

# Markers for immuno-electron microscopy

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The development of techniques for electron microscopic studies of biological specimens has made it possible to visualize fine structural details of intact cells in ultrathin sections. The high magnification power of electron microscopes, along with refined techniques for biological specimen preparation, has enabled a wealth of informations with respect to normal and pathological fine structure of organs. Yet, what is usually detected is a map of distribution of electrons scattered by the macromolecular contents of the ultrathin section of the cells. Because spatial and temporal aspects of cellular processes and the functional or evolutionary significance of the complexity of higher organisms cannot be elucidated simply by comparing fine structures, specific detection methods at the molecular level are required for the identification and localization of individual components, quite analogous to light microscopy for which specific stains have been developed which absorb or emit characteristic optical radiation. For the latter, immunohistological stains with fluorochrome or enzyme labelled antibodies have proved most specific and are most generally used. In electron microscopy, such specific stains will have to scatter electrons in characteristic manner.

In principle, the resolution of the electron microscope enables the demonstration of an antibody molecules which has reacted with its antigen. Yet, unlabeled antibodies are only suitable for immuno-electron microscopic studies of isolated particles when measurable and reproducible changes in density or definite structural changes are obtained. In general terms, for the visualization of specific molecular details in the electron microscope it is necessary to create what is known as defined image contrast: regions of varying electron opacity that allow characteristic differences to be detected and information about the structure to be discerned. This, however, is usually not achieved with intact biological tissues from which ultrathin sections (either cut from resin embedded tissues or cut as ultrathin frozen sections) must be prepared. In both cases, antigen-antibody reactions are not readily to be distinguished from the surrounding matrix.

In order to distinguish cellular antigens the employed antibodies (as well as other molecular probes) must be "labeled" so that the resulting complex becomes visible. Only those substances which lead to significant deflection of the electrons (or to appropriate signals) in the electron microscope and which remain stable under the various steps of tissue preparation are suitable for labelling purposes. Typical marker substances may be classified as (1) primary electron dense molecules such as ferritin; (2) particulate substances detectable by their characteristic shape and size such as plant viruses and colloidal gold; (3) secondary electron dense labels such as enzymes due to specific substrate conversion.

The first successfully applied labeling procedure for immuno-electron microscopy was initiated in 1959, when SJ SINGER was able to link the metalloprotein ferritin covalently to antibodies. Ferritin remained for many years the label of choice in immuno-electron microscopy. Then, another iron-containing marker molecule, Imposil (an iron-dextran with 10% iron), was proposed as electron dense marker. Furthermore, staining techniques with heavy metal labeled antibodies were published, based on direct labeling of antibodies with

heavy metals. Many heavy metals chelate with antibodies. However, in contrast to ferritin, the metal (e.g. uranium) must be bound in large quantities in order to obtain sufficient contrast. For technical reasons, the heavy metal-labeled antibody method has not found wide-spread applications.

Morphologically distinct compounds such as plant viruses (e.g. southern bean mosaic virus) are useful to tag cell surface molecules. The large marker size makes it unsuitable for intracellular labelings. Other direct visible markers are hemocyanin and latex particles, both being useful for cell surface ligands or scanning electron microscopy. In 1971, WP FAULK and GM TAYLOR proposed colloidal gold as electron dense label. In the meantime, colloidal gold could be successfully employed for the detection of molecules at both electron and light microscopic levels using direct and indirect staining techniques. Moreover, this marker proved very useful for immuno-staining of sections prepared by cryo-ultramicrotomy, and, in all cases where antigens resist resin embedment, colloidal gold tagged antibodies can be used for immuno-staining of ultrathin resin sections.

As for light microscopy, radioactive labels may be also used for ultrastructural research. Such labelings are mainly employed in receptor binding studies. The resolution limits of autoradiographic methods (apart from laborious exposure and photographic film technique) as well as hazardous handling of radioactive isotopes are certainly major disadvantages of those methods.

The introduction of enzyme labeled antibodies by PK NAKANE and GB PIERCE (1966) and the development of appropriate cytochemical stainings, e.g. the histochemical horseradish peroxidase reaction by RC GRAHAM and M J KARNOVSKY (1966), proved to be a unique step forward in immunohistology. Enzymes make cellular ligand stainings very sensitive because of the amplifying ability of enzyme molecules. An enzyme is not consumed upon action with its substrate so that the reaction product can accumulate at the ligand site. Direct or indirect enzyme labeling techniques have the advantage over other marker techniques in that the same preparations can be principally applied for both light and electron microscopic studies.

In general, enzymes for labeling purposes should be stable at a given range of temperatures and pH during conjugation, storage and all immunocytochemical steps. In the case of covalent linkage with antibodies, substantial amounts of the enzyme activity must be retained. Furthermore, enzymes with high specific activity and turnover numbers are preferred since with reduced activity the formation of the reaction product may be too slow to remain at the enzyme site which will cause diffusion artifacts. A great number of enzymes have been proposed for cellular labelings from which peroxidase from *horseradish*, glucose oxidase from *Aspergillus niger* and alkaline phosphatase from *E. coli* are the most utilized enzymes for ultrastructural work; other molecules such as heme octapeptide prepared from cytochrome c with peroxidase activity have been proposed (KRAEHENBUHL JP et al., 1974) but are rarely used in immunohistology.

Immuno-staining for electron microscopic studies can be performed by several techniques. The main principles are shortly given in the following,

- preembedding techniques (mainly with enzyme markers): after tissue stabilization by some chemical fixatives, small tissue fragments being prepared by razor blades, tissue chopper/vibratome or by cryo-sectioning are incubated with all the necessary immunohistochemical reagents. Then, tissues are dehydrated, embedded in resin, cut with an ultramicrotome and observed in the electron microscope.
- postembedding technique (mainly with colloidal gold conjugates or protein A-gold complex): after tissue stabilization by some chemical fixatives, tissue fragments are dehydrated (organic solvents, inert dehydration, freeze substitution etc.) and

embedded in resin (epoxy, methacrylate or other resin) following following one of the many published rocedures. Then, ultrathin sections are cut, reacted with the immunohistochemical reagents and observed in the electron microscope.

- ultrathin cryo-technique (mainly with gold labeling): after initial stabilization of the tissue by some fixative, a freeze-protected tissue block is frozen and cut in a cryo-ultramicrotome. Sections are collected on grids, reacted with immunohistochemical reagents and observed in conventional electron microscope.
- Cryo-electron microscopy with frozen-hydrated sections (mainly with gold labeling): tissues or cells which have been *in vivo* labelled are plunge-frozen or high-pressure frozen in special devices. Frozen-hydrated ultrathin sections (cryo-ultramicrotome) or grids containing plunge-frozen cells are loaded onto a cryo-holder maintained at temperatures below -180°C and transferred to the electron microscope. Then, under near-native conditions cell architecture of vitrified cells is carried out by electron microscopy or by electron tomographic analyses. Due to the many technical problems posed by preparation and imaging of vitrified cells or sections, these techniques are far away from being routine.

## **Selected publications for further readings**

Singer SJ (1959)  
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Pepe FA *et al.* (1961)  
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Graham RC and Karnovsky MJ (1966)  
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Faulk WP and Taylor GM (1971)  
Karnovsky MJ *et al.* (1972)  
LoBuglio AF *et al.* (1972)  
Kraehenbuhl JP (1974)  
Molday RS *et al.* (1974)  
Bayer EA *et al.* (1976)  
Dutton AH *et al.* (1979)  
Hearn SA *et al.* (1985)

Full citation of publications is given in chapter *References*

link: <https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf>