

Considerations prior to immuno-staining

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Prior to immuno-staining, specimen sections from paraffin or resin embedded tissues must be rehydrated to allow the preparations to react with antibodies and all the necessary cytochemical substrates in an ordered way. All these treatments follow predefined protocols. All incubation steps and are usually done in buffer solutions.

Because it is known that tissue fixation and the subsequent histological preparations are poorly controlled variables that can alter or damage many antigens, a series of experiments is needed to establish optimal staining conditions. This includes (depending on the antigen under study) a number of attempts to retrieve antigens by “unmasking” epitopes. Furthermore, non-specific interactions between tissue section and immunohistological reagents that may interfere with specific reactions have to be reconciled by the adaption of appropriate means. Apart from endogenous enzyme activities, phenomena such as hydrophobic bindings, ionic interactions and other reasons, which mainly depend on the reagents used, have to be controlled. All these preliminaries are essential for reliable antigen staining.

Conditioning of tissue sections

One of the greatest challenges in immunohistology is the alteration of tissue antigens, f.e. induced by aldehyde fixation. Consequently, methods have to be chosen to partially reverse such changes.

When it was observed that the localization of many antigens can be improved by so-called *antigen retrieval methods* which break fixation induced cross-links in proteins, those antigen retrieval techniques have indeed revolutionized histopathology (HUANG SN, 1975; HUANG SN et al., 1976; SHI SR et al., 1991); see chapter *Retrieval of antigenic determinants*. In summary, a number of further conditioning methods are also important for clear-cut immunostainings (GROSSI CE and MAYERSBACH H V, 1964; STREEFKERK JG, 1972; STRAUS W, 1972; STRAUS W, 1974; KUHLMANN WD, 1975; KUHLMANN WD, 1978; HALL JG et al., 1978; LAURILA P et al., 1978; HEYDERMAN E, 1979; KUHLMANN WD and KRISCHAN R, 1981; WOOD GS and WARNKE R, 1981; DUHAMEL RC and JOHNSON DA, 1985; LI CY et al., 1987; TACHA DE and MCKINNEY LA, 1992; KUHLMANN WD and PESCHKE P, 2006; ROGERS AB et al., 2006).

- Antigen retrieval: Protease induced epitope retrieval (PIER) was the first used method to this approach. PIER methods were quite common until nonenzymatic methods such as heat became introduced. HIER is based on a group of methods using microwave ovens, pressure cooker, steamer, autoclaves or simply hot water baths together with special buffer solutions. For the majority of antigens, heating at high temperature seem to be favorable. A universal method, however, does not exist, and several different HIER buffer solutions should be examined.
- Endogenous enzyme activity: in tissue sections with enzyme activities comparable to the employed marker enzyme, the endogenous activities have to be blocked otherwise

interference with the specific immunostaining can lead to erroneous interpretation (see chapter *Reagents/blocking solutions*). Hydrogen peroxide, methanol or sodium azide are popular means to block endogenous peroxidases. In the case of alkaline phosphatase (AP), intestinal and nonintestinal isoenzymes of AP may produce background reactions. The nonintestinal form is readily inhibited by the inclusion of levamisole in the substrate mixture: the AP isoenzyme from calf intestine which is used as label in immuno-alkaline methods is not inhibited. When the endogenous enzymes are difficult to inhibit, then other marker enzymes may be recommended.

- Endogenous avidin and biotin: false stainings can be due to the interaction of highly charged avidin (in the detection reagents) with oppositely charged cellular molecules. Quite similar, prevention of binding of endogenous biotin with avidin is necessary. Both nonspecific reactions can be partially avoided by preincubation with free avidin or biotin or by incubation in “avidin-biotin detection reagents” which are prepared at high pH (f.e. pH 9) or by the addition of non-fat dry milk (5%). Instead of avidin from egg white, the use of streptavidin from *Streptomyces avidinii* is strongly recommended to avoid nonspecific binding as much as possible.
- Free aldehyde groups: nonspecific binding of antibodies to free aldehyde groups introduced by aldehyde fixatives may disturb the interpretation of immunostaining. This problem is preferably observed with glutaraldehyde as fixative or with prolonged fixation. Blocking reagents are f.e. sodium borohydride, lysine or glycine.
- Fc receptors: Fc receptors which resist to tissue fixation can bind immunoglobulins of the immunohistological reagents. This phenomenon, however, is not a usual problem with paraffin embedded specimens and will occur mainly in frozen sections or cytological preparations of lymphoid cells containing Fc receptors. The use of Fab fragments instead of whole antibody molecules will help to solve this problem.
- Other interactions as reason for background staining: hydrophobic binding, ionic and electrostatic interactions of reagents including protein-protein interactions due to polar groups in tissue components must be mentioned. A variety of blocking solutions containing f.e. buffers with pH different to the pI of antibodies or buffers supplemented with albumin, casein gelatin or nonimmune immunoglobulins of the species which prove the secondary antibodies can be tried.
- Metal precipitations: sublimate containing fixation solutions can lead to irritating precipitation of mercury salts in tissue specimens and should be eliminated prior to microscopy. This step, often called “De-Zenkerization”, can be easily done with Lugol’s iodine either before or after immunostaining. It is sometimes preferable to perform “De-Zenkerization” after incubation in immunological reagents in order to avoid possible deleterous effects of iodine on antigen epitopes.

Procedures of tissue conditioning such as antigen retrieval methods, blocking of endogenous enzymes, formulations of buffer and blocking solutions are given in separate chapters (see chapters *Reagents* and *Laboratory methods*).

Selection of antibodies, conditions of incubation

It is often a matter of personal preference or a question of availability whether polyclonal or monoclonal antibodies should be used in immunohistology. The advantage of monoclonal antibodies is their uniformity. In any case, the quality of antibodies (either monoclonal or polyclonal) is of great importance, thus, careful selection of primary antibodies with respect to specificity is always necessary. It is advised to submit all self prepared and purchased

antibodies to rigorous quality and specificity testing. To this aim, proteome and tissue microarrays are useful tools. They will allow the simultaneous screening of thousands of proteins for possible cross-reactivity. By arraying multiple normal tissues and different tumor types, one can analyze molecular targets and their histological distribution. Finally, the quality of the specific staining against the background can be evaluated (see chapter *Specificity of antibodies*).

For many applications in immunohistology, not only highly specific antibodies are of great importance, the degree of purity with respect to the bulk of contaminating serum proteins is also a matter of concern. Even if hyperimmune sera will work quite well, it can be preferable to use purified or at least partially purified antibody preparations (see chapter *Purification of antibodies by the use of biochemical and immunochemical techniques*).

Furthermore, the conditions of immunostaining method must be optimized in order to obtain reliable results. One of the critical factors is the optimal concentration of the primary antibody. This means that the optimal titer of a new antibody preparation must be determined by titration studies before using it in routine. Users with experience in immunofluorescence techniques will confirm this fact. Once the optimal tissue preparation (especially for paraffin specimens) and the appropriate detection system have been selected, it is important to choose reactive and nonreactive tissues; in this respect, tissue microarrays are greatly useful. Then, one can begin with primary antibody titrations by a series of antibody dilutions and a series of stains. We usually begin with 10 µg antibody/mL buffer solution and dilute down to 0.01 µg/mL or even further depending on the actual type of antibody. In the case of purchased antibodies, one can titrate by beginning with the dilution as suggested by the manufacturer.

Unfortunately, this approach will not guarantee that sections are always and equally well immunostained because one can have tissue sections with more or less antigen. This is due to the typical feature of heterogeneity from cell to cell. Problems of false-positive and false-negative reactions are further treated in the chapter *Artefactual staining in immunohistology*.

There is no rule about the conditions of incubation in primary antibodies and in the detection complex, whether concerning time, temperature or solvents. In research laboratories, one may prefer incubations with highly diluted primary antibody at low temperature for 12-24 hours. In routine practice, however, short incubation times may be needed instead of prolonged periods. Constant mixing of reagents during incubation (f.e. automated immunostainer, orbital rotator) will certainly shorten the times. The incubation temperature can be also varied to some extent. In any case, staining quality with reference to clean and strong stains must be carefully controlled.

Selected publications for further readings

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Full citation of publications is given in chapter *References*

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

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