Discussion). Some that we use routinely include 2.5% glutaraldehyde in 0.12M phosphate buffer (pH 7.4, 1% paraformaldehyde and 1.5% glutaraldehyde in 12.5M phosphate buffer, and 0.8% glutaraldehyde — 0.7% OsO_4 (mixed together just before use and place on ice) in 0.12M phosphate buffer. All processing solutions including the epoxy should be prepared and kept close at hand because solution changes are rapid. The coverslips with culture specimens are transferred to petri dishes as near the size of the slips as possible. Culture medium is added to prevent drying of the specimens. The medium is poured off and replaced with room-temperature fixative (except aldehyde-OsO_4 mixtures, which should be used cold) for 10 min. The fixative is decanted and replaced with 2% OsO_4 in the same buffer as in the aldehyde fixative. Dishes are placed on ice for 10 min. Subsequent steps through 95% ethanol can also be done on ice. If "en bloc" staining is performed (see Discussion), rinse with maleate buffer (pH 5.2) twice for 5 min each, and stain with maleate-buffered uranyl acetate for 15 min. Dehydrate with 70% ethanol, 95% ethanol, and absolute ethanol for 5 min each (two changes of absolute ethanol). If no en bloc staining is done, rinse

osmicated specimens twice for 5 min each with 0.9% saline before dehydration. In all cases, the coverslips should be lifted with forceps after each solution change to prevent previous solutions from being trapped underneath. New solutions should be added quickly when old solutions are poured off, especially in the alcohol series, to prevent drying of the cells.

Embedding

The absolute ethanol is replaced with 50% ethanol–50% epoxy (Epon-Araldite) for 5 min. This solution is poured off and replaced with 100% epoxy (two changes, 10 min each). The dishes can be placed in a 60° C oven for a few minutes during the 100% epoxy changes to assist in evaporating residual ethanol. The dishes should be swirled occasionally to facilitate impregnation. The second change of epoxy is poured off, and fresh epoxy is added so that the slips are covered by a layer 3 mm high. Beem capsules with the pointed ends cut off are inverted over the specimens. The dishes are then placed in a 60° C oven for several hours until the epoxy is tacky; the capsules are then filled to the top with more epoxy and allowed to polymerize for 3 days at 60° C.

Removal

After 3 days of polymerization, the dishes are allowed to cool for several minutes. The capsule is grasped with a pair of pliers, and, with a twisting motion (as in using a screwdriver), the capsule is pulled free. Using a dissecting microscope and reflected light, appropriate areas can be trimmed and thin-sectioned. Cells will be in the first

sections that come off of the knife so care must be exercised in aligning the block face with the knife edge.

Thin sections for electron microscopy (600 Å) are first stained with 2% aqueous uranyl acetate for 10 to 15 min followed by three water rinses and then stained in lead citrate for 1 to 5 min followed by three water rinses.

RESULTS

Examples of specimens fixed with 2.5% glutaraldehyde in 0.12M phosphate buffer and embedded *in situ* as described above are shown in Figs. 1 through 4. These electron micrographs were taken on a Philips 300 electron microscope and printed on AGFA Brovira #2 paper. No major disruption of cell plasmalemma (as would be caused by scraping and centrifugation) is found. Cell-to-cell contacts are readily observable, and microvilli (Figs. 1 through 3) as well as cytoplasmic extensions lying between cells (Fig. 3) are easily studied. High magnification allows visualization of the trilaminar arrangement of unit membranes (Fig. 4) when uranyl acetate staining is used before dehydration.

DISCUSSION

Each of the three basic steps of *in vitro* tissue preparation (fixation, dehydration, and embedding) has special problems unlike those of *in vivo* preparations. When whole organs or slices of organs are fixed by immersion, the rate of penetration is critical. Glutaraldehyde is a

superior fixative, but it penetrates more slowly than formaldehyde. Many laboratories therefore use a combination of glutaraldehyde (prepared from paraformaldehyde) for immersion fixation of biopsy or autopsy specimens and perfuse the fixative when possible for experimental animals. The autolysis incurred with immersion fixation is showed if the tissues are fixed cold. Some cytoplasmic structures such as microtubules may, however, be affected by cold fixation. For *in vitro* work, the penetration rate is not as critical because specimens are usually quite thin (6 to 20 μ layers of cells, for example) and the fixative can reach all of the cells in a matter of seconds. For suspension cultures, again, all cells are quickly exposed to the fixative by adding one volume of double-strength aldehydes in normal-strength buffer to an equal volume of cell suspension before centrifuging the cells into a pellet. Therefore, room-temperature fixatives containing only glutaraldehyde work very well. Indeed, we have noticed very little difference in cell preservation using a wide variety of fixative combinations and strengths. In our experience, "vacuolated" (swollen) mitochondria appear to depend on the cell type rather than on the fixative. Of the cell types tested (see Acknowledgments) using the same fixative (2.5% glutaraldehyde in 0.12M phosphate buffer), BSC-1 kidney cells have only a few swollen mitochondria, neutroblastoma cells have many, and A-9 fibroblasts have a moderate amount. This variation is probably a reflection of the metabolic activity of the particular cell type and possibly a cell-specific reaction to the fixative.

As an alternative to fixing and embedding *in situ*, some investigators scrape the cells off the bottom of the petri dish before or after primary fixation with aldehydes. The cells are usually centrifuged into a pellet for further processing. When we used this technique, monolayer cell cultures were processed to the point where dehydration would begin on the next step. (If any concentration of alcohol is added to the petri dish, cell removal is difficult.) The cells were then scraped off with a piece of rubber tubing, centrifuged, dehydrated, and embedded by transferring the pellet to a Beem capsule. By fixing the cells completely before scraping them off the petri dish, mechanical damage was kept to a minimum, and some cell-to-cell contacts remained although many were destroyed. By contrast, *in situ* fixation and embedding allows maximum tissue preservation, and examination of cell-to-cell contacts is very straightforward.

Before or during dehydration, some investigators use uranyl acetate staining (sometimes referred to as "en bloc") to enhance the trilaminar arrangement of cell membranes. The staining technique that has consistently provided the best results in our laboratory is the maleate-buffered uranyl acetate method described by Karnovsky (1967). Our formula modification of this technique is described in the Appendix. Although en bloc staining is necessary only when studying cell membranes is a focus of the experiment, we routinely use the technique for all specimens because contrast is improved.

When dehydrating *in vivo* specimens for electron microscopy, acetone or propylene oxide is often used as a transitional solvent between the alcohol and the epoxy. Acetone and propylene oxide will, however, dissolve plastic petri dishes. For *in vitro* work, processing from ethanol to

50:50 ethanol-epoxy and then to 100% epoxy works well because the specimen is thin enough to allow the ethanol to evaporate when placed in the oven for polymerization.

The embedding material presents an interesting problem. Many of the low-viscosity plastics that we have tested appear to dissolve or soften the walls of the petri dish, producing a milky appearance in parts of the epoxy. The hardness of the embedded block is important because *in situ* preparations yield only a thin sheet of tissue for sectioning. Therefore, thin sectioning or thick sectioning for light microscopy must be easy to perform from the very surface of the block. Epon and Araldite have consistently given excellent results in our preparations. They do not substantially dissolve the surface of the petri dish, and specimens readily snap free of the carbon-silicon-monoxide-coated coverslips. These plastics also stain in a superior manner for light microscopy (toluidine blue).

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APPENDIX

Stock concentrated buffer (0.48M phosphate; pH 7.4):

16.8 gm K_2HPO_4 (anhydrous)

3.2 gm $NaH_2PO_4 \cdot H_2O$

distilled H_2O to make 250 ml

Fixative (2.5% glutaraldehyde in 0.12M phosphate buffer; pH 7.4):

50 ml 0.48M stock phosphate buffer

10 ml 50% ultrapure glutaraldehyde (Tousimis Research Corp., Rockville, Maryland)

140 ml distilled H_2O

Osmium tetroxide fixative (2% OsO_4 in 0.12M phosphate buffer; pH 7.4):

25 ml 0.48M stock phosphate buffer

50 ml 4% OsO_4

25 ml distilled H_2O

Maleate buffer (pH 5.2) with 1% uranyl acetate:

Add 1.74 gm maleic acid to 100 ml distilled H_2O. Adjust pH to 5.2 with 5N NaOH. Dilute to 300 ml with distilled H_2O. Set aside 200 ml for rinsing cells after osmication. Add 1 gm uranyl acetate to the remaining 100 ml and sonicate until dissolved. The pH of the maleate-buffered uranyl acetate will be approximately 4.5.

Epon-Araldite embedding formula:

30 gm Epon-812

10 gm Araldite-502

18 gm NMA

18 gm DDSA

1 gm DMP-30

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FIGURE LEGENDS

Fig. 1. These two cells are in close contact along a large part of their boundaries (solid arrows). At certain points (hollow arrows), dilated gaps exist between the cells. Microvilli are found in these gaps (A-9 fibroblasts, × 20,550).

Fig. 2. At higher magnifications, microvilli (arrows) lying in intercellular gaps can be studied more closely. The trilaminar arrangement of unit membranes is not quite discernible at this magnification in micrographs reproduced by half-tone. (A-9 fibroblasts, × 48,000).

Fig. 3. Between these two cells (C_1 and C_2) are found two cytoplasmic processes (P_a and P_b). Microvilli (arrows) can be observed lying between the cells and processes. If this specimen had been scraped and centrifuged into a pellet, interpretation of microvilli interrelationships would have been difficult. (A-9 fibroblasts, × 30,780).

Fig. 4. Using uranyl acetate staining before dehydration as described, the trilaminar arrangement of unit membranes is apparent (arrows) in such structures as mitochondria (M) and granular bodies (G). (A-9 fibroblasts, × 120,000).

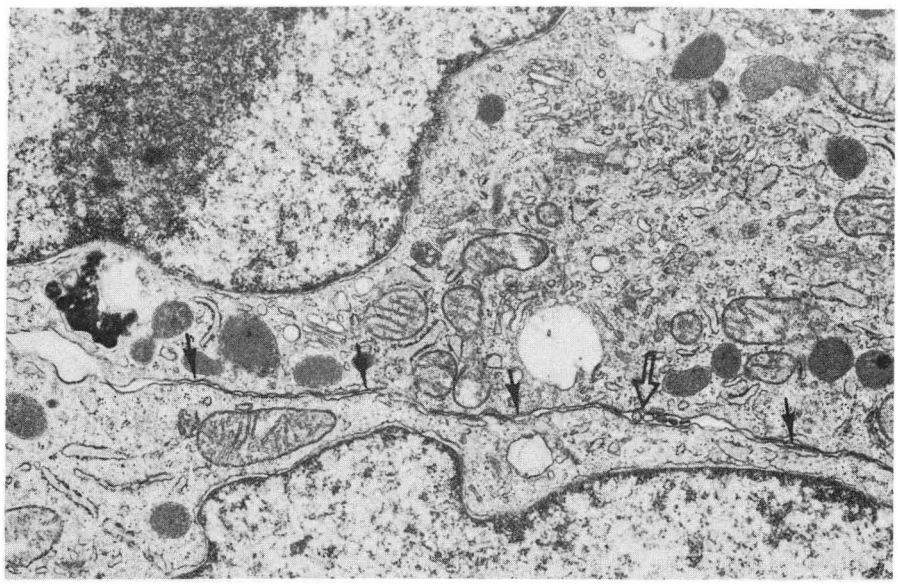

Fig. 1

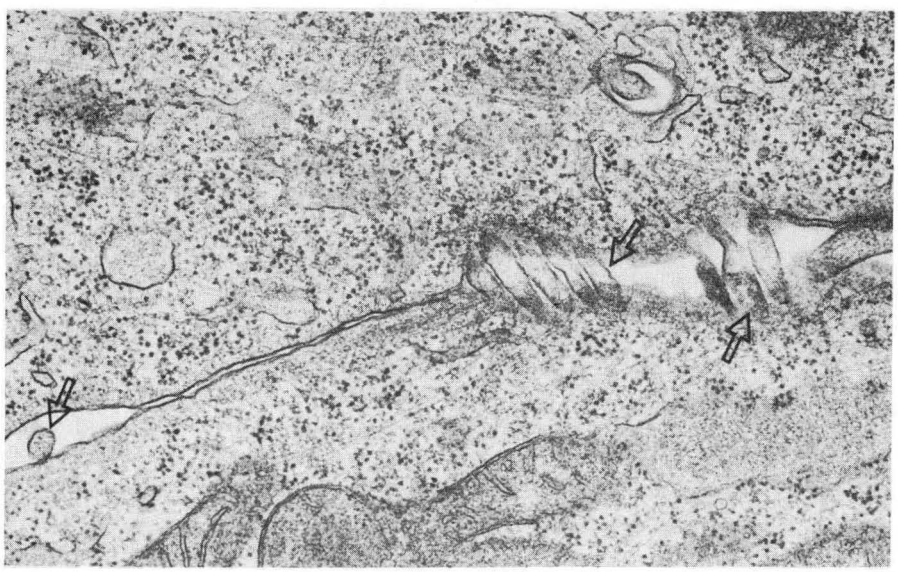

Fig. 2

XBB 776-5739

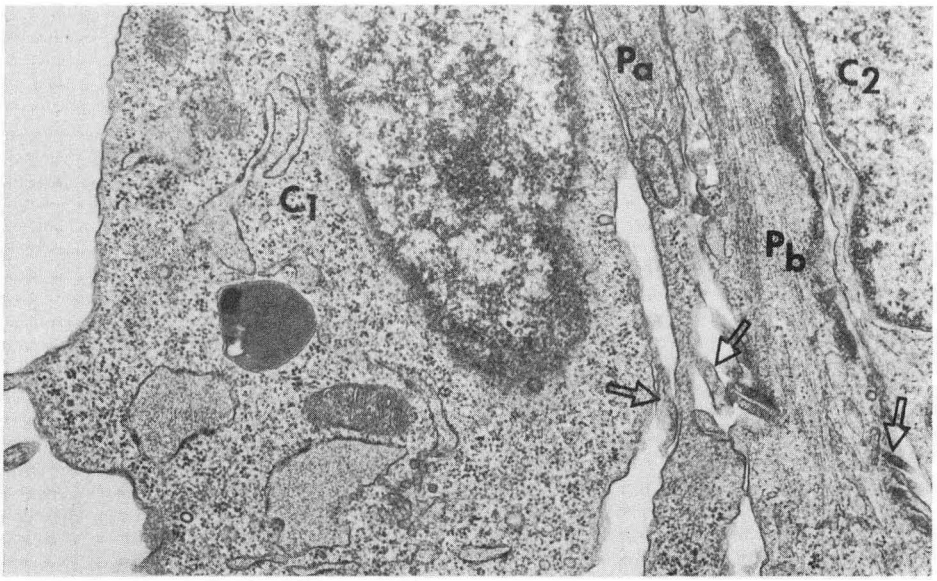

Fig. 3

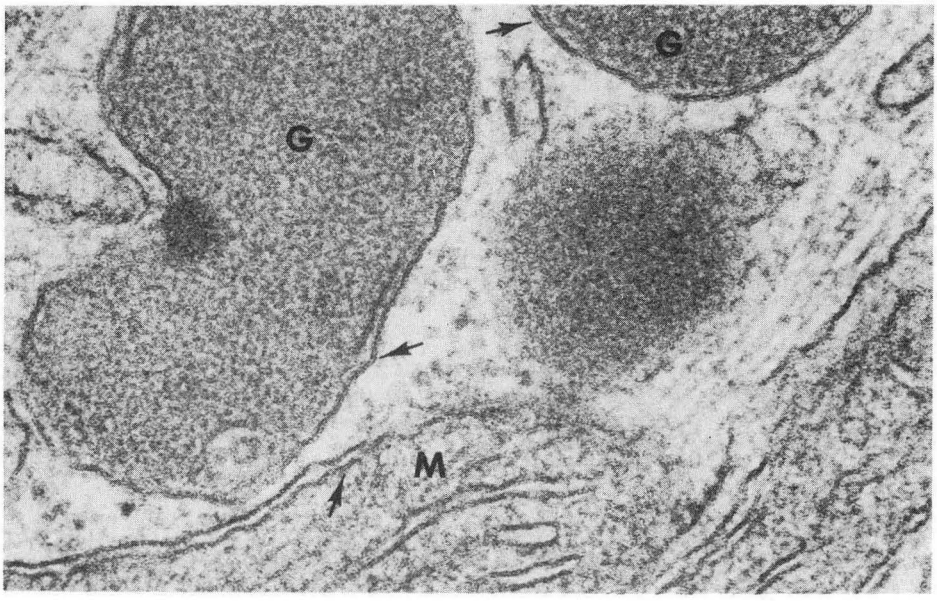

Fig. 4

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