

Cell staining with direct and indirect assay formats

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The fundamental concept of immunohistology is the localization of antigens within tissue sections by means of specific antibodies where the antigen-antibody reaction is detected with a histochemical color reaction visible in the light microscope or with fluorochromes by UV light in a fluorescence microscope. The principle of immunohistology is ascribed to A H COONS, the inventor of the immunofluorescent technique (COONS AH et al., 1941; COONS AH et al., 1942; COONS AH and KAPLAN MH, 1950; COONS AH et al., 1951; COONS AH, 1954; COONS AH et al., 1955; COONS AH, 1958). His original intent was the identification of putative foreign antigens in affected tissues in certain diseases, and soon it became evident that this technique would be of great potential for the detection of all types of antigens in cells. Even if the great impact of this technique on laboratories of experimental cell research, tumor and molecular biology was realized from the very beginning, methods of immunofluorescence were used for a long time only by few specialized research laboratories. Some of the reasons were lack of skill in the preparation of reliable reagents and, more importantly, lack of acceptance by the pathologists.

The immunofluorescent labeling technique of antigens in cells and tissue sections were for decades very suspect in histopathology because pathologists everywhere preferred to rely on classical histological stains; only apologetic approaches to the new way of diagnostics were seen. The great potential of immunohistology became scarcely realized when already abroad laboratories published papers supported by immunohistological studies.

Then, a breakthrough was observed with the introduction of immunoenzyme techniques with special reference to immunoperoxidase methods. More and more laboratories became interested in cellular immunostaining. Within the last 10 to 20 years, histopathologic units could no longer close up the procession of immunohistology. One can expect that in the mean time every histopathologic unit will work with immunohistology.

Much support comes from the manufacturers of biochemical products who offer primary antibodies, link antibodies and a variety of detection reagents including cytochemical substrates. All these activities of commercialization compensate a lot of deficiencies in laboratory skills and immunological know-how. Unfortunately, the laboratory staff is no longer forced to acquire profound knowledge because one must just cope with catalogs to purchase the appropriate reagents. Unwanted consequences may emerge from this behaviour: the laboratory team with the fastest "droppers" (men and women using the ready-to-use reagents in handy dropper bottles) will be ahead to produce a bulk of stainings and to flood the public with all type of papers. The true value of many publications is often difficult to assess.

In order to perform immunohistology as safely as possible, it is critical that histological specimens are prepared carefully and immunohistochemical reagents are reliable and stain reproducibly. The theory of immunostaining is easy. In practice, however, difficulties in achieving consistent results can be encountered due to a variety of factors. At least, one

should be familiar with all histotechnical staining aspects. Yet, it is also important to be aware of the basics of the employed reagents which will include some fundamental knowledge in immunological and the cytochemical reagent preparation. In order to understand pitfalls in immunohistology, the quality of the used reagents is another critical point. Experience and recommendations of laboratories in the use of reagents are quite useful, nevertheless each laboratory has to find out what will work best under its condition. Above all, appropriate quality controls are necessary and should be performed in a continuous process to document the efficiency and specificity of the obtained immunostainings.

There are numerous methods for the localization of antigens (COONS AH et al., 1941; COONS AH et al., 1942; COONS AH and KAPLAN MH, 1950; COONS AH, 1958; AVRAMEAS S and URIEL J, 1966; AVRAMEAS S and LESPINATS G, 1967; Nakane PK and PIERCE GB, 1966; AVRAMEAS S, 1969. Either a **direct** or an **indirect assay principle** can be chosen, and one can select from a large number of **detection and labeling formats** (Fig. 1). The selection of a staining method is based on parameters such as the type of the specimen and the degree of sensitivity required. Even if the choice of a certain method is often a matter of personal preference, under certain conditions, however, one has to adapt a specific staining technique according to the needs of the tissue and the molecules under study.

Fig. 1. Principles of cytochemical localization of molecules in tissue specimens

Target	Biomolecules in cells and tissues ^a (antigenic epitopes, carbohydrates, nucleic acids etc.)				
Probe	Primary antibodies, lectins, oligonucleotides or others				
Detection <i>assay principle and detection format</i>	<div style="display: flex; justify-content: space-between;"> Immunological principle Non-immunological principle </div> <div style="text-align: center; margin: 5px 0;"> <pre> graph TD A[Marker molecules] --> B[Conjugated] A --> C[Not conjugated] B --> D[Fluorochromes] B --> E[Enzymes] B --> F[Luminogens] C --> G[Particles] C --> H[Isotopes] D --> I[Fluorescence] E --> J[Color] F --> K[Light] G --> L[Shape] H --> M[Radiation] </pre> </div>				
Signal	Fluorescence	Color	Light	Shape	Radiation

^a Comments and details on *tissue preparation, antibodies, markers, conjugation methods* and the *staining steps* are made in the relevant chapters

The principles of immunohistological staining can be also applied for the detection of target molecules other than antigens supposed that selective probes are available. With the experience of immunocytochemistry, a number of *non-immunological affinity detection principles* have become developed. For example, molecular affinity bindings of **lectins** and **nucleic acids** (*in situ* hybridization, FISH etc.) evolved from immunohistological detection principles. The detection formats include enzymatic and nonenzymatic labels, avidin-biotin principles, antibody-protein A bindings and other types of ligand bindings.

As many as research and diagnostic laboratories exist as many as different experiences or opinions can be expected with respect to the various steps in immunohistology. Hence, no real standard method exist for immunostaining, but it is advised to follow in the beginning of all experiments with a common “standard” method as formulated by the pioneers in immunohistology. Yet, standard methods are just simplified procedure, f.e.

- Cut sections and mount on slides.
- Deparaffinize (if paraffin) in xylene and rehydrate by graded alcohol to water.
- Perform antigen retrieval (if necessary), rinse in buffer
- Block endogenous enzyme activity (this step can be omitted or may be performed after incubation in primary antibody), rinse in buffer.
- Incubate in primary antibodies, rinse in buffer.
- Incubate in secondary antibodies (labeled link-antibody, detection complex), rinse in buffer.
- Produce signal by chromogen substrate and counterstain if needed
- Proceed for viewing in the microscope.

Direct and indirect detection methods

Generally, assay principles and detection formats can be divided into the following major groups.

- Direct antibody labeled methods: specific antibodies (polyclonal or monoclonal) against the tissue antigen are prepared in a given species, and the detection system (f.e. fluorochrome or enzyme) is covalently linked with the primary antibody (COONS AH et al., 1941; COONS AH et al., 1942; COONS AH and KAPLAN MH, 1950; COONS AH, 1958; NAKANE PK and PIERCE GB, 1966; AVRAMEAS S and URIEL J, 1966; AVRAMEAS S and LESPINATS G, 1967; AVRAMEAS S, 1969). Then, the tissue specimen is incubated with this conjugate. The obtained antigen-antibody complex is directly detected by cytochemical means (enzyme labeling) or by UV light (fluorochrome labeling).
- Indirect antibody labeled methods: specific antibodies (against the tissue antigen) are prepared in a given species (first species); indirect methods exploit the fact that the antibody self will act as antigen (COONS AH, 1958; AVRAMEAS S, 1969; AVRAMEAS S, 1970). In this approach, immunoglobulins from the *first species* (for example *rabbit*) are used as an immunogen for the immunization of a *second species* (for example *sheep*) resulting in antibodies directed against immunoglobulin of the *first species*. Antibodies of the *second species* are then conjugated with a detection system as described for the direct method. Indirect staining techniques are also termed “sandwich” techniques. The method involves incubation with unlabelled primary antibody in the first step followed by incubation with labelled secondary antibodies in the second step. The obtained antigen-antibody complexes are finally detected by the appropriate means, e.g. cytochemically (in the case of enzyme labelling) or by UV light (fluorochrome labels) or another method (depending on the utilized marker molecules).
- Direct and indirect labeled methods with egg yolk antibodies: the yolk of eggs laid by immunized chickens (or ducks etc.) is an excellent source of polyclonal antibodies (see chapter *Antibody molecules and the antigen-antibody interaction*). Immunostaining procedures are in principle as described above (OLOVSSON M and LARSSON

A, 1993), but the advantage of avian antibody production over conventional antibody production in rabbits or other mammals is multiple because (a) chicken housing is inexpensive, egg collection is noninvasive; (b) eggs from immunized chickens provide a continual, daily source of antibody; (c) a single egg yolk contains as much antibody as an average bleed from a rabbit; (d) IgY isolation is rapid; (e) due to the phylogenetic distance, conserved mammalian proteins are often more immunogenic in birds than in mammals; (f) furthermore, chicken IgY does not cross-react with mammalian IgG; and (g) chicken IgY does not bind bacterial or mammalian Fc receptors. Thus, non-specific binding is reduced, and the need for cross-species absorptions is limited, and, after all, egg yolk antibodies can be readily used in combination with mammalian antibodies in multiple antigen stainings.

- Direct and indirect hybrid antibody methods: hybrid antibodies have been proposed for immunostainings and were originally used together with ferritin as marker molecule (HÄMMERLING U et al., 1968). Hybrid antibodies contain two different antibody specificities, i.e. two monovalent Fab fragments which are derived from *two different IgG molecules* and are combined into a bivalent hybrid molecule Fab'₂. One monovalent Fab fragment reacts specifically with the cellular antigen and the other monovalent Fab fragment is reacting specifically with the marker molecule; the tissue is first incubated with hybrid antibodies and subsequently with the marker (ferritin). In another approach, hybrid antibodies may be applied in a sandwich technique in which the hybrids react with the primary antibody (specific for the cell antigen) and then with the marker molecule. Since the hybrid antibody methods rely on immunological binding instead of covalent conjugation of antibody with marker, they may be also classified as antibody bridge methods.
- Antibody bridge methods: antibody bridge methods are modifications of indirect labeling techniques in which no covalent conjugation with the marker substance is needed (MASON TE et al., 1969). There exist numerous possibilities of immunological bridging by use of a spectrum of antibodies from different species and marker molecules including methods of coimmobilization of markers (KUHLMANN WD and PESCHKE P, 1986). The soluble peroxidase anti-peroxidase complexes (PAP) technique is also an immunological bridge method and was originally introduced by LA STERNBERGER (STERNBERGER LA et al., 1970). In the mean time, other preformed soluble enzyme anti-enzyme complexes are available, for example alkaline phosphatase anti-alkaline phosphatase (APAAP) and glucose oxidase anti-glucose oxidase (GAG) complexes.
- Tyramine amplification and polymer-based detection principles: the HRP catalyzed deposition of phenols is the principle of signal amplification (CARD, catalyzed reporter deposition) as described by MN BOBROW (1989). Peroxidase catalyzes the deposition of large amounts of biotinylated tyramine in the vicinity of the antigen-antibody complexes. Deposited biotins are then reacted with streptavidin-labeled enzyme resulting in the deposition of additional enzyme that allows an amplification of the enzyme signal. Another type of signal amplification is the polymeric labeling strategy (e.g. EnVision™ by Dako and PowerVision™ by ImmunoVision Technologies). The detection system is based on a labeled reagent in which link antibodies and marker enzymes are conjugated to a polymer backbone. Specimens are first incubated with primary antibodies, then, in the second step, the labeled polymer is applied (SABATTINI E et al., 1998; SHI SR et al., 1999). With the latter, incubations in link antibody and detection complex are combined into a single step. In routine, this principle has shown the advantages of a simplified staining procedure, high detection sensitivity and high efficiency (see chapter *Staining enhancement and signal amplification*).

- Non-immunological affinity detection principles (labeled protein A or G): the high specific binding of protein A and protein G to the Fc region of immunoglobulins from several species (without affecting the ability of the antibody to bind to its antigen) allow these molecules to be used instead of labeled sandwich antibodies (DUBOIS-DALCQ M et al., 1977; ROTH J et al., 1978).
- Non-immunological affinity detection principles (avidin-biotin): an important variation of the indirect detection methods is the modification of antibodies or protein A and protein G by coupling them with biotin. The extremely high affinity of avidin for biotin and its usefulness in cytochemical studies is well known BAYER EA et al., 1976a, 1976b). Avidin-biotin techniques, e.g. the the avidin-biotin complex (ABC) method and other variants (GUESDON L et al., 1979; HSU SM et al., 1981), are powerful tools in immunohistology. Now, biotinylated labels are very common in a large number of formats for the specific detection of molecules.
- Anticalin detection principles: a novel class of engineered binding proteins, the so-called anticalins (BESTE G et al., 1999; SKERRA A, 2000; SKERRA A, 2003), offers an alternative to the above mentioned detection principles which are based on antibodies, protein A and protein G or the avidin-biotin techniques; the used marker molecules can be the same as in the above cases. Anticalins are derived from natural lipocalins, which present a set of four structurally variable loops on top of a β -barrel scaffold, by employing targetted random mutagenesis and antigen-specific selection techniques. It has been shown that anticalins are well suited for the detection of target proteins on fixed cells. Compared with antibodies, anticalins offer a structural advantage in so far as both their amino and carboxy terminus is sterically accessible and therefore amenable to the construction of fusion proteins with enzymes or other functional proteins by genetic engineering. Anticalins are generated via in vitro procedures and facilitate the preparation of diagnostic tools with tailored effector functions. This provides an easy access to reagents without the need for chemical coupling of the binding moiety with an enzyme, for example.
- Rolling circle amplification (RCA) reporter system, immuno-RCA: rolling circle amplification generates a localized signal which is produced by an isothermal amplification of an oligonucleotide circle. The signal is easily detectable at the site of antibody binding. Because the product of an immuno-RCA reaction remains attached to the immune complex, the method allows its localization within a biological structure (SANO T et al., 1992; ZHOU H et al., 1993; LIZARDI PM et al., 1998; SCHWEITZER B and KINGSMORE S, 2001; GUSEV Y et al., 2001). For example, immunoconjugates are prepared by covalent attachment of RCA primers to antibodies. The latter will bind with the target antigens. Bound immunoconjugates are hybridized with a circle homologous to the RCA primer. RCA reactions are then run in the presence of DNA polymerase and nucleotides; the enzymatic synthesis yields a high molecular weight DNA by rolling circle replication which remains attached to the antibodies. Finally, the amplified DNA can be stained in various ways depending on the applied labeled decorator probe (oligonucleotides, e.g. with biotin, fluorochrome, HRP etc.). In the case of biotin, incubations are performed with streptavidin-HRP conjugate and enzyme substrate. In the case of oligonucleotide-HRP conjugates, the final complex is revealed by enzyme substrate alone.
- Antibody cocktails: the use of a mixture of different specific antibody molecules (either polyclonal or monoclonal antibodies) can be suitable to enhance the detection of a target structure in a histological preparation. Otherwise, antibody cocktails may be used to detect simultaneously different molecules within a tissue preparation; see chapter *Detection of multiple antigens in tissue sections*.

- Double or multiple immuno-staining methods: simultaneous localization of different primary antibodies (i.e. different antigenic entities) by the use of different labels (e.g. the combination of peroxidase and alkaline phosphatase as enzyme markers) or by the use of different chromogens for one and the same enzyme marker (e.g. peroxidase as enzyme label and sequential application of DAB and AEC as enzyme substrates) enable more than one antigen to be detected in the same preparation (NAKANE PK, 1968; MASON DY and SAMMONS R (1978). Then, the use of various fluorescent dyes as well as the combination of fluorochromes with enzymes as markers will allow the detection of multiple antigens. The sensitivity of both staining methods can be significantly enhanced by tyramide signal amplification (TOTH ZE and MEZEY E, 2007). A large number of combinatory possibilities can be imagined for the simultaneous detection of multiple antigens in tissue sections.
- Markers and signal generation: for details on marker molecules such as enzymes and enzyme cytochemical procedures, fluorochromes or other labelings see the respective chapters.

Today, numerous direct and indirect staining principles are in practical use.

- *Direct* and *indirect* stainings by the application of labeled antibodies (primary and secondary antibodies).
- *Hybrid antibody* techniques.
- *PAP technique* and other *antibody-bridge-methods*.
- *Coupled two-step enzyme* methods in which the product being catalized by the marker enzyme is used as substrate by a second indicator enzyme (e.g. GOD-HRP principle).
- *Avidin-biotin* interactions making use of the binding of biotinylated primary antibodies and their detection by incubation with avidin-biotin complexes or by sequential incubation with avidin and biotinylated enzymes (as marker); in the case of enzymes as markers each procedure is followed by enzyme substrate.
- The avidin-biotin procedure being performed in the two-step enzyme method by *coimmobilization* of marker and indicator enzymes at the antigenic tissue.
- Immunolocalizations by the two-step enzyme method in which both enzymes (i.e. marker and indicator enzymes) are coimmobilized by *antibody bridging* of the respectively labeled antibodies.
- Antigen detection by the *coimmobilized two-step enzyme* method including the *PAP technique*: unlabeled primary antibody is sequentially followed by second antibodies (labeled with GOD), PAP complexes, and finally by the substrate.
- *In situ hybridisation* techniques may be performed by various avidin-biotin bindings (*in-situ-avidin*) and by indirect antibody conjugated methods (*in-situ-antibody*).
- *Cryo-sectioning* techniques of unfixed or fixed specimens for light and electron microscopy and the application of either of the above mentioned immunostaining methods.

Detection of multiple antigens in tissue sections

Staining techniques to evaluate the expression of two and more antigens in the same tissue sample, can be performed by the use of distinct protocols, f.e.

- Combination of monoclonal mouse and polyclonal rabbit antibodies.
- A pair of rabbit monoclonal and mouse monoclonal antibodies.

- Use of different fluorochromes (especially with the introduction of new fluorochromes which allow the use of more than one fluorescent probes).
- Comparable to different fluorochromes, simultaneous localization of several antigens in the same cell preparation is possible with the use of different substrates (of the same marker enzyme) which generate different colors or with the use of different enzymes which also can generate different colors.

Immunohistology in practice

The main challenge in immunohistological assays is the specific binding of antibodies to the corresponding antigens. Whether polyclonal or monoclonal antibodies are to be preferred will depend on variables such as the availability of pure antigens for immunization, and personal staff as well as technical equipment for the preparation, purification and control of the needed antibodies. Alternatively, a great variety of specific antibodies (affinity purified polyclonals and monoclonals) can be now commercially obtained. In many cases, such antibodies are already conjugated with a wide spectrum of markers.

One has to recognize that histological preparation is often a limiting factor. Here, especially tissue fixation must be mentioned because tissue antigens are often submitted to significant alteration by the physicochemical means in the course of tissue sampling. Fortunately, enzymatic and nonenzymatic methods have proved useful in attempts to retrieve antigenic epitopes, i.e. to unmask tissue targets prior to immunohistological staining (see chapter *Retrieval of antigenic determinants*).

Direct methods are especially useful for ultrastructural studies of antigens when antigen localization must be performed with tissue preparations prior to embedment and sectioning (so-called *preembedment immuno-staining*). In contrast, *postembedment stainings* are those done with tissue sections prepared from embedded organs (f.e. resins such as Epon or methacrylates). In these cases, indirect immuno-staining is preferred for the reasons described above: they make the immunohistological method very sensitive due to the amplifying effect of the sandwich antibodies and the detection system.

Indirect staining methods make cellular stainings very sensitive and are preferred for the majority of applications. They have the advantage over direct staining methods inasmuch as indirect techniques can be employed to detect immunoglobulins of a given species independent from its specificity for the detection of a range of antigens. Furthermore, primary antibodies are not modified by conjugation procedures and loss of activity can be avoided.

Antibody titer, dilution, temperature, time, pH and salt concentration during the steps of incubation will influence the binding of antibody with antigen. Moreover, phenomena of background staining and prozone effect will be avoided by appropriately diluted antibodies and other cytochemical reagents. This is also economical. Many of the relevant aspects are given previously or will be discussed in the following chapters.

A very important point for successful immuno-staining is the accessibility of the cellular antigen. No serious difficulties are encountered when cell suspensions are submitted to immuno-staining of ligands which are located on the cell surface or when cells can be smeared or centrifuged on slides. Intracellular antigens are made more accessible by disrupting cell membranes. This can be done by tissue fixation with solvents and by sectioning. Furthermore, improved antibody penetration into intracellular spaces can be

obtained by freeze-thaw cycles. We also have experienced enhanced antibody penetration by the addition of detergents (Tween 80, Triton X-100, digitonin, saponin etc.) to fixatives and wash solutions. It must be mentioned, however, that the addition of detergents can be harmful to both the cellular structure and the antigens. It is well known that low-molecular weight reagents can penetrate tissue preparations more readily than high molecular weight reagents. Thus, small marker molecules and antibody (e.g. Fab fragments) conjugates with molar ratio 1:1 are generally of advantage for enhanced penetration into tissue preparations.

Cryostat, paraffin and resin sections as well as cell suspensions, cell smears, monolayer cultures or others are incubated essentially in the same manner (see chapter *Selection of staining protocols*). Under certain conditions, tissue sections must be treated prior to immunostaining, in order to retrieve antigenicity (f.e. after aldehyde fixation) or to remove anorganic precipitates in tissues. For better adherence of tissue sections, one may use bovine serum albumin or silane coated slides; otherwise the use of acetone cleaned slides can be sufficient.

Label-free detection systems

Labeling of proteins such as antibodies is not free of problems because the incorporated label may impair antibody function f.e. by steric hindrance of the antigen binding sites. Furthermore, antibody conjugation procedures may result in an unreproducible labeling efficiency. In all areas involving labeled reporter molecules problems like non-uniform labeling, background noise and signal quenching are observed. In order to overcome these problems, other non-labeling techniques for sensing molecules are under development. The new types of sensors can be categorized into optical, mechanical and electrical sensors. It is expected that an interest into label-free methods will also exist for immunohistological purposes, and one has to evaluate and to adapt (or at least to combine) the modern concepts of clinical chemistry and biochemical research laboratories such as 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. for studying physicochemical properties of the analytes.

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Full citation of publications is given in chapter *References*

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