CHAPTER 10

Immunohistochemistry

IMMUNOHISTOCHEMISTRY NOTES

To start, a great resource for immunohistochemistry (IHC) is Hoffman et al.[24] I highly recommend that you read it if you are interested in learning more about IHC. The protocols that I describe in this chapter are adaptations of the protocols in that paper.

So what is IHC? It is the process of using antibodies to bind a protein in a tissue section and use some chemistry to visualize where that antibody bound (and therefore localize the protein). Immunocytochemistry (ICC) is almost the same thing as IHC; the steps are the same after fixation. ICC examines cell culture preparations and IHC examines tissue sections. Only sticklers like me insist on using the specific terms for the different kinds; most people will just call both IHC and ICC by the same acronym.

Antibody binding in tissue works a little differently from antibody binding in immunoprecipitation or immunoblotting. In those techniques, antibodies bind to proteins that are free in solution or fixed to a membrane. IHC binds proteins that are fixed into some three-dimensional conformation, whether by your tissue fixation solution or by being in their actual site of origin (like receptor proteins are fixed in the membrane). As such, there is a difference in both the number of binding sites and the types of binding sites available. An antibody that works in IHC may or may not work in immunoblotting or immunoprecipitation.

There are many different issues to consider when planning your IHC experiment; we'll discuss each in turn.

Types of IHC Staining

There are currently two main branches of IHC staining: colorimetric and fluorescent. Colorimetric IHC, much as in immunoblotting, forms a colored precipitate at the site of antibody binding to protein. Unlike immunoblotting, however, colorimetric staining can be permanent, as the reaction product is held in place (which can be easily washed off of

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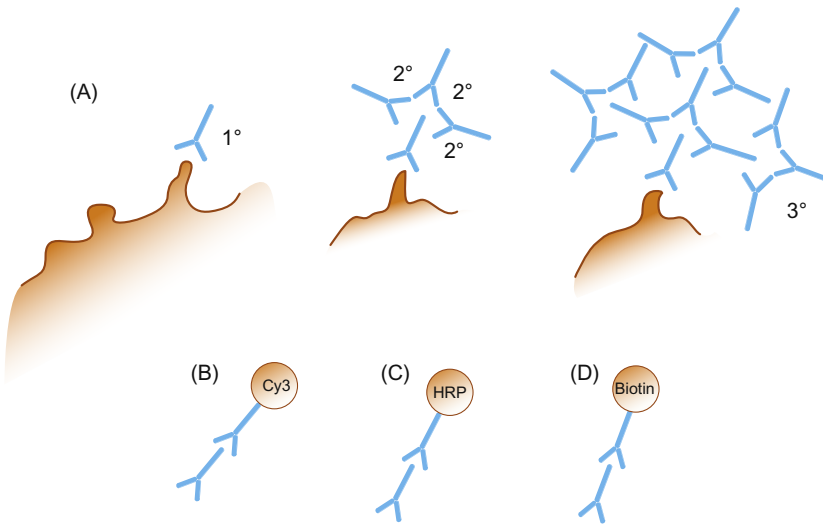
blotting membranes); permanence depends on the stain of choice. Typically, colorimetric staining is used for qualitative experiments ("Where's my protein?"). While some groups claim to pursue quantitative colorimetric staining, I don't buy it: colorimetric staining works by enzymatic action and therefore the reaction continues until there are no more reagents or there is a loss of physical space to deposit more reactant, and either condition can make it appear to have more or less protein.

Many colors are available for colorimetric staining (Vector Labs sells a number of good reagents). I typically perform colorimetric staining with 3,3-diaminobenzidine (DAB) with nickel salts (Ni-DAB). DAB, by itself, stains the entire tissue slice a light brown, while a darker brown indicates the presence of your protein. Ni-DAB stains black and white and is easier on my eyes to see the difference between staining and not staining (black and white tissue slices are easier to examine than gradients of brown); additionally, Ni-DAB stains slightly blue and brown if your titration isn't optimal, and this really helps to decide how much antibody to use. The other benefit of DAB (nickel or not) is that the product is permanent, while some other colorimetric stains are not permanent. Colorimetric stains may need a counterstain (like cresyl violet or methyl green) in order to see structures appropriately.

Fluorescent IHC is when your antibody (primary, secondary, tertiary, or streptavidin, or whatever you use to bring in your signal molecule) is conjugated to a fluorescent molecule. Fluorescence is, in my opinion, preferable for quantitation and multiple labeling experiments in IHC. However, while fluorescent IHC reactions are shorter overall and easier to perform, there is a big cost: the fluorescence is dying out as soon as you're done with the experiment. Even with antifade reagents, after a month of being kept in the dark and in the refrigerator, fluorescence is lost from slides, gone, repeat the experiment. As such, it's usually best to check your fluorescent IHC either the day you finish the experiment or within a week of doing so; if you choose to count after a couple of days (some report that this lowers background noise), then choose a consistent timeframe for examination so that any quantifiable changes are not due to differences in fluorescent quenching. The two available fluorescent counter stains are 4′,6-diamidino-2-phenylindole (DAPI) or propidium iodide (PI). Both of these bind to DNA and therefore stain nuclei: DAPI stains blue and PI stains red/orange. If you're using a red/orange fluorophore, then don't use PI. The resolution of these counter stains depends on the thickness of your slice; slices as thick as 50 μm can hold three or more layers of cells, and therefore complicate analysis of the counterstain.

Secondary Antibodies and Blocking Buffers

As in immunoblotting, we use blocking reagents to block nonspecific antibody binding in the tissue, so that the staining we observe is just due to our primary antibody. Typically, BSA, casein, and normal serum are used as blockers. There are commercial blockers available, but I don't like to use them because I don't know what's in them. If they happen to include BSA, then I can use goat antibodies all day long and not know why I'm getting such high background signal. If they include rabbit serum, then my rabbit antibodies will look especially terrible and I won't know because the block solution's components are protected information! I use serum to block and try to use secondary antibodies from a single host (at least within a single reaction; see Multiple Labeling section). Avoid using milk as a tissue blocker; it has so many different protein components that stick all over the tissue slice that you get some background and/or ugly slices (including biotin, which will give false positives in an avidin–biotin complex (ABC) reaction, and phosphoproteins, which can give false positives for phosphorylated targets).[25] You can test different blockers for your reaction, but I've had good experiences just using serum from the secondary antibody host animal (e.g., use normal goat serum to block if you're using a goat secondary antibody).



Amplification of the Signal

Amplification of the signal is admittedly more common in IHC than in immunoblotting. So what do I mean by amplification? Let's say

that you have a protein of interest that has three binding sites for your primary (first) antibody (all of these numbers are examples). If you directly conjugate some signal molecule to your primary antibody, there will be three signal molecules (max) per copy of your protein of interest. Three signal molecules don't generate a lot of signal. Well, let's add a secondary antibody to the mix, and say that there's four sites for the secondary antibody to bind your primary (there's typically a lot more, but this is an example). If each secondary antibody has a signal molecule conjugated to it, there are now 12 signal molecules (max) per copy of the protein of interest (see figure above). I routinely use a form of amplification called the ABC reaction (comes in a kit; get the unmixed kits, if possible). Avidin is found in egg whites, but streptavidin (from *Escherichia coli*) is a much more stable form of avidin. I use a biotinylated secondary antibody, and each biotin has four binding sites for avidin. Free biotin will bind avidin, and these will continue to bind each other in solution until there is a huge complex of molecules with signal molecules on them (HRP conjugated to streptavidin, in fact). I have also used streptavidin alone with a biotinylated secondary antibody (no free biotin, therefore not an ABC reaction) to amplify my signal; in keeping with the above example, streptavidin amplification would look like this: 3 primary binding sites $\times$ 4 secondary binding sites $\times$ 4 avidin binding sites = 48 signal molecules per protein (max). There are also kits incorporating tyramide amplification, which amplifies the signal greatly.

Multiple Labeling

What kind of experiment are you performing? Are you hoping to examine multiple proteins in the same tissue section? While that can be done, there are some additional considerations for that experiment. First, multiple labeling experiments work much better if your primary antibodies are made in different hosts. There are ways to "convert" a primary antibody to another "host" but are far more cumbersome than using antibodies from a different host. For example, one can use goat anti-rabbit Fab fragments to convert a rabbit antibody to a "goat" antibody and use anti-goat antibodies conjugated to a signal molecule to get data. Second, multiple labeling experiments work best if your secondary antibodies come from the same host. As an example: mouse anti-tyrosine hydroxylase and rabbit anti-calbindin primary antibodies are used with goat anti-mouse and goat anti-rabbit secondary antibodies. This combination will stain well together (different

hosts for primary antibodies) with low background (one host for the secondaries means that I can block with only goat serum; if secondaries were from different hosts, I would need normal serum (plural *sera*) from each, and the secondaries might cross-react with the different sera). Multiple labeling works best with fluorescent detection; while colorimetric staining can work with multiple labeling, colorimetric staining is forming a precipitate, and one precipitate might crowd out the other (both in terms of not being able to see one precipitate, and physical space for the chemical reaction). There are some who perform multiple labeling with colorimetric systems and have found ways to use the same enzyme (HRP or AP) for both stains,[26] since HRP and AP have different sensitivities. Additionally, while this may be elementary, double-check that your secondaries (or whatever you're using) are conjugated to different fluorophores that show up on different parts of the visible spectrum (both excitation and emission spectra); any bleed over in light emission will give false positives.

ANTIBODY TITRATION PROTOCOL (NI-DAB)

I begin with this protocol when I acquire a new antibody. This gives me an idea of what titers work for staining and which do not. This protocol (mostly) follows the advice of Hoffman et al.[24]

1. Remove tissue slices from cryoprotectant and place into PBSX.
2. Three washes, 10 min each ($3 \times 10'$), in PBSX to wash out cryoprotectant solution.
3. 15′ wash in 1% hydrogen peroxide (most fixatives) or 1% sodium borohydride (if acrolein or glutaraldehyde was present in the fixative).

> *In my experience, this is an important step. We wash in peroxide/$NaBH_4$ to decrease background noise in the ABC reaction. Ni-DAB precipitates are formed by the reaction of HRP (from the kit) with peroxide. Red blood cells also express a peroxidase, and applying peroxide this early exhausts the ability of that peroxidase to catalyze reactions. The borohydride is used because acrolein forms free aldehydes as part of its fixation process; these aldehydes can bind antibodies and give false positives. Borohydride reacts and frees aldehydes from solution in addition to reacting with peroxidase. You'll see bubbling either way. On another note, 30% hydrogen peroxide is not as stable at 3% hydrogen peroxide. 3% is the bottle you get from any pharmacy for less than $2 USD. I buy the 3% and don't have to worry about degradation of 30% peroxide, which has caused me to get false negatives because the Ni-DAB precipitate never formed.*

4. $3 \times 10'$ washes in PBS.
5. 60 min in blocking solution.

   As described above, I prefer to use serum for my blocking solution. I have noticed absolutely no difference in blocking quality between 1% and 10% serum solutions for most antibodies. Most protocols recommend 10%; I only use 1% serum unless it becomes obvious that I need to do otherwise.

6. Put tissue into primary antibody solutions. I typically run a logarithmic curve of antibody dilutions: 1:1000, 1:3000, 1:10,000, 1:30,000, 1:100,000, and 1:300,000.

   While it may be tempting to avoid performing the whole curve, my experience has been that some antibodies work at much lower titers than many researchers expect. A goat anti-calretinin antibody from Chemicon that I've used didn't even show staining until the 1:100,000 dilution. Unless under extreme duress, just test the whole curve. This step is part of why I'm skeptical about the titers reported by different companies.

7. Leave tissue in primary antibody solution for 48 h at 4 °C.
8. After the 48 h incubation, $3 \times 10'$ in PBSX. Save antibody, if desired.
9. 2 h in secondary antibody solution.

   Most people use secondary antibodies at concentrations like 1:200 or 1:600. I think both of these are a waste of secondary. I use 1:1000 and have seen no difference in staining between 1:500 and 1:1000. I could probably use less, but secondary antibodies are comparatively cheap.

10. $3 \times 10'$ PBSX.

    Because most ABC solutions require 30 min to incubate, I prepare the solution just before I start these washes.

11. $60'$ in ABC solution. I use much less solution than the company recommends; see Solutions and Notes.

    The company says to use drops; some protocols say to use a 1:100 dilution of A and B solutions. I use 45 μl of A and 45 μl of B per 10 ml of total solution; much less than recommended to use, but my picture quality is great every time.

12. $2 \times 10'$ in PBS.
13. $5'$ in acetate buffer.

14. 20′ in Ni-DAB solution.

    While most people think 20 min is insane, allowing the reaction to proceed for such a long period of time (including 48 h of primary incubation) allows me to use much lower titers of antibody than otherwise. Most people only want to leave antibodies on tissue overnight; just try 48 h and see if your picture and antibody titer improve.

15. 5′ in acetate buffer.
16. 2 × 10′ in PBS. Mount tissue on slides (make sure they are flat!), allow to air dry overnight. Follow with coverslipping protocol, moving through graded ethanol solutions, then delipidizing tissue using xylenes (or available alternatives). Apply some adhesive and put coverslip on, gently pressing on coverslip to remove bubbles. Allow to dry (hours) and view under light microscopy.

    Staining should appear black on a white or very light background. Sometimes the DAB itself lightly stains different structures, acting as its own counterstain. If staining is brown or blue-black, that titration is not optimal (typically the titer is too high). Note that positive and negative controls (like omitting the primary antibody) are very important here, because Ni-DAB can generate a lot of extra noise that, if staining is very weak, would be indistinguishable from a weak signal. This noise is present even after filtering the Ni-DAB solution.

ANTIBODY TITRATION PROTOCOL (IMMUNOFLUORESCENCE)

This follow-up to the Ni-DAB titration follows the advice of Hoffman et al.[24]

1. Remove slices from cryoprotectant and place into PBSX.
2. 3 × 10′ PBS to wash out cryoprotectant.
3. 60′ in blocking buffer.
4. Place tissue into primary antibody at 10×, 30×, and 100× the amount of antibody that you determined was optimal using the Ni-DAB titration. If you want to use a biotinylated secondary with fluorophore-conjugated streptavidin, use 10×, 20×, and 30× the amount of antibody that you determined was optimal using the Ni-DAB titration.

    This part can require additional titrations, but I have typically found that company datasheets give a much higher titer (more concentrated) than I successfully used in my own IHC. The additional titrations are usually worth it.

5. Save antibody if desired. $3 \times 10'$ in PBSX.

   If using a monoclonal antibody, this is only to reuse the antibody. If you are using a polyclonal antibody, running the antibody through a reaction once is a cheap form of affinity purification: the various antibodies have already bound the nonspecific proteins and parts that they could, and so (in theory) the remaining antibody is specific for your target. You could, alternatively, do a similar purification by incubating your polyclonal antibody in tissue where the target is knocked out.

6. 2 h in secondary antibody solution.

   Some people increase the amount of secondary antibody because they're performing immunofluorescence; I still use 1:1000 and it works just fine. I have seen no difference in staining between 1:500 and 1:1000 dilutions of the secondary antibody.

7. If using streptavidin, wash $3 \times 10'$ in PBSX, followed by $60'$ of streptavidin.

   I use the fluorescent streptavidin at 1:1000, just like the secondary antibody.

8. $3 \times 10'$ PBS, mount tissue. Allow to air dry (20 min), cover tissue with antifade reagents, and place coverslip on slide. Gently press on coverslip to remove bubbles. Allow to air dry (20 min or overnight) and apply nail polish (or some plastic adhesive) to edges to seal slide. View under fluorescent microscopy within a week.

   Antifade reagents are crucial but not perfect. Even with antifade reagents, my FIHC signal is gone if left in the dark and in the cold for a month. While you can get a counterstain (like DAPI) included in the antifade reagent, it's not necessarily a good idea if you want to pursue confocal microscopy. The excess DAPI in the reagent can overwhelm the microscope's detection ability; use a separate DAPI reaction if you want the counterstain or at least check if the microscope you wish to use can handle the DAPI.

Staining should appear brighter than background fluorescence. Adjust the fluorescent lamp output so that you see immunoreactivity higher than background noise. Use the lowest amount of antibody possible while getting the same amount of immunoreactivity.

IHC PROTOCOL

The IHC protocols are the same as the titration protocols, except at the titer you calculated for your own antibodies.

CRESYL VIOLET COUNTERSTAIN (NISSL STAIN) PROTOCOL[27]

1. For free-floating sections, wash slides briefly in dH_2O to remove any residual salts.
2. Immerse slides through two 3′ changes of 100% ethanol.
3. Immerse slides through two 15′ changes of 100% xylenes (or alternative).
4. 3×10 min in 100% ethanol.
5. Wash in dH_2O briefly.
6. Stain in Cresyl Violet solution for 15 min.
7. Rinse in dH_2O briefly.
8. Wash in 70% ethanol briefly.
9. 2 min in differentiation solution.
10. Two 3′ changes of 100% ethanol.
11. Two 3′ changes of 100% xylenes, coverslip. Allow to dry in fume hood.

Any counterstain should be undertaken with an understanding of the original stain involved. For instance, DAB precipitates are insoluble in water and alcohol but are sensitive to pH. Older xylenes and alcohols can have pH changes that affect DAB deposits, especially if those deposits are on the surfaces of cells. Slices can show excellent Nissl staining while your original staining has disappeared.

IHC CONTROLS

1. *Negative controls*: control tissue (knockout tissue, tissue that doesn't express the protein, cell lines that don't express the protein) can be useful to identify spurious binding of the primary antibody. Remember that knockouts aren't always perfect: virally induced knockouts aren't 100% effective and may still show some staining (if they don't infect every cell), while some knockouts still generate a truncated (nonfunctional) protein that may still be recognized by your antibody.

2. *Positive controls*: control tissue that does express your protein of interest can identify primary antibody problems, including low antibody titers (primary and/or secondary) or blocking issues.
3. *Peptide preincubation control*: while incubating the antibody with the antigenic peptide before exposing the antibody/peptide mixture to tissue (called a peptide preincubation control) has been a popular choice, it's not necessarily a good control. If you don't see staining after a peptide preincubation control, it can mean the following:
   a. The antibody doesn't bind spuriously, as it bound the peptide completely.
   b. The antibody has a higher binding affinity for the free peptide than whatever it did bind in your tissue (which can still be nonspecific!).
   c. You preincubated in the wrong conditions and now the antibody has no activity (to solve this, simply leave both your antibody with peptide and your antibody alone solutions in the same conditions).

   If you see staining after a peptide preincubation control, it can mean the following:
   a. The antibody binds spuriously, as it didn't bind the peptide completely.
   b. The antibody has a higher affinity for the fixed target than the free peptide.

   I consider the preincubation control to be better than nothing, but the results are ambiguous without further testing; combine it with other controls.
4. *Omit primary antibody*: tests if the secondary antibody or other signal-generating components in the reaction bound to your tissue and gave false positives or background noise.
5. *Omit primary and secondary antibodies*: identifies spurious binding from additional signal amplification components (such as endogenous biotin giving a false positive for the ABC reaction). Unnecessary if omitting the primary antibody doesn't lead to any staining, noise, or otherwise.
6. *Single-color controls*: for multiplex fluorescent IHC, this can identify if your antibodies are binding each other instead of the target

proteins, and if your fluorophores are "bleeding over" into each other's channels (and therefore giving you false positives).

7. *Fixation controls*: some antibodies will not find their targets if the tissue is fixed with incompatible fixative. Additionally, some fixations are just not as good as others, and it can be useful to have a "known good" set of fixed tissue to compare to new tissue to determine if the tissue fixation was acceptable.
8. *Multiple antibodies against different parts of the target*: expensive, but useful, as it is in immunoblotting. If you have multiple antibodies that target different parts of the same protein (one targets the C-terminus, and the other an internal portion, for example), check if their staining patterns are the same. If so, that's a good indication of specificity. Combine with other controls.
9. *Colocalization with mRNA*: combine IHC with *in situ* hybridization (ISH) to see if your antibody staining overlaps with the mRNA staining (though each stain may be in a different compartment of the cell, depending on your target). A very convincing control, if difficult to pull off, because optimal ISH conditions may be incompatible with optimal IHC conditions.
10. *Use antibody in immunoblotting to validate antibody*: this control can be useful but has some drawbacks. This control is considered a success if your immunoblot has bands at the correct weight(s). However, immunoblotting and IHC use antibodies to identify targets in very different scenarios: immunoblotting examines a protein fixed to a membrane, while IHC examines a protein fixed within tissue. Many antibodies that work for one technique don't work for the other, which can give a false negative. Further, antibodies that are specific for one technique can have spurious binding when used in the other technique. This control can also take a lot of time, as you would need to perform controls for both techniques in order to truly assess specificity. While this can suggest specificity, it is best combined with other controls.

IHC Troubleshooting

Issue	Possible Cause	Possible Solution
High background:	Nonspecific binding.	*Test antibodies on positive and negative control tissue; increase blocking reagent or get new antibodies. You can also incubate secondaries at 4° C.*
	Fluorophores have similar emission wavelengths.	*Choose fluorophores with nonoverlapping spectra; it'll make the experiment go much more smoothly.*
	Endogenous biotin.	*Only some tissues have this problem; use a biotin/avidin blocking solution before primary antibody incubation.*
	Avidin introduced into system (issue for ABC amplification).	*This only really happens if you study chickens or you put the tissue in a gelatin/egg yolk mixture and got some egg white contamination. Choose a different amplification technique or start over.*
	Insufficient blocking.	*Increase block time or reagent concentration (1–5% serum, for example).*
	Incorrect antibody titer.	*Perform antibody titration. Some antibodies need high titers to show up, some don't show up until very dilute titers.*
	Primary antibodies bind to each other instead of targets.	*Use single-color/single-antibody controls to determine if this is the case. Don't mix the antibodies again if you know they bind each other.*
Low signal:	Poor or over-fixation.	*Compare newly fixed tissue to known good tissue to determine if this is the case. Start over if that's the case.*
	Excessive blocking.	*Lower block times or reagent concentrations (from 5% to 1% serum, for example).*
	Incorrect antibody titer.	*Perform antibody titration. Some antibodies need high titers to show up, some don't show up until very dilute titers.*
	Not enough signal amplification.	*Not typically an issue when using the ABC kit, but you can add tertiary amplification (streptavidin, tertiary antibodies, and tyramide kits).*

SOLUTION RECIPES

DAB solution (3.3 mg/ml)

DAB	330 mg
Acetate buffer	100 ml

Aliquot and freeze solution at –20°C. Can reuse aliquots after thawing. Avoid exposure to light.

$NiSO_4$ solution (416.7 mg/ml)

$NiSO_4$*$6H_2O$	41.67 g
Acetate buffer	100 ml

Aliquot and freeze solution at –20°C.
Can reuse aliquots after thawing.

AB solution (0.4% Triton X-100)

Triton X-100	800 ml
1× PBS	200 ml

PBSX (0.1% Triton)

5× PBS	200 ml
Triton X-100	1 ml
dH_2O	To 1 L

1% serum solution

Normal serum	200 µl
1× PBS	To 20 ml

ABC solution

A	45 µl
B	45 µl
AB	To 10 ml

1% sodium borohydride

$NaBH_4$	0.3 g
1× PBS	To 30 ml

Streptavidin solution

Streptavidin	Varies
AB	Varies

Cresyl violet solution

Cresyl violet acetate	1 g
Acetate buffer	To 500 ml

Acetate buffer

Na acetate	1.45 g
dH_2O	100 ml

pH to 7.5–7.6. Make fresh.

1× PBS

5× PBS	200 ml
dH_2O	To 1 L

5× PBS

Na_2HPO_4	9.2 g
NaH_2PO_4	2.35 g
NaCl	40.9 g
dH_2O	To 1 L

Antibody solution

Normal serum	200 µl
AB solution	To 20 ml

Vary the amount of solution and antibody based on your needs.

Ni-DAB solution

$NiSO_4$ solution	2.4 ml
DAB solution	2.4 ml
Acetate buffer	35 ml

Filter Ni-DAB solution in a Whatman filter (1 or 3 works) before use. Add 300 µl of 3% hydrogen peroxide just before use.

1% hydrogen peroxide

3% H_2O_2	10 ml
1× PBS	To 30 ml

Differentiation solution

Glacial acetic acid	4 drops
95% ethanol	200 ml