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An improved procedure for immunoelectron microscopy: Ultrathin plastic embedding of immunolabeled ultrathin frozen sections

(cryo-ultramicrotomy/osmium fixation/colloidal gold immunolabeling)

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ABSTRACT **Ultrathin frozen sections are ideal substrates with which to carry out immunolabeling experiments in electron microscopy. However, the ultrastructural delineation in positively stained frozen sections has not been as detailed as in conventionally osmium-stained and plastic-embedded sections. We now describe a simple technique in which immunolabeled ultrathin frozen sections are subsequently treated with osmium tetroxide, dehydrated, and then embedded in plastic by impregnation with a monomer to the thickness of the section, followed by polymerization of the monomer. By this technique ultrastructural definition as good as that of conventional plastic sections is achieved, while the high density and specificity of immunolabeling characteristic of ultrathin frozen sections are retained.**

In previous work from this laboratory (1–5), we have developed techniques for the preparation of ultrathin frozen sections of fixed biological specimens for high-resolution immunolabeling experiments in electron microscopy. The immunolabeling of ultrathin frozen sections has many advantages over other procedures of immunocytochemistry: (*i*) the immunolabeling reagents have access to any antigen exposed by the sectioning; (*ii*) such access is not reduced by the presence of a polymeric embedding agent filling the spaces in the section; (*iii*) the ultrastructure of the specimen as well as the antibody-binding capacity of its antigens are maximally retained by the chemically mild procedure for specimen preparation; (*iv*) the level of nonspecific labeling is low compared to that in conventional plastic sections used as substrates; and (*v*) immunolabeling and high-resolution localization of two or more components on the same specimen can be carried out (6, 7). However, a disadvantage has been that the ultrastructural delineation in the ultrathin frozen sections after positive staining with heavy metal ions (3) has not been as detailed as is routinely obtained with conventional sections prepared by osmium fixation and plastic embedding. Treatment of ultrathin frozen sections with osmium fixatives does not produce characteristic osmium staining, because, as is well known, reduction of osmate ion does not occur in aqueous solutions but only during the process of replacement of the aqueous medium by organic solvents for conventional plastic embedding.

We now have devised an immunolabeling technique that combines the best features of ultrathin frozen sections and of osmium-stained plastic sections. In the first part of this technique, ultrathin frozen sections of glutaraldehyde-fixed (or similarly fixed) specimens are prepared, mounted on electron microscope grids, and immunolabeled as in our previous procedures (3, 4). Colloidal gold immunolabeling reagents are often used because of their large electron density, although in favorable circumstances, ferritin or iron-dextran (6) labels can be employed. In the second part of the technique, the immunolabeled mounted sections are then treated with osmium fixative, and solvent replacement is performed (during which the classical osmium staining occurs). This is followed by impregnation with an appropriate liquid monomer to a thickness corresponding to that of the frozen section. Polymerization of the monomer is then carried out. We refer to this second part of the technique as "ultrathin plastic embedding." These mounted plastic-embedded sections are then directly examined in the electron microscope. By this technique, we show that ultrastructural delineation essentially equivalent to that of conventional plastic sections is achieved, while the high degree of specificity of immunolabeling of ultrathin frozen sections is maintained. Therefore, this technique should be very useful in immunoelectron microscopy.

MATERIALS AND METHODS

Specimen Preparation. For ultrastructural studies, the duodenum and pancreas of Sprague–Dawley rats were dissected, cut into small blocks in 2% glutaraldehyde/0.1 M cacodylate buffer (pH 7.2), immersed in the fixative for 2 hr, and then stored in 0.01 M phosphate/0.15 M NaCl, pH 7.2 (P_i/NaCl) at 4°C until sectioning was to be performed. For immunolabeling experiments, the liver of a fasted rat was fixed by portal perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer/0.1 M lysine/0.1 M Na *m*-periodate, pH 7.2 [PLP fixative (8)]. After 5 min of perfusion, small blocks were cut and immersed in the same fixative for 4 hr at 4°C and then were stored in 0.4% formaldehyde in P_i/NaCl until ready for sectioning.

Cryo-Ultramicrotomy and Immunolabeling. The fixed specimen blocks were infused with 2.3 M sucrose, rapidly frozen, and ultrathin-sectioned in the frozen state as described (1, 3, 4), by using glass knives with a DuPont–Sorvall ultramicrotome MT-2 equipped with the LTC-2 cryoattachment (DuPont Instruments, Sorvall Division). Gold-colored sections were transferred onto Formvar films on 300-mesh electron microscope grids. For immunolabeling of the liver sections, the primary reagent was affinity-purified rabbit antibody to rat serum albumin, used at 10 μg/ml, and the secondary reagent was a colloidal gold adduct of affinity-purified goat antibody to rabbit IgG. Colloidal gold particles of 6- to 8-nm diameter were prepared by treatment of 0.005% $AuCl_3$ in water adjusted to pH 7–8 with 0.2 M K_2CO_3, with increments of freshly prepared 0.01 M Na borohydride (unpublished data). The adduct was made by adding the goat antibody in 10 mM Tris buffer (pH 8.5) to the colloidal gold solution at a final concentration of 50 μg/ml (9).

Osmium Fixation and Ultrathin Plastic Embedding. Ultrathin frozen sections (including those that were immunolabeled) mounted on electron microscope grids were placed face down on drops of 0.5%–2% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.2) on a sheet of Parafilm for 10 min,

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Abbreviation: PLP fixative, 4% paraformaldehyde/0.1 M phosphate buffer/0.1 M lysine/0.1 M Na *m*-periodate, pH 7.2.

then washed on drops of 7% sucrose, and post-stained for 20 min on a drop of 0.5% uranyl acetate in barbital/acetate buffer, pH 5.2 (10), containing 5% sucrose. The grids with mounted sections were then placed in small wells, dehydrated by immersion for 2 min each through a series of aqueous ethanol solutions from 40% to 95% and then in 100% ethanol, and finally infused either with 2% Epon 812 in ethanol or with LR White acrylic resin (London Resin, London). After infusion, the grids were blotted between two disks of Whatman no. 50 hardened filter paper to remove excess monomer. (Undiluted Epon is too viscous to make a uniform film after blotting.) Polymerization was effected overnight in a vacuum oven at 60°C. After hardening, the specimens were sometimes post-stained with 2% aqueous uranyl acetate and 0.04% alkaline bismuth subnitrate (11). The grids were examined in a Philips Model 300 transmission electron microscope at 80 kV.

RESULTS

Representative electron micrographs of ultrathin sections of rat pancreas and intestine are shown in Figs. 1 and 2, respectively. These sections were prepared by ultrathin sectioning of frozen glutaraldehyde-fixed tissues, followed by osmium fixation and ultrathin plastic embedment in LR white acrylic resin. These micrographs are intended to show the ultrastructural delineation that can be achieved by this technique, and immunolabeling of these sections was not carried out. The detail in these micrographs is very similar to that observed in conventionally prepared plastic sections. In particular the rough endoplasmic reticulum, mitochondrial cristae, Golgi saccules and contents, and zymogen granules in the pancreatic acinar cell (Fig. 1) are well preserved features, and coated vesicles can be discerned (arrows, Fig. 1). In the intestinal epithelial cell (Fig. 2), the junctional elements in the brush border are well visualized, as are the actin filaments within and extending inward from the microvilli.

Although glutaraldehyde is the most widely used fixative for ultrastructural preservation of specimens, it inactivates a significant number of protein antigens (4). PLP is a fixative that has been recommended (8) for immunolabeling experiments where glutaraldehyde is unsatisfactory. PLP fixation was tested in the course of a series of immunolabeling experiments with rat liver. In Fig. 3 is shown an ultrathin frozen section of the PLP-fixed tissue that was immunolabeled with colloidal gold for serum albumin, then osmium-fixed, and ultrathin-plastic-embedded in Epon. These are not necessarily the optimal conditions for the immunolabeling of albumin, but Fig. 3 illustrates that the gold labels are easily discerned in the Golgi apparatus and associated vesicular structures as well as in the rough endoplasmic reticulum. Little or no nonspecific labeling is observed over mitochondria or the nucleus. At the same time, the ultrastructure is at least as well preserved as in liver tissue that was conventionally PLP-fixed, post-fixed with osmium tetroxide, and embedded in plastic (8, 12). Elements of the endoplasmic reticulum, Golgi apparatus, and mitochondrial cristae are well preserved and visible.

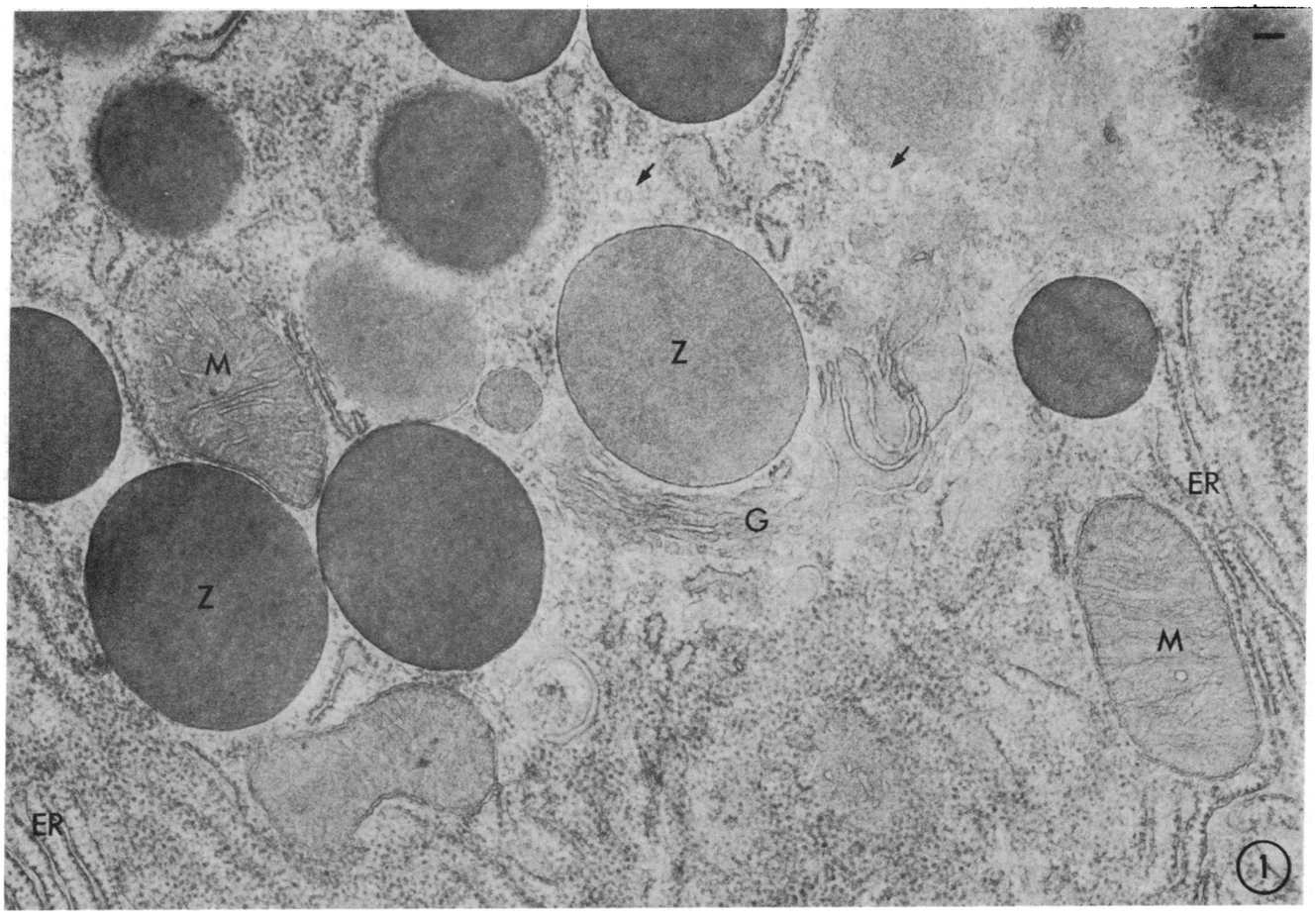

FIG. 1. Frozen section of glutaraldehyde-fixed rat pancreatic exocrine cell treated with osmium tetroxide, stained with uranyl acetate, dehydrated in ethanol, embedded in an ultrathin layer of LR White acrylic resin, and, after the polymerization of the resin, stained with alkaline bismuth acetate. As a whole, the ultrastructure appears very similar to that of conventional plastic-embedded sections of the pancreas. G, Golgi apparatus; ER, endoplasmic reticulum; M, mitochondria; Z, zymogen granules. Arrows show coated vesicles. (Bar = 0.1 μm.) (×38,000.)

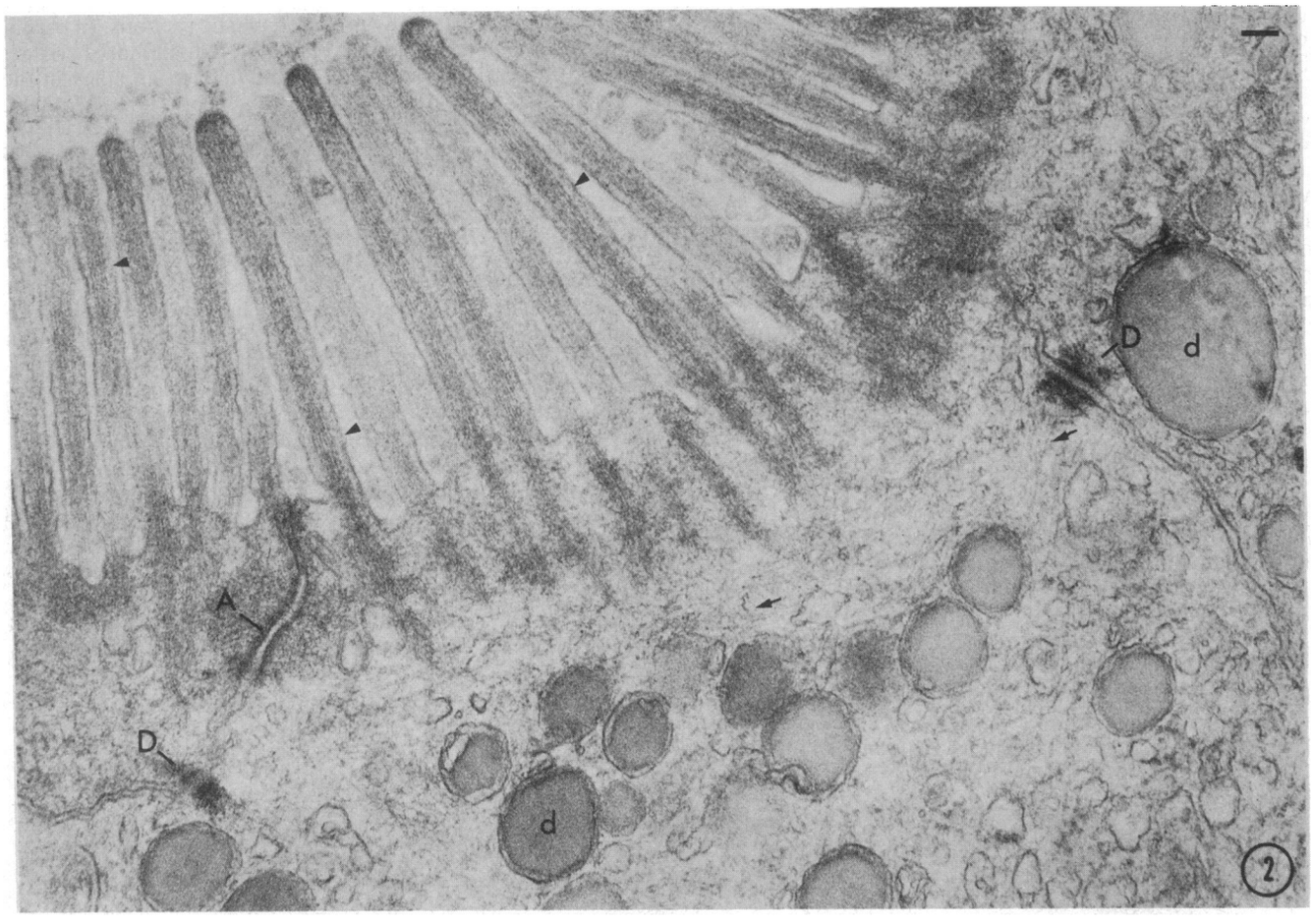

FIG. 2. Frozen section of glutaraldehyde-fixed rat intestinal epithelial cell treated with osmium tetroxide, dehydrated in ethanol, embedded in an ultrathin layer of LR White acrylic resin, and, after the polymerization of the resin, stained with uranyl acetate and alkaline bismuth subnitrate. As a whole, it resembles conventional plastic-embedded sections of the epithelial cell. A, zonula adherens; D, desmosomes; d, lipid droplets. Arrowheads show actin filaments in microvilli, and arrows show intermediate filaments. (Bar = 0.1 μm.) (×47,000.)

DISCUSSION

The technique of ultrathin plastic embedding of immunolabeled ultrathin frozen sections that is described in this paper has evolved after examining a number of variables of fixation, staining, and the nature of the monomer used. These variables will only be discussed briefly here.

The conditions used for the initial fixation of the specimen, before the preparation of ultrathin frozen sections, are determined by the nature of the antigen and requirements for effective immunolabeling, as has been thoroughly analyzed elsewhere (4). Two different initial fixation procedures are illustrated in this article, glutaraldehyde and PLP. The former gives better ultrastructural preservation, and the latter gives better retention of antigenic activities (8). Both fixation procedures were found to allow satisfactory ultrastructural preservation and delineation upon subsequent treatment with osmium tetroxide and ultrathin plastic embedding.

With respect to staining of the specimen, treatment with 0.5% osmium tetroxide for 10 min, followed by 0.5% uranyl acetate for 20 min imparted adequate contrast to the specimen upon subsequent dehydration and embedding. On such specimens small colloidal gold particles used as immunolabels could readily be detected (Fig. 3). Ferritin immunolabels, however, were difficult to discern within the matrix of the specimens (not shown).

Several monomers were examined for the suitability of their polymers as ultrathin plastic embedding agents. Ultrathin frozen sections were osmium-stained, dehydrated, and impregnated with Lowicryl HM-20 (13), SPURR epoxy resin, Epon 812, or LR White acrylic resin. Lowicryl HM-20 gave poor contrast even after post-embedment staining with uranyl and lead salts. Undiluted SPURR and Epon 812 did not give uniform plastic-embedded sections after polymerization, probably due to the large viscosities of the monomer preparations. Solutions (10%) of SPURR in ethanol yielded uniform plastic-embedded sections, but severe shrinkage distortions were observed in the specimens. Satisfactory results were obtained with either 2–10% solutions of Epon 812 in ethanol (Fig. 3) or with undiluted LR White acrylic resin (Figs. 1 and 2).

The technical advance that is described in this paper is an important one for immunocytochemistry. In our previous studies with ultrathin frozen sections, the contrast provided by osmium staining could not be realized, and positive staining was kept low in order to discern ferritin and iron-dextran immunolabels (14, 15). This may still be the technique of choice in circumstances where a high density of immunolabeling is of primary concern, because, in our hands using a variety of different procedures, we have not been able to achieve as high a density of labeling with colloidal gold adducts as with ferritin on the same specimens. However, by the method of ultrathin plastic embedding and the use of colloidal gold immunolabeling reagents, the high degree of specificity of immunolabeling characteristic of ultrathin frozen sections is achieved, while the detailed ultrastructural delineation of conventional osmium staining and plastic em-

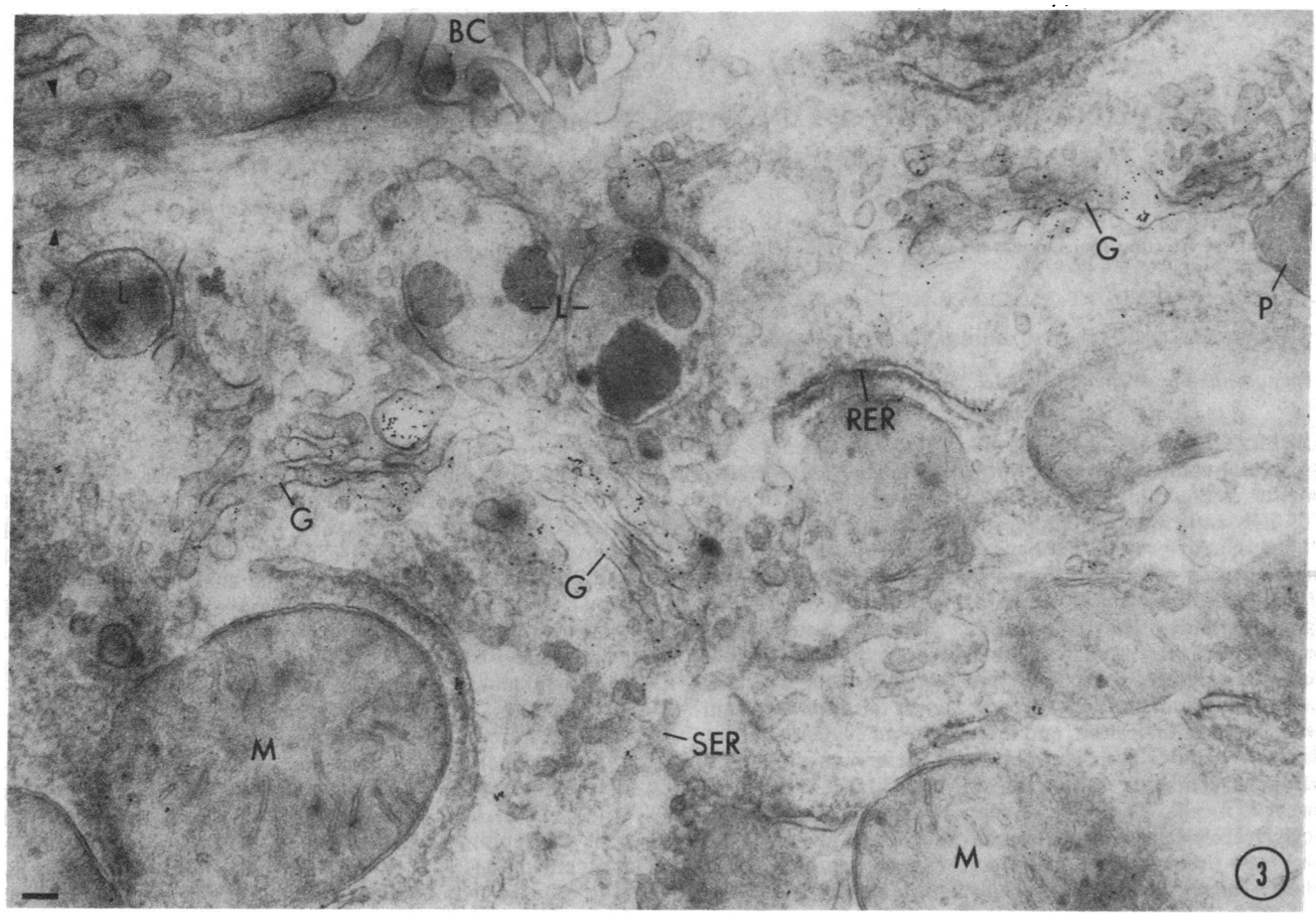

FIG. 3. Frozen section of rat liver fixed by the PLP method. It was immunostained first with rabbit anti-rat serum albumin and then with goat anti-rabbit IgG conjugated to 6- to 8-nm gold particles. Subsequently, the section was fixed with glutaraldehyde, treated with osmium tetroxide, stained with uranyl acetate, and embedded in 2% Epon 812 in ethanol. After the grid was left in air and ethanol was removed from the mixture, the ultrathin layer of the plastic was polymerized. Gold particles are readily recognizable in the cisternae of the Golgi apparatuses (G) as well as in some areas of endoplasmic reticulum (RER, SER). M, mitochondria; L, lysosomes; P, peroxisome; BC, bile canaliculus. Arrowheads show actin filaments in the subcortical areas. (Bar = 0.1 μm.) (×45,000.)

bedding is attained. Therefore, this procedure provides a powerful new method for high-resolution immunoelectron microscopy.

The procedures we are constrained to use here do not do full justice to the originals submitted.

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1. Tokuyasu, K. T. (1973) *J. Cell Biol.* **57,** 551–565.
2. Tokuyasu, K. T. & Singer, S. J. (1976) *J. Cell Biol.* **71,** 894–906.
3. Tokuyasu, K. T. (1980) *Histochem. J.* **12,** 381–403.
4. Singer, S. J., Tokuyasu, K. T., Dutton, A. H. & Chen, W.-T. (1982) in *Electron Microscopy in Biology*, ed. Griffith, J. D. (Wiley, New York), Vol. 2, pp. 55–106.
5. Tokuyasu, K. T. (1983) *J. Cell Biol.* **97,** 562–565.
6. Dutton, A. H., Tokuyasu, K. T. & Singer, S. J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 3392–3396.
7. Geuze, H. J., Slot, J. W., Van der Ley, P. A. & Scheffer, R. C. T. (1981) *J. Cell Biol.* **89,** 653–665.
8. McLean, I. W. & Nakane, P. K. (1974) *J. Histochem. Cytochem.* **22,** 1077–1083.
9. DeMey, J. (1983) in *Immunocytochemistry: Practical Applications in Pathology and Biology*, eds. Polak, J. M. & Van Noorden, S. (John Wright, Bristol, England), pp. 82–112.
10. Kellenberger, E., Ryter, A. & Sechaud, J. (1958) *J. Biophys. Biochem. Cytol.* **4,** 671–678.
11. Riva, A. (1974) *J. Microscopie* **19,** 105–108.
12. Novikoff, P. M., La Russo, N. F., Novikoff, A. B., Stockert, R. J., Yam, A. & LeSage, G. D. (1983) *J. Cell Biol.* **97,** 1559–1565.
13. Carlemalm, E., Garavito, R. M. & Villiger, W. (1982) *J. Microscopy (Oxford)* **126,** 123–143.
14. Geiger, B., Dutton, A. H., Tokuyasu, K. T. & Singer, S. J. (1981) *J. Cell Biol.* **91,** 614–628.
15. Tokuyasu, K. T., Dutton, A. H., Geiger, B. & Singer, S. J. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 7619–7623.