Immunohistological marker molecules

WOLF D. KUHLMANN

Division of Radiooncology, Deutsches Krebsforschungszentrum, 69120 Heidelberg, Germany
Laboratory Diagnostics & Cell Science, 56112 Lahnstein

Immunohistology as well as a wide range of other biomolecular techniques depend on the use of labeled antibodies. Labelings such as radiolabels, fluorophores or enzymes may be used as reporters in distinct assay formats. Immunohistology in its proper meaning will depend on procedures which enable the identification and characterization of cellular structure and function in situ. Hence, the respective probe (antibody) must be "labeled" in some way so that the antigen-antibody reaction becomes clearly visible. Marker substances for labeling purposes are for example those which lead to distinct colors (light microscopy) or which lead to defined electron scattering (electron microscopy). Milestones in light microscopy were the introduction of fluorochromes by AH COONS and coworkers (1941) and, in the case of electron microscopy, the conjugation of the metalloprotein ferritin with antibodies by SJ SINGER (1959).

Most interestingly, the success of immunofluorescence paradoxically induced the need of further developments, principally due to inherent disadvantages in the use of fluorescein labeled antibodies (e.g. acquisition of a fluorescence microscope, bleaching of the fluorochrome, combination with conventional histology). In the search for alternatives to AH COONS' immunofluorescence, significant progress was achieved with the development of immunoenzyme techniques, notably, the conjugation of horseradish peroxidase (HRP) with antibodies. The development of enzyme markers for immuno-staining has greatly facilitated all immunohistological investigations. Quite comparable to different fluorochromes, simultaneous localization of several antigens in the same tissue section is possible with the use of different substrates or enzymes by the generation of different colors.

In the early times of immunoenzyme labeling, HRP was the enzyme of choice because HRP was available in a pure form. Furthermore, HRP proved stable under various conjugation conditions. Furthermore, chromogenic substrates and cytochemical prodedures became available leading to stable colored reaction products at the enzymatic reaction site, i.e. the localization site of the tissue antigen.

Even if in the early days of peroxidase labeling, HRP conjugates appeared not to be better than the fluorochrome conjugates, the applicability of immunoperoxidase methods to routinely processed tissues on the one hand and the easily detectable reaction products in the light microscope on the other were the main reasons that this technique was rapidly agreed for research and diagnostic purposes. During the subsequent years, a number of refinements of the immunoperoxidase and other enzyme labelings followed. For appropriate use, however, the selection of a direct or an indirect staining method is generally steered by the molecule to be detected and by the tissue itself.

The successful detection of a binding event depends on a sufficiently strong signal. In early immunofluorescent studies (together with the microscopes available at that time), a single fluorophore was usually difficult to be detected, and multiple fluorophores and binding events were needed. Thus, signal amplification methods have been developed which increased the

signal intensity per binding event in order to improve the limit of detection. For example, more than one labeled detecting antibody can be used for the detection of a bound analyte in "sandwich" assays; the analyte specific detecting antibody can be detected with several secondary labeled antibodies. Then, in enzyme assays, the enzyme itself will give rise to signal amplification because an enzyme is not consumed upon action on its substrate and can process many substrate molecules. A similar approach to labeled secondary antibodies is the use of biotinylated target specific antibodies which become bound by several labeled avidin/streptavidin molecules.

In principle, all enzymes can be used in light or electron microscopy which produce, upon action on their substrates, colored and insoluble or electron dense products. Until now, the most employed enzymes are (1) *horseradish* peroxidase, (2) *E. coli* alkaline phosphatase and *Aspergillus niger* glucose oxidase. Our own and other experiments have shown that especially horseradish peroxidase (HRP) is an ideal enzyme for immunohistological studies. Apart from their use in cytological assays, enzymes are very versatile labels in many other quantitative and qualitative immunoassays which include a number of different binding studies on the basis of lectins, protein A, biotin-avidin, nucleic acids and others.

In principle, the resolution of the electron microscope enables the demonstration of an antibody molecule which has reacted with its antigen. Immuno-electron microscopy dates back to the early forties of the last century when antigen-antibody reactions were observed for the first time in an electron microscope (ANDERSON TF and STANLEY WM, 1941; ARDENNE M et al., 1941). In these experiments, mixtures of virus particles and the corresponding immune sera were just deposited on grids. Even if the employed techniques were not as sophisticated as those used today, they were indeed the precursors of modern dispersive immuno-electron microscopy.

Yet, unlabeled antibodies are only suitable for the characterization of isolated particles when measurable and reproducible changes in density or definite structural changes are obtained. In order to distinguish cellular antigens the employed antibodies must be usually "labeled" so that the resulting antigen-antibody complex becomes visible. Three types of markers may be used.

- Primary electron dense molecules (e.g. ferritin, heavy metals).

- Particulate substances which can be detected by their size and shape (e.g. plant viruses, colloidal gold particles).

- Molecules which become electron dense by chemical conversion (e.g. enzymes).

Thus, antibody conjugates with *direct* and *indirect* visible markers can be prepared. The first successfully applied labeling procedure for immuno-electron microcopic work was initiated in 1959 when SJ SINGER was able to link the metalloprotein ferritin covalently to antibodies. Ferritin is usually prepared from horse spleen (but is also commercially available) and contains approx. 20% iron within its protein shell. The spherical ferritin molecule has a diameter of about 110 Å, its good electron scattering power is conferred by the large iron content. This makes individual ferritin molecules readily visible in the electron microscope; while the electron density of ferritin is due to the iron core; the shape of ferritin molecules can be outlined by the procedure of classical negative staining. Another iron-containing marker molecule for the preparation of antibody conjugates is Imposil (an iron-dextran molecules) which is commercially available.

Staining techniques with heavy metal-labeled antibodies (direct labeling of antibodies with heavy metals) were also proposed (PEPE FA, 1961; STERNBERGER LA et al., 1966). Later on, morphologically distinct compounds such as plant viruses were tried (HÄMMERLING U et al.,

1969). In the mean time, numerous other marker molecules such as hemocyanin or latex particles were published (KARNOVSKY MJ et al., 1972; LOBUGLIO AF et al., 1972). Especially, colloidal gold particles with different sizes (FAULK WP and TAYLOR GM, 1971; ROTH J et al., 1978) proved useful in electron microscopy for postembedding immuno-stainings and in techniques based on cryo-ultramicrotomy.

Regardless of whether a direct or an indirect immunostaining method will be used, the most crucial decision is the choice of a suitable label; most commonly employed labels with their advantages and disadvantages are summarized in Table 1.

Table 1: Choice of labels in immunohistology

Label	Application	Detection	Advantage	Disadvantage
Fluorochromes	Immunohistology FACS (cell sorting)	Fluorescence microscope Flow cytometry	High resolution Long shelf life of conjugates	Autofluorescence Quenching Low sensitivity
Enzymes	Immunohistology (LM and EM [a])	Chromogenic substrates for light and electron microscopy	High sensitivity Long shelf life Direct visualization for light and electron microscopy	Endogenous enzymes Lower resolution than fluorochromes Substrates can be hazardous
Radioisotopes	Immunohistology Radioautography (LM and EM)	Fine grain radio-autographic film technique	Easy to label (f.e. *in vivo* labelling of monoclonal antibodies; cell receptor binding) High sensitivity	Short half-life Potential hazard Low resolution
Metals, metallic nanoparticles	Immunohistology (LM and EM)	Direct visualization in EM (ferritin, colloidal gold) Double labelling in EM (use of different gold sizes) Direct visualization in LM (colloidal gold, with or without silver enhancement)	Easy to label (colloidal gold) High sensitivity Direct visualization for light and electron microscopy (colloidal gold)	Lower resolution than fluorochromes Low shelf life of conjugates

[a] LM (light microscopy; EM (electron microscopy)

Once an original cytochemical method has been published, technical improvements and variations in the substrate formulas were usually tried thereafter in order to achieve either higher resolution or higher sensitivity than with the original procedure (see Chapter *Immuno-staining*). Some of those improvements proved indeed very sophisticated and useful for a wide range of applications. For example, classical cytochemical tools such as the diaminobenzidine (DAB) cytochemistry can be successfully combined with fluorescence methods involving the use of fluorescent dyes for the photooxidation of DAB which gives an insoluble, colored and electron dense reaction product: when a fluorophore is exposed to high-intensity photon illumination, the excited state of the fluorophore can generate reactive singlet oxygen by energy transfer which in turn will oxidize DAB (MARANTO AR, 1982). By

this technology, some disadvantages of fluorescent labeling can be overcome, i.e. the inability of the original fluorescence technique for correlated light and electron microscopic studies.

The inherent disadvantages of FITC such as rapid bleaching and, more importantly, optical limitations of conventional microscopy so far (resolution, diffraction effects etc.) have almost limited immunofluorescent studies in the field of modern cell research. This, however, has changed dramatically within the last two decades with the development of numerous new techniques such as confocal, deconvolution, ratio-imaging, total internal reflection and other microscopical applications together with recent technical advances in computers, lasers and detectors (see Chapter *Fluorochromes in selective cell analysis*). In parallel, a large variety of new fluorochromes became introduced which allow the use of more than one fluorescent probes. These latest advances in fluorescence allow to reveal both structure and dynamic features in living material (including the dynamics of the structure itself) or in fixed material. Moreover, Scanning Near-field Optical Microscopy (SNOM) overcomes hitherto limitations of all farfield methods by a proximity method based on scanning the sample relative to an optical probe of sub-wavelength size at a distance of a few nanometers.

The choice of label is for some techniques straightforward. For example, certain fluorescent labels are essential for cell sorting techniques (FACS, fluorescence activated cell sorting) and for high-resolution immunohistology in confocal scanning microscopy (LSCM). In other models, the choice of label can be a matter of personal preference. In histopathology, immuno-stainings are most satisfactorily performed with enzyme labeled antibodies. In electron microscopic studies, the detection of molecules is done either with primarily electron dense markers (f. e. metals such as iron as used in ferritin conjugates or colloidal gold particles adsorbed to antibodies) or with enzymes which generate electron dense reaction products. Enzyme labels offer the advantage of an instant visual result of great sensitivity. Quantitative assays, however, are more difficult to perform with enzyme labels as compared with fluorescent labels due to the difficulty to measure the rate of enzymatic reaction in order to calculate the amount of bound enzyme.

Label-free detection systems

Labeling of antibodies is not free of problems because the incorporated label may impair its function f.e. by steric hindrance of the antigen binding sites. Furthermore, antibody conjugation may result in unreproducible labeling efficiency. Problems such as non-uniform labeling, background noise and signal quenching can be observed in all areas in which labeled reporter molecules are involved. In order to avoid these problems, non-labeling techniques for sensing molecules are under development. The new types of sensors can be categorized into optical, mechanical and electrical sensors. One can expect that label-free methods will be also of interest in immunohistology. For this purpose, modern concepts of clinical chemistry and biochemical research including mass spectrometry (ZHU W et al., 2003), surface plasmon resonance (PATTNAIK P, 2005), atomic force microscopy (KIENBERGER F, 2006), quartz micro-balance technology (MARX KA, 2003; MARX KA et al., 2003), electrical detection (ZHENG G et al., 2005) etc. should be evaluated.

Selected publications for further readings

Anderson TF and Stanley M (1941)
Ardenne M *et al.* (1941)

Coons AH *et al*. (1941)
Coons AH *et al*. (1942)
Coons AH and Kaplan MH (1950)
Coons AH (1954)
Coons AH (1958)
Libby RL and Madison CR (1947)
Riggs JL *et al*. (1958)
Singer SJ (1959)
Beutner EH (1961)
Pepe FA (1961)
Pepe FA and Finck H (1961)
Pepe FA *et al*. (1961)
Almeida J *et al*. (1963)
Sternberger LA *et al*. (1965)
Avrameas S and Uriel J (1966)
Nakane PK and Pierce GB (1966)
Sternberger LA and Donati EJ (1966)
Sternberger LA *et al*. (1966a, 1966b)
Avrameas S and Lespinats G (1967)
Hämmerling U *et al*. (1969)
Sternberger LA *et al*. (1970)
Faulk WP and Taylor GM (1971)
Kuhlmann WD and Avrameas S (1971)
Karnovsky MJ *et al*. (1972)
LoBuglio AF *et al*. (1972)
Kraehenbuhl JP *et al*. (1974)
Molday RS et al. (1974)
Horisberger M and Rosset J (1977)
Mason and Sammons (1978)
Roth J *et al*. (1978)
Dutton AH *et al*. (1979)
Hsu SM et al. (1981)
Cuello AC *et al*. (1982)
Maranto AR (1982)
Deerinck TJ *et al*. (1994)
Marx KA (2003)
Marx KA *et al*. (2003)
Zhu W *et al*. (2003)
Pattnaik P (2005)
Zheng G *et al*. (2005)
Kienberger F (2006)

Full citation of publications is given in chapter *References*
link: https://www.kuhlmann-biomed.de/wp-content/uploads/2020/12/References.pdf

© Prof. Dr. Wolf D. Kuhlmann   *09.12.2008*