Immunohistochemistry-Paraffin Tips & Tricks

Immunohistochemistry (IHC) is a common laboratory technique used to visualize proteins in tissue or tissue sections with the help of antibodies conjugated to enzymatic or fluorescent labels. For clarification of the terminology, it is important to differentiate this technique from the imaging of proteins in cells, a method referred to as immunocytochemistry (histo = tissue versus cyto = cell). Depending on how the immunostaining signal is visualized, the IHC method is classed as fluorescent or chromogenic IHC. In the past, chromogenic IHC was nearly exclusively performed, but recently due to increasing multiplexing needs (visualizing several proteins simultaneously in one tissue sample) fluorescent IHC is on the rise.

IHC is used from basic to clinical research, for example, to determine disease states and morphological abnormalities of human and animal tissues. Depending on how the tissue has been prepared, the IHC method is known as IHC-frozen (tissue has been snap frozen in liquid nitrogen, isopentane or dry ice) or IHC-paraffin (tissue has been fixed in formaldehyde and embedded in paraffin wax; FFPE). In both IHC-frozen and IHC-paraffin experiments the tissue or sections of the tissue are mounted on slides prior to staining. This is in contrast to the IHC-free-floating technique where the entire IHC procedure is performed in liquid to increase antibody binding and penetration; slide mounting only takes place upon experimental completion (McCollum no date). IHC-free-floating appears to be most popular in neuroscience research. When analysis of the tissue by electron microscopy is desired, the tissue is often embedded in acrylate resins such as glycol methacrylate (GMA), a technique referred to as IHC-resin (Howat et al. 2005).

Due to the significant differences in the described IHC procedures, it is essential to confirm that your primary antibody has been tested/validated in the intended IHC procedure before beginning your experiment. In addition to the availability of antibody reagents, your protein target itself may dictate the tissue preparation and IHC methods utilized. For example, for phosphoprotein visualization, dedicated preservation methods are required to conserve the post-translationally modified state before staining (Mueller et al. 2011).

Staining of FFPE Tissues

To support troubleshooting and confirm the specificity of the antibody staining, we recommend inclusion of both positive and negative controls in your experimental design. It is also critical to familiarize yourself with the expected staining pattern and your microscope set-up to ensure that you can detect the antibody staining.

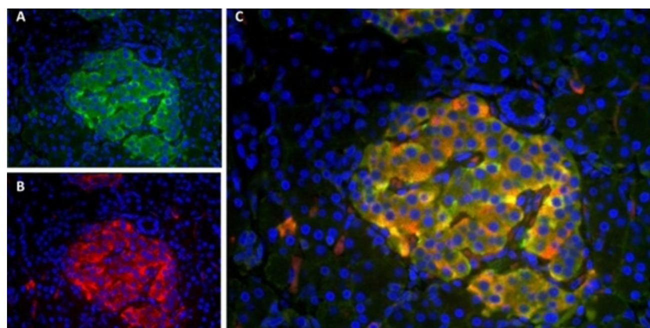

Fig. 1. Localization patterns of insulin and amylin in human pancreas section. A, immunofluorescence staining of paraffin embedded human pancreas using Guinea Pig Anti-Pig Insulin Antibody (Cat. #5330-0104G) and **B**, Mouse Anti-Human Amylin Antibody (#MCA1126). **C**, Merged image showing co-localization of the two proteins. DAPI was used as nuclear counterstain (blue).

20-Step IHC-Paraffin Protocol Example

1. Mount and section samples
2. Heat sections on the specimen slide to improve adherence
3. Remove paraffin and rehydrate the tissue
4. (Optional) Perform heat induced or protease induced epitope retrieval
5. Wash
6. Block endogenous peroxidases/phosphatases (for enzymatic labels) and biotin (for biotin/avidin detection systems)
7. Wash 1x in phosphate buffered saline (PBS)
   Use Tris-buffered saline (TBS) instead of PBS for detecting phosphoproteins or when using alkaline phosphatase (AP) conjugated antibodies
8. Block non-specific binding sites
9. Wash 3x in PBS or TBS
10. Incubate with primary antibody
11. Wash 3x in PBS or TBS
12. (Optional) Incubate with secondary antibody
13. Wash 3x in PBS or TBS
14. (Optional) Incubate with amplification reagent
15. Wash 3x in PBS or TBS
16. Incubate with DAB or other substrate solution (for enzymatic labels only)
17. Wash in ddH_2O
18. Counterstain
19. Dehydrate tissue sections (for organic mounting media only)
20. Mount coverslip

Tips for Step 4 – Perform Antigen/Epitope Retrieval

Tissue fixation methods often result in antigen masking, which subsequently impairs antibody binding and therefore protein detection. The effects of tissue fixation can be partially reversed by performing epitope retrieval, also known as antigen unmasking.

Two types of epitope retrieval have been established; heat based (HIER; Heat-Induced Epitope Retrieval) or enzyme based (PIER; Proteolytic-Induced Epitope Retrieval).

Table 1. Comparison of epitope retrieval methods.

	HIER	PIER
Method Overview	Can be performed using autoclaves, heating plates, hot water baths, pressure cookers, microwaves or steamers*	Can be performed using enzymes such as pronase, proteinase K, trypsin or pepsin
Mode of Action	Restores secondary and tertiary epitope structures	Degrades the peptides masking the epitope
Popularity	Very popular	Less popular compared to HIER Reason: may induce changes to the specimen's morphology

* Kim et al. 2016, Ward and Rehg 2014

Consider these guidelines to determine if and how to perform antigen retrieval

- Perform a literature search to determine what retrieval techniques and conditions other researchers have used to successfully detect your protein of interest. This search also allows you to familiarize yourself with the expected localization/distribution pattern
- Check if the antibody supplier or IHC resources recommend a specific antigen retrieval protocol (for example we have a dedicated protocol for the antigen retrieval of the murine F4/80 antigen)
- If no specific antigen retrieval method is recommended for your specific protein, try HIER first (see Table 1)
- For HIER, initially start with a neutral pH antigen retrieval buffer, such as Antigen Retrieval Buffer, pH 8.0 (#BUF025A). Compare against tissue for which the antigen retrieval step has been omitted
- Alternatively, if no specific protocol is recommended, start with commonly used antigen retrieval buffers such as 10 mM citrate buffer (pH 6.0) and Tris-EDTA buffer (pH 9.0). You may also want to compare different buffers to determine the optimal conditions
- In addition to pH, consider optimizing the temperature and duration of the HIER procedure. Ideally test various pH, temperature and time parameters
- To eliminate staining artifacts created by the HIER process, compare against a control sample for which no HIER treatment was performed
- Bespoke treatment conditions may be required for the detection of certain antigens such as 5'-bromo-2'-deoxyuridine (BrdU) incorporated into DNA. For BrdU staining experiments, treatment with hydrochloric acid or nucleases is frequently performed to facilitate anti-BrdU antibody binding (Liboska et al. 2012)

Tips for Step 6 – Block Endogenous Enzymes and Biotin

To avoid staining artifacts it is important:

- To block endogenous peroxidases and phosphatases prior to using AP/horseradish peroxidase (HRP) antibody conjugates
- For blocking of endogenous peroxidase activity, use hydrogen peroxide solutions such as Bio-Rad's ready-to-use Peroxide Blocking Reagent (#BUF017B; 3% (v/v) hydrogen peroxide). However, for the detection of certain antigens, such as CD4, this percentage of hydrogen peroxide may be too harsh (Kim et al. 2016)
- To block endogenous biotin when using avidin/biotin or streptavidin/biotin detection systems. For this specific purpose Bio-Rad offers a ready-to-use Avidin/Biotin Blocking Reagent (#BUF016)

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- To block endogenous AP activity supplement the substrate solution with levamisole (Ponder and Wilkinson 1981). According to Ponder and Wilkinson (1981), 20% acetic acid may also be used. However, this method may not be suitable for all antigens (Ponder and Wilkinson 1981)

Tips for Step 7 – Wash

To reduce non-specific binding and background staining, sufficient washing is essential throughout the IHC procedure.

PBS is a commonly used wash buffer but not recommended for the detection of phosphoproteins. This is because the antibody raised against the phosphorylated protein may bind to the phospho groups present in the buffer rather than to the phospho antigen itself. TBS is a commonly used substitute in such experiments, as well as when phosphatase labeled antibodies are used.

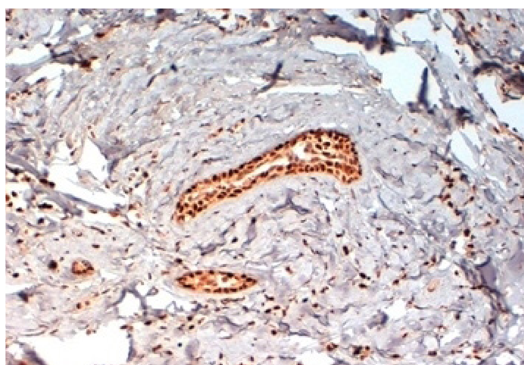

Fig. 2. Staining of progesterone receptor phosphorylated on serine residue 190. FFPE human breast cancer tissue stained with Mouse Anti-Progesterone Receptor (pSer190) Antibody (#MCA2406). Detection was performed using a HRP polymer and DAB as the substrate. Antigen retrieval method: HIER, citrate buffer (pH 6.2).

Tips for Step 8 – Block Non-specific Binding Sites

To prevent non-specific antibody binding to tissue surface structures such as Fcγ receptors, blocking is traditionally performed prior to incubation with the primary antibody (Kim et al. 2016).

Although the impact and merit of this blocking step has recently been questioned by Buchwalow et al. (2011), we recommend including a blocking step in your experimental design.

Blocking advice:

- Block with serum from the same species as the tissue; the immunoglobulins present in the serum will bind to the receptors present on the tissue
- Alternatively, use normal serum from the same species as the one in which the secondary antibody was generated
- Block with 10-20% normal serum
- Never block with normal serum from the same species that the primary antibody was generated in. This could lead to blocking of reactive sites or higher background
- If serum is unavailable, use bovine serum albumin, non-fat milk or gelatin
- Do not block with milk when using biotin-avidin detection systems. Milk contains biotin and therefore will result in staining artifacts/high background (Kim et al. 2016)

Tips for Step 10 – Incubate with Primary Antibody

- Check the manufacturer's datasheet to confirm that the antibody has been tested in IHC-paraffin. If the antibody has not been tested in IHC-paraffin but in another method such as IHC-frozen, do not assume that the antibody will automatically work in IHC-paraffin
- Consider using a polyclonal antibody when first establishing an IHC protocol. Although antigen retrieval is possible, the efficiency of the process is variable and certain epitopes might still remain inaccessible. Therefore a polyclonal antibody, which recognizes a multitude of epitopes due to its heterogeneous nature, provides a definite advantage over a monoclonal antibody which recognizes a single epitope. However, monoclonal antibodies have the advantage of batch-to-batch consistency and specificity, which often leads to lower background staining
- Prior to performing the experiment, perform a literature search to determine the spatiotemporal localization of your protein of interest (in healthy and diseased tissue). For a preview of the expected staining pattern, the antibody datasheet or other web resources may also be consulted. Knowledge of the localization patterns also assists in selecting the optimal counterstain
- When using an antibody for the first time, always determine the optimal antibody dilution by titrating the antibody. You may also want to test different incubation periods and temperature conditions (4°C versus RT or 37°C)
- Should you encounter high background staining or suspect your primary antibody to be non-specific, consider including a concentration matched isotype control antibody (for example Mouse IgG2b Negative Control #MCA691XZ) or pre-immune serum in your experimental design

Tips for Optional Steps 12 and 14 – Incubate with Secondary Antibody and/or Amplification Reagent

- Especially for multiplexing experiments, it might be tempting to use directly conjugated primary antibodies to mitigate the risk of non-specific secondary antibody binding. Although these antibodies are suitable for the detection of certain high abundant targets, we generally advise using secondary antibodies in IHC experiments. Multiple secondary antibodies bind to a single primary antibody, thereby leading to signal amplification

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- To reduce the risk of background staining from non-specific secondary antibody binding, select cross-adsorbed/pre-adsorbed secondary antibodies. Also, include a secondary only control in your experimental design to control for this type of background
- For very low abundant proteins, we suggest using biotinylated secondary antibodies in combination with conjugated avidin (to form an avidin-biotin complex; ABC). Since a single avidin molecule can simultaneously bind up to four biotin molecules, this method results in higher signal amplification. Labeled streptavidin is now commonly used as a substitute for avidin in the Labeled Streptavidin Biotin (LSAB) method (Ramos-Vara 2005). Signal amplification can also be achieved by using the Peroxidase Anti-Peroxidase (PAP) and Alkaline Phosphatase Anti-Alkaline Phosphatase (APAAP) methods
- When using biotinylated primary or secondary antibodies, ensure that you have blocked endogenous biotin prior to primary antibody incubation (see tips for step 6)
- For tissues rich in Fcγ receptors, consider using fragment secondary antibodies such as Rabbit F(ab')2 Anti-Mouse IgG:HRP (#STAR13B). This type of fragment antibodies lacks the Fc region, thereby mediating Fcγ receptor interactions
- When selecting fluorophore conjugates, ensure that you are able to excite and detect the fluorophore optimally. Therefore, review the excitation and emission spectra of your fluorophore of interest as well as the lasers and filters of your microscope
- To reduce photobleaching (chemical destruction of a fluorescent dye), select photostable fluorophores belonging to new generation dyes such as Alexa Fluor and DyLight Fluor. Although still used in imaging applications, traditional dyes such as FITC are highly susceptible to fading/photobleaching and should therefore not be your first conjugate choice
- While spectral separation unmixing software has significantly advanced in recent years, for multiplexing experiments, we still recommend that you review the excitation and emission spectra of fluorophores in advance to minimize spectral overlap (Lavdas no date). Selecting fluorophores with no or very little overlap minimizes the risk of one fluorophore getting detected in another fluorophore's channel, a process commonly referred to as bleed-through, cross-talk or cross-over

Tips for Step 13 – Wash

Consider adding detergents such as 0.05% Tween 20 to the wash buffer to reduce high background staining or non-specific antibody binding. However, these detergents may not be compatible with the markings of hydrophobic pens that aid the compartmentalization of liquids on the slide.

Tips for Step 16 – Incubate with DAB or Other Substrates

With the increasing multiplexing and quantification needs, more sophisticated IHC technologies such as multiplexed ion beam imaging and mass spectrometry immunohistochemistry are being developed (Levenson et al. 2015, Angelo et al. 2014). Despite these advances, conventional chromogenic IHC is still routinely performed.

In contrast to fluorescent labels, enzymatic labels such as HRP and AP require addition of substrates. These are also referred to as chromogens, and when added to the enzyme, produce colored precipitates.

- Different enzyme/chromogen combinations produce different colored precipitates (Tables 2 and 3). A HRP/DAB (3,3'-Diaminobenzidine) reaction results in a brown precipitate, while using TMB (3,3',5,5'-Tetramethylbenzidine) as the chromogen for HRP yields a blue-green staining (van der Loos 2010). To achieve the desired precipitate color, determine the optimal enzyme/chromogen combination at the experimental design stage
- When choosing enzyme/chromogen combinations, precipitate color is not the only important selection criterion. To preserve the precipitate, ensure compatibility with your mounting medium of choice (see tips for step 20)
- Enzyme and substrate reaction efficiencies vary significantly. Therefore, less efficient reactions may result in lower staining intensities, which will impact primary antibody titrations (Van der Loos 2010). This is especially important when designing experiments with more than one chromogenic label. Ensure that you select the most efficient enzyme/chromogen combination for the least abundant antigen
- When simultaneous detection of more than one antigen is desired, confirm that your final precipitates and counterstains are easily spectrally distinguishable (see tips for step 18)

Table 2. HRP substrates and precipitate colors.

HRP Substrates/Chromogens	Precipitate Colors
AEC (3-Amino-9-Ethylcarbazole)*	Red*
DAB (3,3'-Diaminobenzidine)*	Brown*
DAB with $NiCl_2$**	Purple blue**
DAB with $CoCl_2$**	Dark blue**
TMB (3,3',5,5'-Tetramethylbenzidine)*	Blue-green*

* Van der Loos 2010
** Hsu and Soban 1982

Table 3. AP substrates and precipitate colors.

AP Substrates/Chromogens	Precipitate Colors
Fast Blue BB*	Blue*
Fast Red TR*	Red*/**
New Fuchsin***	Red***

* Lauter et al. 2011
** IHC World, no date a
*** IHC World, no date b

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Tips for Step 18 – Counterstain

Counterstains provide contrast as well as knowledge about localization within the tissue sample (for instance by visualizing nuclei or filamentous actin).

What to consider when selecting counterstains?

- Ensure that the counterstain can be easily spectrally distinguished from your precipitate color or fluorescent label. For example, when using red emitting fluorophores, select a blue nuclear counterstain such as DAPI or Hoechst 33342 (Tables 4 and 5)

Fluorescent counterstains

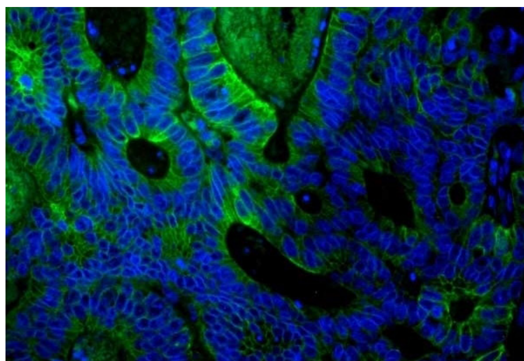

Fig. 3. PureBlu™ Nuclear Fluorescent Dye Hoechst 33342. Paraffin section of human colon adenocarcinoma (heat induced antigen retrieval with citrate buffer (pH 6), blocked with 10% FCS) stained with Mouse Anti-Human Cytokeratin 18 Antibody (#MCA1864H, green). Nuclei were counterstained with PureBlu Dye Hoechst 33342 (#1351304, blue).

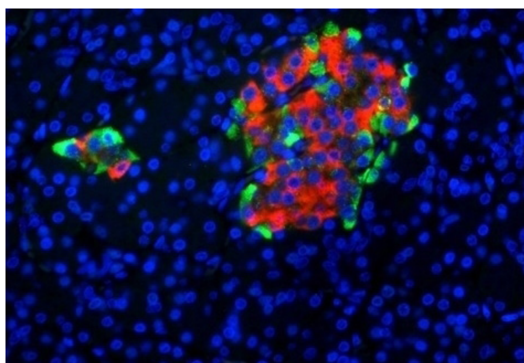

Fig. 4. PureBlu Dye DAPI. Paraffin section of human pancreas stained with Guinea Pig Anti-Pig Insulin Antibody (#5330-0104G, red), Mouse Anti-Human Chromogranin A Antibody (#MCA4773, green) and PureBlu Dye DAPI (#1351303, blue).

- To save time, consider using pre-made mounting media already containing counterstains

Table 4. Commonly used chromogenic counterstains.

Chromogenic Counterstains	Colors	Counterstain for
Eosin	Red	Proteins containing cationic groups*
Fast Red/Kernechtrot	Red	Nuclei
Hematoxylin (4 types - Harris's, Mayer's, Carazzi's and Gill's)	Blue	Nuclei
Methylene blue	Blue	Nuclei
Methylene green	Blue/green	Nuclei
Toluidine blue	Blue	Nuclei

* Kim et al. 2016

Adapted from Kim et al. 2016 and Paul no date. Fast Red/Kernechtrot has been included in the table according to IHCWorld (no date c)

Table 5. Commonly used fluorescent counterstains.

Fluorescent Counterstains	Colors	Counterstain for
DAPI	Blue	Nuclei
DRAQ5	Red	Nuclei
Hoechst 33258/33342	Blue	Nuclei
Propidium iodide	Red	Nuclei
SYTOX Green	Green	Nuclei
Phalloidin	Conjugate dependent	Filamentous actin

Tips for Step 20 – Mount Coverslip

Mounting media are essential when long term storage of slides is required (for instance when the slides are to be stored for future reference purposes). They enable permanent adherence of the coverslip to the slide, thereby protecting the tissue specimen from damage while simultaneously adding contrast during microscopy.

Mounting media categories:

1. Aqueous/water-based mounting media (hydrophilic; examples of aqueous mounting media include glycerine-glycerol and glycerine jelly) (Ravikumar et al. 2014)
2. Organic-solvent based mounting media (hydrophobic; examples of organic solvent based media include Euparal and Canada Balsam) (Ravikumar et al. 2014).

Mounting media can be further subcategorized into solidifying media or those that stay liquid (Microbehunter.com no date). In general, organic-solvent based media solidify while water-based ones remain liquid.

Factors to consider:

- Aqueous-mounting media are compatible with both fluorescent and enzymatic labels (Ravikumar et al. 2014). It is important to confirm that the medium is clear before application as cloudiness is a possible indication of bacterial or fungal growth (Kiernan no date). To mitigate the risk of contamination, ensure that the medium contains bacteriostatic agents (Ravikumar et al. 2014)

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- Organic mounting media should exclusively be used for mounting chromogenic IHC slides (Ravikumar et al. 2014). However, even for these slides, exemptions exist as some precipitates (for example by reactions with AEC) are soluble in organic mounting media (Howard and Kaser 2014). For these types of precipitates and substrates aqueous mounting media should also be used
- For wide-field microscopy, mounting media that solidify should be used (North 2006)
- To visualize 3D structures or to obtain 3D tissue information, mounting media that remain liquid are required (North 2006). When using this type of mounting media, the coverslip edges must be sealed with nail polish to avoid leaks (North 2006)
- Slides should be stored in the dark to prevent photobleaching/fading. The risk of photobleaching may be further reduced by selecting mounting media containing antifade reagents (North 2006). As certain fluorophores are incompatible with antifade reagents, it is important to first confirm suitability before using this type of mounting media

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