

Introduction

The point of immunohistochemistry is to detect and specifically label antigens (mostly proteins) in biological tissue. It makes use of the vertebrate immune response which produces antibodies that recognize and bind to specific antigens. An antigen of interest is injected into an animal (e.g., a rabbit or a mouse). The cheaper way to go from there is to harvest polyclonal antibodies from serum of the animal, the more elaborate one to fuse myeloma cells with B cells from the animal's spleen into so-called hybridoma cells, separate them into cell lines, screen them for their specificity and then harvest *monoclonal* antibodies. Either way, a lot of testing for specificity (Western blots, etc.) has to be done, but I'm going under the assumption here that this has been done for the antibody you want to use and the only objective is to actually stain the tissue. Once the antibody detected an antigen, it has to be visualized to be useful for microscopy. Non-fluorescent methods are usually based on enzyme reactions and lead to dark precipitates that can easily be detected in conventional brightfield transmission microscopy. I am only going to consider fluorescent applications here. In almost all cases, the fluorescent tag is not put directly on the primary antibody. Instead, so-called secondary antibodies are used. Secondary antibodies are raised against immunoglobulins of the donor species in a different species. For example, if the primary antibody was raised in a rabbit, you could use an antibody against rabbit IgG raised in goat (goat anti-rabbit). The advantage is that the secondary antibodies are generic (can be used against any primary antibody from the target species). In addition, it allows you a free choice of fluorescent (or other) tags. Finally, as secondary antibodies are polyclonal and therefore detect more than one epitope, more than one fluorophore can be coupled to each target protein and the signal is amplified. To amplify even more, you can use a secondary antibody conjugated to an amplifier (like biotin), and then use a third step to bind multiple fluorophores to that.

Critical considerations for immunohistochemistry are tissue fixation, permeabilization to allow the antibody to penetrate the tissue, and minimization of unspecific staining.

Fixing

The point of tissue fixation is to kill the tissue and repel microorganisms to stop autolysis by proteolytic enzymes and bacterial digestion. In addition, it increases the strength and rigidity of the tissue to preserve morphology. The main mechanism of fixation is denaturing and crosslinking of proteins. Actually, there are types of tissue fixation that have been used for ages in everyday life, namely "pickling" in alcohol or vinegar. Precipitating or denaturing fixatives like ethanol, methanol, acetone, or acetic acid reduce the solubility of proteins, disrupt hydrophobic interactions responsible for their tertiary structure, and precipitate and aggregate them. For the purpose of preserving tissue structure such

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fixatives are not very effective because they obscure the epitopes for antibody detection and cause a lot of shrinking and swelling. Therefore, almost all fixation for scientific purposes is done with aldehydes, which act by crosslinking proteins. Most commonly used are formaldehyde and glutaraldehyde. Glutaraldehyde crosslinks proteins much better than formaldehyde, but because of its size it doesn't penetrate the tissue as easily and, more importantly, it causes a lot of background fluorescence. It is particularly good for electron microscopy, but not a good choice for immunohistochemistry, particularly in wholemount fluorescence applications. The fixative of choice for standard immunohistochemistry is formaldehyde, which has good tissue penetration and doesn't cause background fluorescence. It acts primarily through lysine-crosslinking. Usually it is purchased as paraformaldehyde, a polymerized version of formaldehyde that comes as a white powder and is then dissolved in buffer and used at 4%.

Tissue penetration

To ensure good penetration of the antibody into the tissue, it is advantageous to remove lipids and maybe even permeabilize extracellular matrix. How critical this is depends on the tissue and there are a number of different ways to achieve that. The easiest and most gentle way is to use buffer solution with Triton-X, a detergent ("soap"). This is usually sufficient for the STG, which is a fairly small and not very densely packed tissue. If it is not sufficient, the next thing to try is to dehydrate in an ethanol series and then to rehydrate again. Significantly more efficient (but even harsher on the tissue) is to apply an organic solvent (methyl salicylate, benzene, toluene, or xylene) between the ascending and descending ethanol series. If all of that is not enough, you can use enzymes like collagenase. The insect neuroanatomy literature should be full of examples of how to do that.

Blocking

The signal-to-noise ratio in antibody staining is dependent on many things. Optimizing the concentrations of primary and secondary antibodies is important, but there will be large differences between different antibodies, so there is little general advice to give. However, a substantial part of non-specific staining can be blocked by Normal Serum. Usually, 5-10% normal serum is used. It should come from the same host species as the secondary antibody. The idea is that the IgG in serum occupies "sticky" binding sites to prevent non-specific binding. It therefore shouldn't come from the same host species as the primary antibody. Another possibility for universal blocking is bovine serum albumin (BSA), but we usually go with normal goat serum (because our secondary antibodies are usually from goat).

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The actual protocol

Preparation: Antibodies and some of the other solutions are expensive, so you want to minimize the liquid volume. If you start out in a physiology petri dish, repin the ganglion in a Sylgard dish you keep for that purpose and don't mind getting cut up. You don't want the fixative etc. to come into contact with a dish you want to reuse for physiology. I recommend making some dishes with a fairly thin Sylgard layer. After fixing and the first washes, cut the Sylgard around the ganglion so you can lift out a small block of Sylgard with the STG pinned to it. Stick a minuten pin into one corner of the block that you can use as a handle. You can now transfer the block between small vials for the rest of the protocol, instead of having to use 10-20 ml volume in a petri dish.

- 1) Fix in paraformaldehyde (4% in buffer). Do this under the hood. Leave for ~2h. 1h is probably fine, too. However, never fix overnight. "Overfixing" has a tendency to obscure epitopes and hardens the tissue too much. Instead of making fresh paraformaldehyde every time, I prefer using EMS 16% vials that I dilute to 4% in buffer.
- 2) Discard the paraformaldehyde (waste bottle under fume hood!) and wash 5 x 10 min in 0.1M phosphate buffer solution + 0.3% Triton-X.
- 3) Cut out the block of Sylgard and transfer to vial with the primary antibody (at whatever concentration) in Triton-buffer and 10% Normal Goat Serum. For most antibodies, 12h (overnight) should work fine. More than 2 nights usually is bad because it increases background. If available, put the vial on a shaker or rotator in the coldroom.
- 4) Wash in Triton buffer 6 x 10 min. You can either do that by replacing solution in the vial, or probably more effectively by transferring the Sylgard block into a fresh vial every time.
- 5) Incubate in secondary antibody in buffer (no Triton needed from here on). We usually use Alexa-conjugated ones at concentrations between 1:400 and 1:800. There is some sense that lower concentration for 2 nights give you better stains than higher concentration for 1 night, but we haven't tested that rigorously. Either way, do this in the dark (fluorophore bleaching!), or at least with aluminum foil wrapped around the vial.
- 6) Wash in buffer 6 x 10 min.
- 7) If you are lazy and don't care too much about the general shape of the tissue, you can now unpin the STG (easiest way is to cut all nerves proximal to the pins), transfer it to a microscope slide and mount in glycerol or any glycerol-based solution containing anti-fading agents.
- 8) If you want to do it right, you should mount in methyl salicylate. Instructions for clearing and mounting are in a separate document.