

Practical aspects in quality control of immunohistology

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Quality control is of great importance, and it was stressed in the previous chapter (*Specificity and Standardization of Immunohistology*) that reliable immunohistology is only obtained by intensive work with control sets which include reagents as well as tissue preparations. However, it must be born in mind, that even if all test schedules will run perfectly, one can be overtaken by artefacts which may be not easily explained.

Artefactual immunostaining

Artefacts are so-called *false positive* (ectopic immunostaining) and so-called *false negative* staining reactions (mainly loss or denaturation of molecules from in vivo sites, but also non-reactivity of the employed reagents). Hence, artefacts can depend on the used immunocytochemical reagents as well as on the type of tissue preparation. The most pertinent sources of artefacts include

- **Antibody preparation:** immune sera are often the main source of false positive staining due to unwanted antibodies or due to peculiar molecular activities of the antibody molecules themselves (binding of Fc portion of immunoglobulins to Fc receptors). Apart from the specific antibodies, immune sera may also contain
 - natural antibodies
 - contaminating antibodies
 - cross-reacting antibodies
 - heterophile antibodies
- **Marker substances:** molecular heterogeneity and impurities give rise to uncontrolled conjugation. In bridging techniques, impurities will equally lead to by-reactions with uncontrollable staining effects
- **Degree of purification:** in the case of conjugated antibodies, contamination by unconjugated antibodies will give false negative staining results
- **Tissue preparation:** tissue sampling/preparation plays a major role in false positive and false negative staining results. Strong and weak fixations must be reconciled, f.e.
 - aberrant diffuse staining of cell or tissue compounds caused by necrotic areas in the tissue or by physical and chemical injury of the organ by inappropriate organ (or tissue section) handling
 - false negative results can be derived from too strong a fixation leading to denaturation of tissue molecules
 - false negative staining may also be due to diffusion of molecules (and often together with aberrant diffuse staining)
 - dehydration and embedment can lead to further denaturation and, thus, add to false negative staining reactions

- false positive results are to be expected by weak fixation due to diffusion of molecules; be aware that diffusion of molecules prior to fixation (e.g. in damaged, apoptotic or necrotic areas) will also result in false positive ectopic staining
- **Endogenous molecular activities:** molecular interaction of tissue molecules with incubation media, f.e.
 - in the case of enzymes as markers, endogenous enzyme activities can interfere with specific immunostaining
 - endogenous biotin-avidin-binding activity
 - binding of Fc portions of immunoglobulins to FcR on certain cell types (Fc receptors, membrane glycoproteins, f.e. leucocytes) with different class, subclass and species specificity
 - Protein A binding to tissue immunoglobulins
- **Undesired staining, non-specific adsorptions:** interaction of tissue components with reagents of the incubation media, often concurrent phenomena,
 - hydrophobic interaction (protein-protein, protein-immunoglobulin)
 - ionic and electrostatic interactions
 - non-immunological protein-protein interactions mainly attributed to pH and ionic environment in the incubation media
 - label-binding, f.e. affinity of peroxidase (HRP) for cell membranes or affinity between carbohydrate components of peroxidase (HRP) and other tissue structures (see also *Endogenous molecular activities*)
- **Enzyme substrate diffusion:** generally, enzymes with high specific activity and turnover number are preferred. With reduced enzyme activity the formation of the reaction products may be too slow to remain at the enzyme site, thus causing diffusion artefacts.

In the beginning of immunohistological staining, specificity can be broadly rated by some easily performed schedules:

- immunohistological staining should only occur with tissue preparations which contain the appropriate antigen; staining must be limited to that antigen
- staining should be inhibited if the conjugate (or primary antibody) is fully absorbed with homologous antigen, but not if different antigens are employed for absorption
- no tissue staining must be observed with nonimmune IgG
- staining by conjugated antibodies (direct method) should be inhibited by pretreatment of the tissue with unconjugated antibody, the so-called blocking test.

Even if such testing is usually not sufficient, one can obtain by microscopical inspection an overall view on the quality of the histological preparation. With some experience it is possible to discern lack of staining, overstaining or background staining. The manufacturers of immunostaining kits provide troubleshooting guides with their products which are useful in the beginning. With growing experience, the histologist will become familiar with the problems of his methods.

Simplified troubleshooting in immunoenzyme labeling

Some major problems in immunohistology are listed in the following tables. Because of the complex nature of this technique unexpected results and pitfalls are often difficult to interpret. The mentioned problems and the proposed solutions are certainly not complete,

and many of the staining problems will occur concurrent. It is evident that control experiments always include sets of positive and negative controls.

- **Problem: weak or no staining**

Possible source	Solution
Inactive primary or secondary antibodies	Replace with new batch of antibodies; aliquot antibodies into small volumes, and if stored in freezer (see manufacture's instructions) avoid repeated freeze and thaw cycles
Dissociation of primary antibody during washing and subsequent steps of incubation	Replace with higher affinity antibodies
Inactive secondary reagents (secondary antibodies, ABC reagents etc.)	Replace with new batch of secondary reagents
Incompatible primary or secondary antibodies	Check specificity of the applied primary antibodies (antigen binding); use secondary antibodies that will interact with primary antibodies (f.e. correct anti-species antibodies)
Concentration of primary antibodies/secondary reagents too low	Increase reagent concentrations; run dilution tests to determine optimal dilution for optimal signal to noise ratio
Incompatible or defective enzyme substrate	Replace with new batch of reagents; use compatible enzyme substrate
Inadequate antibody/secondary reagents incubation time	Increase antibody/secondary reagents (antibodies, ABC reagents etc.) incubation times
Inadequate enzyme substrate incubation time	Increase enzyme substrate incubation time; check substrate composition, make shure that all necessary components are added; choose a cytochemical enhancement method
Reagents used in wrong order or incubation steps omitted	Check protocols and procedures used
Lack of antigen staining (<i>see also Loss of antigen</i>)	Check tissue/tissue type: is the cell/tissue type known to express the antigen, and is the processed tissue of the correct species origin? Check protein expression (f.e. in situ hybridization); level of protein/antigen expression may be too low to be detected by the employed method, choose a higher sensitivity staining system
Loss of antigen (<i>see also inadequate tissue or section preparation</i>)	Change fixation protocol to monitor antigen preservation (overfixation, loss by diffusion); when antigen is destroyed by enzyme chenching before primary antibody incubation, block endogenous enzyme activity after primary antibody incubation
Inadequate tissue or section preparation	Choose by trial and error different fixatives, fixation schedules and embedding methods; choose an appropriate antigen retrieval method (no single

	demasking protocol exists for all applications); frozen sections: prepare sections in different ways to control antigen preservation
Incompatible counterstain or mounting media with enzyme substrate (e.g. use of organic solvents)	Chromogen partially dissolved by organic solvents, use water-based counterstain and mounting media. Use chromogen that is stable in organic solvents
Incomplete removal of paraffin (or other embedding media)	Check deparaffinization procedure (or removal of other embedding media), use fresh reagents (xylene, xylene substitute)

- **Problem: overstaining**

Possible source	Solution
High concentration of primary and secondary antibodies	Run dilution tests to determine optimal dilutions for optimal signal to noise ratio
Cross-reactivity of antibodies with tissue components (compare with <i>high background</i>)	Use blocking solutions to dilute the reagents, pretreat tissue with blocking solution prior to incubation; use preadsorbed immune sera/antibodies. Use an isotype control of irrelevant specificity in place of the secondary antibody
High concentration of secondary reagents (e.g. ABC reagents) and enzyme substrate	Choose by trial optimal concentration of each component, perform incubation with appropriately prepared enzyme substrate media
Inadequate long incubation time	Determine optimal incubation time for each incubation step, i.e. primary antibodies, secondary antibodies, secondary reagents (ABC reagents), enzyme substrate
Non-specific binding of primary and secondary reagents to tissue, hydrophobic and ionic interactions	Use blocking solutions (normal serum, bovine serum albumin, avidin-biotin blocking reagents), increase their incubation times; use adsorbed secondary antibody
Inadequate blocking serum	Check protocols, blocking serum and link antibody should be from the same species; substitute blocking serum by a serum-free blocking solution
Inadequate incubation temperature	Reduce incubation temperature
Inadequate inhibition of endogenous enzymes	Select appropriate quenching procedure
Inadequate tissue or section preparation (diffusion artefacts)	Choose different fixatives, fixation schedules and embedding methods to monitor diffusion of antigen; be aware of passive uptake of molecules within damaged or necrotic areas. Frozen sections: prepare sections in different ways to control antigen localization
Inadequate rinsings and washings	Check washing protocol and optimize washing times; washing solution may require higher concentration of saline or detergent

Sections too thick	Cut thinner tissue sections
Sections dried out	Avoid drying of sections during all staining steps

- **Problem: high background, debris on sections**

Possible source	Solution
Possible sources include those described above (see <i>Overstaining</i>)	See <i>Overstaining</i> . Check staining reaction on negative and positive control slides
Inadequate washing steps	Wash at least 3 times between each incubation step
Cross-reactivity of secondary antibodies	Use preadsorbed secondary antibodies (f.e. rabbit anti-rat IgG may cross react with mouse tissue), i.e. use mouse adsorbed rabbit anti-rat IgG on mouse tissue
Mouse antibodies on mouse tissue	Treat with <i>mouse-on-mouse blocking reagent</i> prior to incubation with primary antibodies
Inadequate blocking of endogenous enzymes	Select an appropriate enzyme quenching procedure or select another enzyme as marker molecule; alternatively, employ immunogold-silver staining
Endogenous biotin	See <i>Overstaining</i>
Diffusion of antigens (specific background)	Damaged/necrotic tissue areas: passive uptake of molecules (diffusion artefacts) can be monitored by double stainings or sequential staining of sections: stain one section for the antigen under study and stain another section for an antigen that normally does not occur in this area. Use different fixatives, fixation schedules and embedding methods to monitor diffusion of antigen; be aware of passive uptake of molecules within damaged or necrotic areas. Frozen sections: prepare sections in different ways to control antigen localization
Debris and precipitates on sections	Check quality of tissue and tissue sections, check specimen preparation (partial drying?); check enzyme substrate (if too old, reagents will precipitate out of solution); use new xylene bath (xylene too old and dirty)
Excessive counterstaining	Check incubation time of counterstain; optimize concentration of counterstain and staining time, use other counterstains

Simplified troubleshooting in immunofluorescent labeling

Some major problems in immunofluorescent labeling are listed below. Many of the reasons for unexpected results in immunofluorescent stainings are the same as those listed above for the immunoenzyme techniques. This holds especially true for the use of primary and

secondary antibody systems as well as for the preparation of tissues and tissue sections. Hence, the same control strategies can be used, irrespective of the applied staining methods, i.e. using the *direct labeling* technique (in which the primary antibody is labeled with signaling molecules) or an *indirect labeling* technique (secondary reagents labeled with fluorophore molecules). Inherent properties of the immunofluorescence technique are mainly related to the phenomenon of autofluorescence of the tissues and to the wavelength/energy characteristics of the used fluorochromes with their excitation and emission spectra (derived from various electronic levels), the Stokes shift and the fluorophore stability. All this needs careful attention.

- **Frequent troubles in immunofluorescence**

Observation	Solution
Autofluorescence (cell structures and metabolites)	<p>Prepare control specimens under the same conditions as the immunostained sections, i.e. all incubation steps are performed with all reagents except that the fluorochrome is omitted (use of unlabeled primary antibodies and unlabeled secondary reagents, respectively).</p> <p>Check tissue preparation including fixation, mounting and embedding media; omit or replace by other media.</p> <p>Monitor autofluorescence with optical filters, dual-wavelength correction, computational image correction.</p> <p>Use quenching solutions and histological stains.</p> <p>Select other fluorochromes or fluorescence techniques (e.g. FRET)</p>
Weak fluorescence	<p>Check buffer pH (pH too low, e.g. pH < 7.2); bleached fluorochrome, incubate with new batch of fluorochrome conjugate; new batch of fluorescent substrate (inactive or inadequate substrate).</p> <p>Check optical arrangement of illumination (Köhler illumination); use illumination with appropriate wavelengths (f.e. high pressure mercury lamp, laser light); check the runtime of the lamp and replace if illuminator necessary.</p> <p>Select proper objective lenses for fluorescent work, use high NA objectives.</p> <p>Poor sensitivity when molar F/P ratio is too low, prepare conjugates with F/P ratios in the range of 2.0-3.0</p> <p>Be aware of photobleaching; use anti-fading reagent (f.e. DABCO for FITC experiments); limit the amount of irradiation</p> <p>Use quartz cover glass</p>
High fluorescence	<p>Inadequate high F/P ratio, use conjugates with lower molar F/P ratio; check pH of washing buffer</p>
Background fluorescence	<p>FITC labeled antibodies and other conjugates should not contain free fluorochrome (dialyse or purify by chromatography).</p>

Use conjugate with appropriate F/P ratio.

Check washing protocol and washing buffer (pH too high), optimize washing times; replace embedding medium; use qualified immersion oil.

Check illumination and microscope filter system

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>

