A SIMPLIFIED METHOD FOR MASS PRODUCTION OF ELECTRON MICROSCOPE SPECIMENS FOR HISTOCHEMISTRY

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A simplified method for mass production of electron microscopic specimens for histochemistry without using any special instrument is introduced. This is also useful for a very small amounts of tissues. The method prevents the loss of thick sections during the treatment and it does not produce any nonspecific staining. The wide future application is expected.

In applying electron microscopic enzyme histochemical techniques for various organs, it is generally accepted that the diced block incubation causes the concentration difference of the incubation medium and produces reaction artifacts (10). When metal salts are used as capturing reagents, such possibility would be heightened, especially when lead salt is used severe artifacts are produced (5). In the study of alkaline phosphatase activity of the kidney convoluted tubules, for example, if kidney tissues are incubated as diced blocks, the reaction products are observed on the microvilli of the proximal convoluted tubules at the superficial layer, on the microvilli and on the basal infolding of the proximal convoluted tubules at the second layer, only on the basal infolding at the third layer, while no reaction products are found anywhere at the innermost layer. Such a phenomenon was first interpreted as the result of the concentration gradient of the fixatives which penetrated from the surface of the diced blocks (12). Later it became apparent that this was not the result of the concentration gradient of the fixative, but was attributable to the procedure of diced block incubation (6, 11). At the present time, it is maintained that the real localization of the alkaline phosphatase activity in the kidney convoluted tubules is limited at the microvilli, whereas the reaction products on the other cell organellae are regarded as artifacts. Accordingly, the routine electron microscopic histochemical techniques generally employed are to cut non-frozen or frozen sections from unfixed or fixed tissues, using vibratome (1, 13), tissue sectioner (14), automicrochopper (10), cryostat (17), or freezing microtome (8), and to incubate them in the incubation media at certain temperatures for certain intervals and to postfix with osmium tetroxide, to dehydrate through graded series of alcohol and propylene oxide, to embed in Epon, to cut ultrathin sections, and to observe with or without electron staining.

In preparing specimens for electron microscopic histochemistry, the high viscosity of the epoxy resin is very troublesome. Though the epoxy resin has such

FIG. 1. Schematic drawing of the incubation, postfixation, dehydration, penetration and embedding procedures. Details in the text.

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- FIG. 2. Photographs of several glass tubes covered with a sheet of lens paper and tied with a suture respectively.
- FIG. 3. The method of picking up thick sections by pressing lens paper attached to the glass tube to the edge of the microtome knife.
- FIG. 4. Thick sections are sandwiched between two sheets of lens paper by covering with a new sheet on the sections.
- FIG. 5. Thick sections were incubated in a beaker containing a histochemical medium kept in a thermostat bath.
- FIG. 6. Postfixation, dehydration and penetration are carried out in the same container.
- FIG. 7. Photograph showing the procedure of epoxy resin infiltration. Mélangeur shaker is used to promote resin penetration by oscillating the glass tubes up and down in the Epon mixture.
- FIG. 8. Photograph showing the embedding procedure. Sandwiched tissues are taken out by opening the outer sheet of lens paper and are embedded into silicone rubber mould.
- FIG. 9. Light photomicrograph of a sheet of Nikon lens paper. Many fibers are mingling together. It is apparent that through these pores, fixatives, incubation medium, dehydration solvents and embedding media can pass freely into sandwiched tissues.
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advantages that it is resistant for electron beam irradiation, it keeps the fine structure of cells and tissues and it gives the dignified impression for tissues embedded with it. It has such disadvantages that 30–40 μ thick sections for histochemical reaction do not sink at the bottom of vessels during the penetration of sections with propylene oxide and epoxy resin mixture or epoxy resin alone, and some thick sections come up to the surface of Epon and are discarded, so that repeated centrifugations are required to prevent floating. Also it is a well-known fact that a simple centrifugation of low speed does not improve the percentage of loss of thick sections. In order to save such a trouble, a special carrier which is covered with fine metal meshes at the bottom is devised (16). This paper deals with a simplified method for mass production of electron microscopic specimens for histochemistry by using such simple materials as commercially available lens papers and glass tubes to prevent thick sections from losing during dehydration and penetration.

MATERIALS AND METHODS

One end of a glass tube with a diameter of 10 mm and a length of 15 cm is covered with a sheet of lens paper (Nikon Opt. Co.) 3 cm $\times$ 3 cm in size. The lens paper is fixed by tying with a suture (No. 2–No. 5) around the glass tube (Figs. 1 and 2). Thick sections cut with a freezing microtome are picked up onto the lens paper by touching the end of glass tube on the blade surface (Figs. 1 and 3). Another sheet of lens paper is placed on the sections, thus the specimens are sandwiched between the two lens papers (Figs. 1 and 4). The glass tube is then ready to be processed through routine histochemical incubation, washing, dehydration and penetration. In this way, many glass tubes belonging to several experimental groups can be bounded with a rubber band and placed in a same incubating medium (Figs. 1 and 5). For example, 50 ml of incubating medium is enough for about 25 different experimental groups. Incubating medium can pass freely through the lens papers. The procedures of postfixation, dehydration and penetration can be processed in the same beaker by changing the content (Fig. 6). Penetration of resin is promoted by connecting the bundle of glass tubes to the stalk of Mélangeur

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- FIG. 10. Electron photomicrograph of the peroxidase activity of a mitotic guinea pig heterophilic leukocyte. Reaction products are localized in the primary granules. Pseudoperoxidase activity of the immature erythrocyte is also observed. Unstained section. $\times 9,200$
- FIG. 11. Electron photomicrograph of the peroxidase activity of guinea pig heterophilic leukocytes (h) and eosinophilic leukocyte (e). Reaction products are localized in the primary granules of the heterophilic leukocyte and in the eosinophilic leukocyte; reaction products are confined to the granule matrix, whereas central cores are free of reaction products and gives an opposite image as compared with normal eosinophilic granules. Unstained section. $\times 8,000$
- FIG. 12. Electron photomicrograph of the acid phosphatase activity of a guinea pig myeloblast. Reaction products are localized at Golgi lamellae. Doubly stained with uranyl acetate and lead citrate. $\times 22,000$
- FIG. 13. Electron photomicrograph of the acid phosphatase activity of rabbit heterophilic leukocyte. Reaction products are observed in the primary granules. Doubly stained with uranyl acetate and lead citrate. $\times 36,000$

shaker (Fig. 7) and the specimen containing glass tubes oscillate up and down in the beaker. After the penetration, the edge of the outer lens paper of the glass tube end is cut with a razor blade (Fig. 1) and the sections are freed into the Epon mixture. Thus, they are embedded in gelatin capsules or silicone rubber moulds (Fig. 8). If thick epoxy resin mixture does not penetrate into lens paper by clogging the mesh after a long time of penetration, the outer lens paper should be changed with a new one. The mesh of a lens paper is shown in Fig. 9.

RESULTS

Some electron photomicrographs taken from specimens processed through this method are demonstrated. These are peroxidase activity of the guinea pig heterophilic leukocytes and eosinophilic leukocyte by Graham and Karnovsky (Figs. 10–11) (4), acid phosphatase activity of the guinea pig myeloblast (Fig. 12) and rabbit heterophilic leukocyte (Fig. 13) by Barka and Anderson (2), thiamine pyrophosphatase activity of the guinea pig heterophilic leukocyte by Novikoff and Goldfischer (Fig. 14) (9), arylsulfatase B activity of the rabbit blood platelet by Goldfischer (Fig. 15) (3) and dialyzed iron staining of human lymphocytes and erythrocyte by Wetzel et al. (Figs. 16–17) (18). The results are quite satisfactory in the view of electron microscopic histochemistry.

DISCUSSION

The procedure reported here is a simplified method for histochemistry devised by the present authors and requires neither special instruments nor facilities. It is a very convenient method handling many kinds of experimental groups at once. It is also a very excellent method for a small amount of tissues preventing the loss of specimens during preparation procedures. At the present time, for the demonstration of various enzymes in the electron microscopic level, block incubation is not a recommending method from the view point of preventing the removal of nonspecific staining (6, 11). Thus the incubation in a form of thick sections is essential. However, the incubation of thick sections has such a disadvantage that it causes the loss of sections during dehydration and penetration. Furthermore, epoxy resin mixture used for the embedding medium has a high viscosity, which acts disadvantageously for keeping the sections during the preparation procedures. A low speed centrifuga-

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- FIG. 14. Electron photomicrograph of the thiamine pyrophosphatase activity of a guinea pig heterophilic leukocyte. Reaction products are localized at Golgi region. Doubly stained with uranyl acetate and lead citrate. $\times 20,000$
- FIG. 15. Electron photomicrograph of the arylsulfatase B activity of a rabbit blood platelet. Reaction products are observed at the rims of the azurophil granules. Unstained section. $\times 45,000$
- FIG. 16. Electron photomicrograph of dialyzed iron staining of a human lymphocyte (1) and an erythrocyte (e). Reaction products are observed at cell surfaces of these cells. Unstained section. $\times 30,000$
- FIG. 17. Electron microphotograph of dialyzed iron staining of a humal lymphocyte. Reaction products are confined to the cell surface and cytoplasmic vacuoles. Unstained section. $\times 36,000$

tion of epoxy resin mixtures containing sections can not prevent the loss of specimens. The one of the main purpose devising this procedure is to prevent the loss of thick sections during the preparation process. Organic solvents such as ethanol and acetone which are used as a routine procedure do not break, nor melt the lens papers. Incubation media, organic solvents, fixatives and embedding resin mixture can pass freely through coarse meshes of the lens papers. Accidentally, after a long time penetration with epoxy resin, the surfaces of a few lens papers were covered with sticky epoxy resin, which caused the blockage of the penetration of resin through the lens paper mesh. In such cases, covered lens paper should be replaced with a new one. In aforementioned conditions, the insufficiency of the penetration of the resin has not been encountered in this laboratory after many times experiments. The results obtained using this method are satisfactory enough in the view of electron microscopic histochemistry. Another method to prevent the loss of free thick sections is to use the low viscosity resin which is resistant to electron beam damage and keeps the cell fine structure well. To fulfil this purpose, low viscosity epoxy resin introduced by Spurr (15) several years ago is worth trying. Not only this resin is low viscous, but is mixed accurately with a gravimetric method and the polymerization time is shorter. Good results are obtained in this laboratory (7).

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