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Immunocytochemistry at the Electron-Microscopic Level

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various cryoultramicrotomes which are commercially available. The cutting temperature appears to be influenced more by the type of specimen than by any other factor, and it is clear that the actual process of cutting is far from understood. Thicker sections are prepared in a similar fashion although the cutting temperatures are somewhat warmer (−35° C to −85° C). It is necessary to provide some sort of support for the sections and single cell and organelle isolates.

Ideally this support should be electron transparent, conductive and make a minimal contribution to the signals generated in the section.

Depending on whether the analysis is to be carried out using energy dispersive or wavelength dispersive spectrometers, it may be necessary to apply a conductive coating to the specimen. This coating layer is usually 10-15 nm of carbon or aluminium deposited in a clean environment on the specimen maintained at least at −130° C. Sections which are analysed using diffracting spectrometers usually require a conductive coating because of the higher beam current used in this method. This is, however, not always the case and is the exception rather than the rule with specimens analysed using energy dispersive spectrometers.

Whether or not the specimens have to be coated, it is necessary to transfer them to the electron beam instrument which is to be used for the examination and analysis. This transfer must be done under conditions which ensure that the specimen remains frozen-hydrated and does not melt, sublime or become contaminated. Similarly, it is necessary to ensure that the specimen is kept sufficiently cold and clean during the actual process of analysis. Changes in the mass of the specimen, either by contamination or sublimation of ice, can seriously distort the experimental result and elemental mass ratios. The specimens are maintained at a low temperature inside the microscope during examination and analysis. The specimen temperature should not be allowed to exceed −140° C during analysis, and lower temperatures are preferable.

The environment surrounding the specimens must be carefully monitored during all phases of preparation, examination and analysis. Constant checks must be made on the temperature of the specimen, the total pressure and partial pressure of water in the system and on the level of residual gases surrounding the specimen. This is necessary, certainly in the initial phases of any investigation, to ensure that the specimen remains in the frozen-hydrated state.

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KRAEHENBUHL J. P.*, RACINE L.*, PAPERMASTER D.** and SCHNEIDER B.**. *Immunocytochemistry at the electron microscopic level.*

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Introduction

Since the early sixties, a number of reports have been published concerning immunocytochemical techniques for the detection of intracellular constituents at the electron microscope level. The initial studies were made possible by the introduction by Singer *et al.* (1, 2) of ferritin-tagged antibodies which could be visualized directly in the electron microscope. From all the procedures described later, immunocytochemical methods at the electron microscope can be classified into two main categories: 1) preembedding staining and 2) postembedding staining techniques.

In the former, tagged antibodies or antibody fragments are allowed to penetrate fixed cells and to interact with antigenic sites prior to embedding and thin sectioning. Antigens are usually visualized by virtue of an electron-opaque reaction product generated by enzyme-tagged antibodies.

In the postembedding staining technique, thin sections of fixed and embedded tissues are prepared prior to the detection of antigenic sites with immunological reagents which consist either of antibodies coupled to electron dense particles (ferritin) or enzymes. The main practical limitation of the preembedding staining procedure has been the problem of obtaining tracers of sufficiently small size to allow ready penetration of all barriers present in fixed cells. These barriers include the plasma membrane of the cells as well as the membranes bounding intracellular compartments. Even the smallest reagents available, *i.e.* Fab antibody fragments coupled to a hemeoctapeptide (M.W. of the conjugate ~ 50,000) do not diffuse freely through the cell membranes thus leading to negative results.

For the postembedding staining procedure, no diffusion restrictions are to be expected because the immunological reagents are applied directly to the surface of thin section. When the tracers employed are particle tagged antibodies, the resolution of the technique is expected to be high in that one particle (ferritin) is associated with one antigenic determinant in a stochiometric relation.

For this reason, the results are amenable to quantitation by morphometric analysis of particle density on the section surface.

Problems in the postembedding staining technique, however, concern the supporting matrix used to obtain thin sections. Usually organic matrices such as Epon, methacrylate or polyethylene glycol have been used, sometimes preceded by osmium fixation. Such reagents can be expected to alter seriously the conformation of proteins and hence to compromise antigenicity. These problems have been overcome by the use of protein embedment (3, 4) or ultracryotomy (5). The development of direct labeling of tissue in a hydrophilic matrix of glutaraldehyde cross-linked serum albumin provides a new approach to localization which does not depend on further manipulation of the section to expose antigens and restrict protein perturbation to aldehyde treatment.

This method satisfies the following requirements:

1) The antigens are stabilized in the tissue by aldehyde fixation which prevents their deplacement and secondary relocation. False positive results are consequently minimized.

2) The antigens are available for interaction with the immunological reagents, since during sectioning the cell compartments are opened and diffusion barriers are removed. Thus the risk of false negative results is reduced.

3) Tissue processing and aldehyde fixation do not alter seriously protein conformation, as reflected by the preservation of the antigenicity of several proteins (6).

The different steps involved in the preparation of material for immunocytochemical localization of intracellular antigens are summarized as follows:

Tissue processing

Low concentration of glutaraldehyde is desirable for primary fixation, since fine structure is well preserved and antigenicity is not seriously altered (6, 7). Routinely, small pieces of tissue are fixed for 4 hrs at room temperature in 0.5% glutaraldehyde buffered with 0.1 M Na cacodylate pH 7.4 containing 5% sucrose. The fixed tissues are then embedded in serum albumin, dehydrated, glutaraldehyde cross-linked according to a modification of the procedure of Farrant and Mc Lean (3, 4). The details of the procedure have been published elsewhere (8). Thin strips containing pieces of tissue are cut from the disk of cross-linked protein, dessicated overnight and mounted with Epoxy cement to Epon dummy blocks. Thin sections are harvested on grids covered with Formwar and carbon.

Immunological reagents

In our studies we have chosen to use indirect localization sequences in which a common tagged second step antibody is used to reveal antigenic sites which have interacted with specific first step antibodies.

Preparation of specific antibody fragments (1st step reagents). Animals are immunized either with antigens purified by conventional biochemical means or with antigens contained in a strip of polyacrylamide gel after sodium dodecylsulfate polyacrylamide gel electrophoresis as recently reported (9, 10). Antibody fragments are then extracted from the antiserum by pepsin digestion of the antiserum, incubation with the appropriate insolubilized antigen, and elution of the specific antibody fragments with a chaotropic agent such as 3.0M. KSCN (8).

Preparation of tagged antibodies. In an attempt to overcome the loss of antibody activity which accompanies conjugation, we have devised a solid phase coupling procedure, in which the antibody active site is involved in a specific interaction with its antigens during conjugation and consequently should be protected. In addition, the antigen is insolubilized

on a solid support which facilitates the mecanics of the conjugation procedure and allows the extraction of specific antibody fragments (7, 11). Homogeneous preparations of $F(ab')_2$ fragments directed against the 1st step reagent and coupled to ferritin are characterized by a $F(ab')_2$ to ferritin ratio of ~ 5, a MW of ~ 850,000 daltons and a retention of over 80% of the antigen binding activity.

Biotin-avidin complex. A new approach to two stage immunocytochemistry has been made possible by the work of Heitzmann and Richards (12). Biotin is coupled to the 1st step antibody fragment. Its detection by avidin-ferritin is rapid and stable as predicted by its high binding constant. The approach provides a new and useful tool for localization since the preparation of first stage antibodies will not be restricted by the availability of appropriate second stage antibodies.

Examples

Localization of secretory proteins in the bovine exocrine pancreatic cells (7). This system was chosen to validate the postembedding staining procedure on a complex cell type because it has been established in biochemical and functional studies that secretory proteins are in high concentrations in zymogen granules. The results suggest that all zymogen granules and all Golgi complexes contain chymotrypsinogen, trypsinogen, carboxypepties, DNase and RNase.

Detection of opsin and a high molecular weight protein in the photoreceptor cells of frog retina (9, 10). Specific binding of antiopsin antibodies indicates that opsin is localized in the discs of rod outer segment and in the Golgi zone of the rod cell inner segments. In addition, using morphometric analysis (13) quantitatively different labeling patterns were observed on the outer segments of rods and cones. Because these quantitative variations extend on the entire outer segment, it was possible to identify the cell which had shed its discs into adjacent pigment cell phagosomes. Finally the large molecular weight protein was restricted to the incisures of the rod outer segments (10).

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Donelli G. *Diffraction and optical filtering of electron micrographs as methods for fine structure analysis of biological membranes.*

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The processing of electron microscopic data using coherent optical systems, proposed for the first time in 1964 as a method of micrograph analysis of periodical structures (1), has been widely developed in the last ten years (2-6). The fundamental advantage of the optical processing techniques over other different processing methods comes from the data's being in most cases already available in such a form as to be directly introduced into the optical system. Any degradation of the electron microscopic data due to « translation » procedures can be thus avoided. Optical processing with coherent systems