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TO RESIN OR NOT TO RESIN IN IMMUNOCYTOCHEMISTRY

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Abstract

Hydrated resinless sections produced by a variety of methods (cryoultramicrotomy or polyethylene glycol techniques) appear to be excellent specimens for post-embedding immunocytochemistry at the electron microscopic level. A perplexing problem is the lack of apparent penetration throughout the section thickness of particulate probes such as ferritin or colloidal gold. This report draws attention to the possible causes for this phenomenon namely the size of the probe and the possible effects of fixation or processing. Smaller probes, gentler fixation or permeabilization procedures and increased incubation times all seem to be logical approaches to increasing penetration of immunoreagents into thick hydrated sections.

Key Words: Immunocytochemistry, hydrated sections, colloidal gold

The past decade has seen a tremendous increase in the use of immunocytochemical techniques at the electron microscopic level. Perhaps the main reason for this surge of interest has been the introduction of particulate probes (e.g., colloidal gold) that are recognized easily at the electron microscopic level. One needs only to scan the literature to see that the number of papers each year has increased from a scant few in 1974 to over hundreds in 1985.

Other important technological advances also have contributed to the use of immunocytochemistry at the electron microscopic level. These include primarily the development and refinement of methods such as cryoultramicrotomy (1-4), the introduction of protocols designed to preserve better, or to make antigenic sites more accessible to the immunoreagents (5-8).

The two most common approaches for post-embedding immunocytochemistry have been the use of hydrated sections (resinless) or sections of nonextractable resin embedded tissues processed in ways that permit a greater retention of antigenicity (e.g., low temperature dehydration and embedding, 5-8). It seems logical that hydrated, resinless sections of tissues would permit "better" localizations of antigens in all cell compartments than cells embedded in nonextractable resins. Because the matrices used to support the tissue during sectioning are absent (e.g., the ice in cryosections, or the matrix in polyethylene glycol embedded tissues) all cell compartments are theoretically "open" and freely accessible to the immunoreagents. Penetration in these specimens was thought initially not to be a major problem.

It appears however, that even with hydrated, resinless sections labeling of cell antigens occurs largely at the surface of the section, as it does in immunostained

resin embedded tissues. This occurs regardless of the methods used to prepare the sections. This is puzzling and has perplexed immunocytochemists. What could be the causes for such surface labeling of antigenic sites in hydrated resinless sections? Some points of immediate consideration are the physical characteristics of the probes (e.g., size, diffusion rates) and the manner of tissue fixation. Of the two the latter may be of far more importance, although all contribute to the vexing problem of greater penetration i.e., more complete labeling. Smaller electron dense probes, gentler fixation techniques and long incubation times would appear to be needed to solve these problems.

Before proceeding to the discussion of hydrated sections, let us consider for a moment some aspects of preembedding immunocytochemistry of cells. There is no doubt that nonparticulate probes such as those used in the now standard PAP method can, in fact, penetrate even thick slices of tissues (e.g., 20-30 μm thick). The literature is repleate with such examples (e.g., see ref. 9). In many instances the resolution of these techniques is excellent with localizations confined to a single cell compartment (e.g., portions of the Golgi complex). However, this technique is not used extensively for fibrillar components due to the apparent lack of resolution that is needed in many investigations (e.g., cytoskeletal-associated antigens). The reaction product may be fuzzy or flocculent and discrete localizations characteristic of particulate probes appear not within the scope of this technique. Nonetheless, the approach has served the purpose extremely well over the past years and continues to be an important light and electron microscopic technique.

Preembedding techniques (i.e., vibratome sections of tissues) cannot be used effectively with the particulate probes irregardless of the fixation protocol. Penetration of the particulate probes does not occur to any great extent due to the size of the probe and the barrier posed by the cell membranes. On the other hand, preembedding of cells in monolayer culture is possible provided the cell (the plasma membrane and cytomatrix) is prepared or "opened" sufficiently to allow penetration of the immunoreagents. Whereas it is always the goal of immunocytochemists to preserve structure and antigenicity, this is not always possible. To open the cell or to make it permeable to the immunoreagents, it has been necessary to use various detergents (e.g., Triton-X 100, saponin) in combination with stabilizing buffers and/or fixatives. Such extraction procedures if used judiciously, can retain cell structure, thus insuring the possibility of more "complete" labeling of the antigen in question (see ref. 10 for an excellent example of preembedding immunocytochemistry using immunogold techniques). Frequently much of the cytoplasm, including membranes and the cytomatrix is removed in these procedures. If glutaraldehyde is not used, even at low concentrations, then the ultrastructure assuredly will suffer. A notable example would be the use of solvents, e.g., acetone or methanol, as fixatives. While these have been employed as fixatives for light microscopy, the ultrastructure is not preserved adequately. This may serve the purposes at the light level but certainly would not be acceptable at the EM level, especially when considering the cytoskeletal associated proteins, for example.

Thus it appears that one way to insure penetration of the probes into tissue sections is to distort, extract or otherwise disrupt cell structure to such a degree so as to allow labeling of the antigenic sites. This could lead to problems, when, for example, the antigen in question may reside on a short, thin cytoskeletal or cytomatrix filament, (e.g., cross-bridges between microtubules). Many of the antigenic sites may be lost inadvertently with the removal of the cytomatrix structure. This can be controlled to some extent with the extraction protocol.

Using nonextraction fixations and a removable resin technique (i.e., polyethylene glycol embedding, 11-13), we have achieved frequently what appears to be almost complete labeling throughout the section thickness. Glutaraldehyde-fixed (e.g., 0.5%) mouse testis was embedded in polyethylene glycol, sectioned, mounted, divested of the embedment and processed for indirect immunocytochemistry by the immunofluorescence or immunogold techniques (11-13).

At the fluorescence microscopic level using nonparticulate probes (e.g., IgG fluorescein) one gets the impression of complete labeling. For example, Fig. 1 is a pseudo stereo image of dividing spermatogonia stained for tubulin. The image was recorded by focusing the microscope at different levels in the section. The immunoreagents appear to have penetrated the full thickness of this 1 μm thick section as evidenced by the detection of the fluorescence signal at the various levels in the section. This is a consistent observation in these and even thicker immunofluorescence preparations. Whereas this "complete" and in-

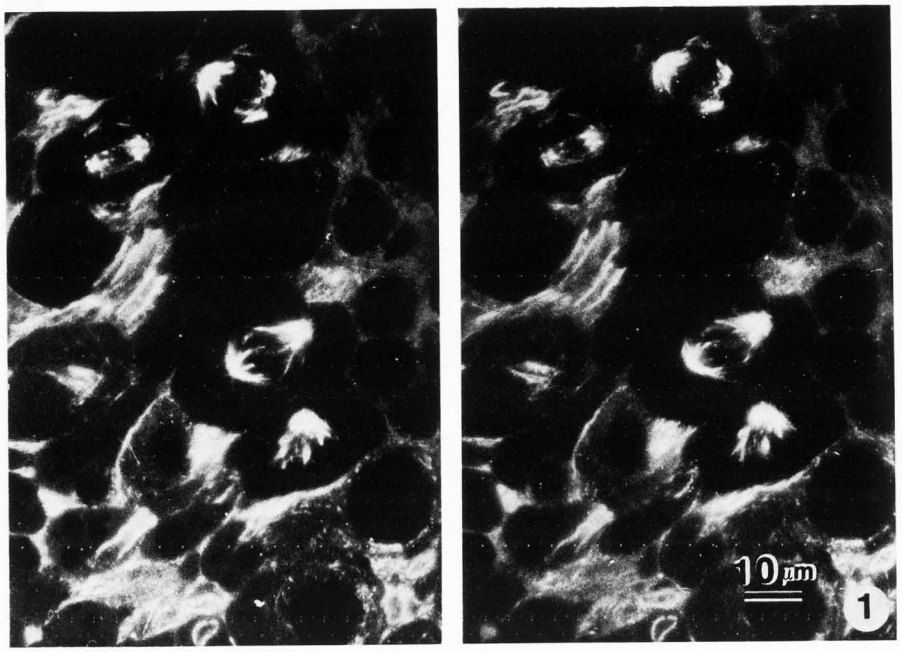

Figure 1. Pseudostereo image of dividing spermatogonia from the mouse seminiferous epithelium stained for tubulin by immunofluorescence method after polyethylene glycol embedding (11-13). This image shows that microtubules can be detected at all levels of the section suggesting complete penetration of the immunoreagents throughout this 1 µm thick section.

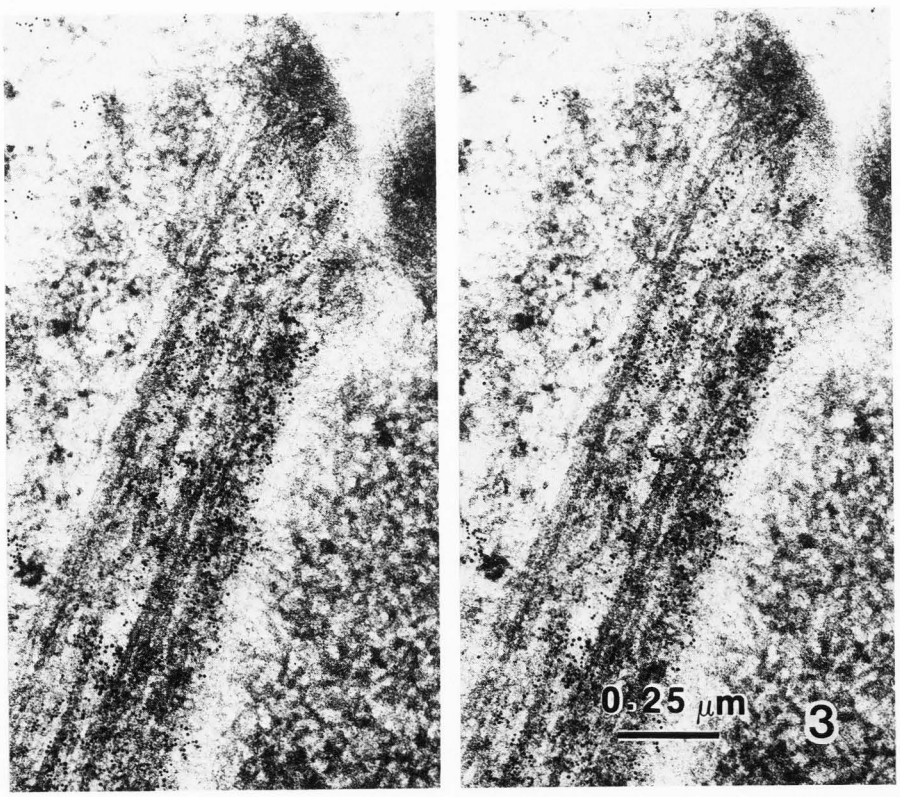

Figure 3. Image of a portion of the microtubules encircling the spermatid nucleus from mouse testis stained for tubulin after embedding in LR White acrylic resin (8). Immunogold was the secondary probe and the microtubules are seen labeled along their lengths. Only those microtubules at the cut surface are labeled.

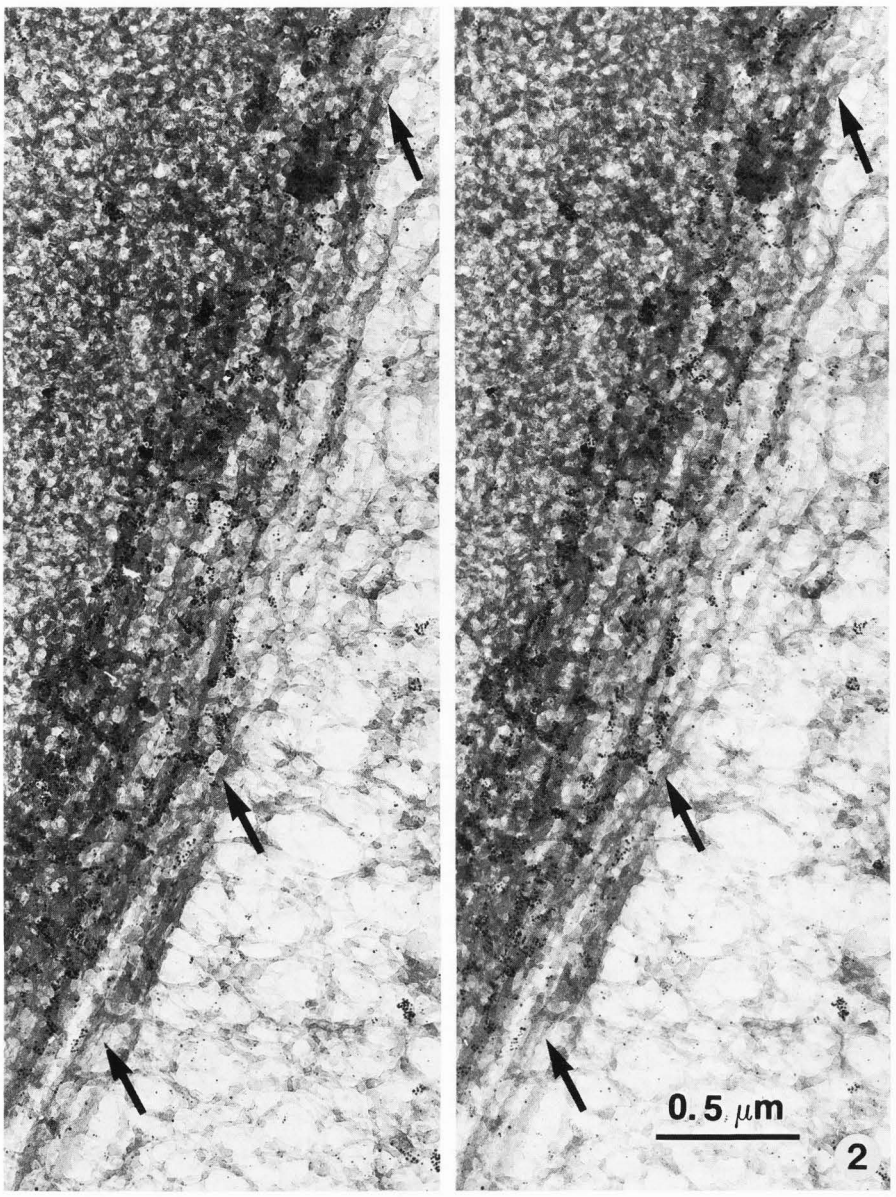

Figure 2. Stereo image of a developing mouse spermatid stained for tubulin by the immunogold method after poly-ethylene glycol embedding (11-13). This section was dried by the critical point process to visualize better the cytoskeleton and the colloidal gold label. This image shows that the microtubules are labeled almost completely throughout the section thickness. The microtubules on the lower surface, however, appear not to be labeled suggesting incomplete penetration of the immuno-reagents (most likely the secondary colloidal gold probe).

depth labeling may be valid, the possibility exists that the labeling is not complete and the resolution of the microscope insufficient to resolve the discontinuity in labeling. That is, while the label may be detected by fluorescence microscopy it cannot be resolved at the limit needed to show incomplete labeling. Thus, partially or sparsely labeled microtubules, for example, are visualized as being completely labeled.

This image however does seem to favor the former, i.e., complete labeling and a probable reason for this is that the secondary probe (e.g., goat antirabbit-IgG-FITC) is non-particulate and is to penetrate throughout the section. The situation is similar to the preembedding PAP methods. In addition, the sample also may be distorted sufficiently during sectioning, or subsequent processing. PEG is rather brittle and may fracture at the surface thus exposing additional antigenic sites.

At the electron microscopic level this "distortion" also is noted. Unlike other hydrated section techniques, (e.g., cryosections) PEG sections frequently are dehydrated after immunogold labeling and dried by the critical point process (12,13). This permits the visualization of the cytomatrix and other cell organelles unencumbered by electron scattering embedding matrices (e.g., epoxy resins). These specimens yield

very good contrast. The example in fig. 2 is such a specimen viewed in stereo to illustrate the depth of the label in the section. The structure imaged is a portion of a developing spermatid from the mouse testis. The elongated nucleus is surrounded by a band of longitudinally oriented microtubules. In stereo, note that the microtubules are labeled almost completely to the lower surface of the grid, and appear to follow the contour of the nucleus. Labeling appears to be almost complete.

Two other interesting features can be recognized in this stereo image. The first is that there appears to be a large area to the right of the nucleus that is devoid of structure, and second, that the microtubules are not labeled completely along their surfaces. The large gap is probably one reason for the success of labeling of this cell. That is, the section was distorted sufficiently to expose the microtubules, thus allowing the labeling. The second point, i.e., is the sparsity of labeling along the length of the tubules. This probably is the result of the antigenic sites either being masked by the components of the cytomatrix and or were "modified" during fixation, dehydration or embedding procedures, or some of the sites may simply have been destroyed by the procedures.

Fig. 3 shows a different preparation. Similarly fixed tissue was dehydrated and embedded in an acrylic resin (i.e., LR White, see ref. 8). Sections were cut and mounted on grids and processed for routine immunocytochemistry by the immunogold method. In this example the embedding matrix of specimen was not removed from the section (once polymerized, LR White is a crosslinked polymer). The section was stained in the conventional manner for electron microscopy (e.g., uranyl acetate and lead citrate). The example is similar, i.e., microtubules of the spermatid are labeled with the colloidal gold indicating the presence of tubulin. Here too, labeling is not complete along the length of the microtubules, but in this instance this is not unexpected. Many of the microtubules are still embedded in the resin and only those sites exposed during the cutting process are accessible. Nonetheless the labeling is prominent and the ultrastructure is more reminiscent of conventionally embedded specimens than those critical-point dried.

These examples and the foregoing considerations highlight some of the problems of immunolocalizations, especially for the finer components of the cytoskeleton and cytomatrix. First if the resin is present, then only those sites exposed in the sectioning process will have a chance to be labeled. For abundant proteins such as actin or tubulin, or proteins within membrane-bounded structures (e.g., zymogen granules, Golgi apparatus), then this approach is likely to result in good success if the antigens are not destroyed by processing. Second, if however the antigen is not abundant and not exposed in each section, then post embedding labeling of resin sections undoubtedly will be sparse and could lead to a misinterpretation of the results. Third, it seems logical therefore that only a hydrated, resinless section approach would be the method of choice especially for those "minor" components of the cytomatrix (e.g., microtubule or microfilament associated proteins). Fourth, while a hydrated section may be the logical approach, to label small structures otherwise not accessible in resin sections, the problem of increasing penetration must be further addressed and overcome. If not, then the practical limits of the post-embedding technique may have been reached. Nonetheless the approach is still a significant advance over the methods employed in the past decades.

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