Immunohistochemistry in the Rodent Brain: A Broad Lab Manual for Preparing Tissue and Using Antibodies to Robustly Reveal Antigens

by

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Disclaimer: I am still working on putting in citations and references over time. I had originally intended this handbook to be an internal document when I started it. But, now that I am making it available externally, this necessitates making it clear who should be credited with what ideas, where possible. For the vast majority of the examples and protocols described in this document, I am ***not*** the person who originally developed them. This document is just a place where I have gathered my notes on so many IHC-related readings and ideas over the years. If you are unsure who to credit, please use a web search to find the relevant article(s). If you are crediting me from something you read here, please ask first – I don't want someone else's hard work misattributed to me!!!

Tips: You can use the search tags in this Contents section, denoted in brackets, to jump to the sections of interest quickly by hitting Ctrl and F, then typing in one of those tags (Cmd F on Macs). Additionally, if you activate the Navigation Pane, you can skip to the heading of your interest that way.

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Part 1: Tissue Processing and Staining Procedures

I. Introduction [INR]

The following is a guide for preparing and staining brain specimens using immunohistochemistry (IHC) and/or immunofluorescence (IF). This guide is not comprehensive, and any guide should always remain open to updates as things change in a lab. The old phrase "if it ain't broke, don't fix it" only goes so far; IHC protocols always have either room for improvement or room for tweaks to fit specific project goals, as long as such changes are justified. Herein you will find various protocols, amplification techniques, order information for new supplies, recipes for new reagents, and much commentary for those who have questions that are not easily answered by literature/web searches. I have been using these reagents and techniques for immunofluorescence for the latter half of my grad work, and this work has yielded consistent staining results.

This guide only includes a little troubleshooting advice – there are many troubleshooting guides online, and some errors may be more easily revealed by comparing notes with researchers from other labs. In that line of thought, do make sure that you are taking extensive notes, as such notes not only allow for easier identification of mistakes, but also ensure proper planning and consistency in the staining process. One should have three sets of notes for every staining project – those describing the staining plans prior to executing them, those showing how the staining was actually executed (as this may not be identical to initial plans), and those describing the analysis of tissue and staining results.

II. Perfusion and Post-Fixation [PRF]

NOTES: Read your lab's notes for how to perform a transcardial perfusion if you are unfamiliar with the procedure. The following procedure is what I had used in my graduate student and post-doc years. The perfusion procedure is important for removal of blood cells from the vasculature in the brain, as well as delivering the fixative solution into the brain internally. Some labs extract whole brains without perfusion and just do the "post-fix" (which is just incubation in a fixative solution), but this will typically result in tissue that is less usable for IHC staining.

1. Ensure that you have one of the following:
    1. Gravity perfusion setup: Two 1 L flasks each with hose attachments. The type of flask you should use is depicted to the right. Also, two clips that will pinch each hose to prevent fluid from flowing. Pick clips that apply a decent amount of clamping pressure, but not so much that they would crush and rupture the hoses. **Start with both hoses clamped before proceeding!**
    2. Pump perfusion setup: two 1 L beakers plus a perfusion pump and one hose.
    3. If you have neither, two large syringes (50 mL or greater) can be used instead. But, be aware that this approach will wear down your hand strength due to the sustained force



required on the syringe plunger. Consider using a syringe pump if you have one on hand.

2. If using either setup with a hose, you will need to insert part of a 10 mL syringe inside of the other end of the hose. Remove the plunger, cut off most of the back (perhaps down to the 0.3 mL mark, insert this into the hose. The hose's inner diameter should fit extremely tightly around this syringe - no leaks, and very unlikely to dislodge under pressure.
3. Obtain a large needle (18-21 gauge) to attach to your syringe tip. **Keep the needle guard**.
4. Remove the needle guard, and cut off about 1 cm from the end of that guard. Reattach the guard to the needle.
    1. You've now created a "stop" that will prevent the needle from being inserted too deeply during the perfusion procedure.
5. Set up everything within a fume hood. Grab the necessary surgical tools, a basin to collect blood and perfusate, and a sturdy grating to put on top of that basin.
    1. For the grating and basin, people often use a standard-size plastic rat cage and its accompanying metal top.
6. Fill the first beaker/flask with 0.9% saline (NaCl).
    1. Dissolve 4.5 g NaCl in 250 mL distilled water. Does not need to be sterile. Scale up for every rat you are perfusing.
    2. The saline should be at room temperature or near body temperature, near pH 7.4, and can be more effective with vasodilators and/or anti-coagulants added. See Part 2, Section IV of this manual.
7. Fill the second beaker/flask with freshly made (within the past 5 days) 4% paraformaldehyde (PFA) solution.
    1. See Appendix F for the recipe to make this solution. *Must* allocate time to make this in advance.
    2. Assume you will use 250 mL or greater per rat. Usually ~150 mL is expended for a ~400 g rat over a 15 minute perfusion.
    3. Can also be at room temperature or warmer during the perfusion procedure to prevent vasoconstriction.
8. Attach needle + guard to your saline source.
9. In any hoses & syringes, flush out any air and major air bubbles.
10. Anesthetize subject, open chest cavity when ready, and clamp descending artery.
11. Create incision or cut in the rat's right atrium of the heart (top left from our point of view.
    1. Serves as a drainage point so that the fluid we are perfusing can have an outlet somewhere.
12. Transcardially perfuse rat with saline solution by pushing the needle into the rat's left ventricle (right side from our point of view, piercing the apex of the heart). Flush is adequate when paws, lungs, and nose turn white. Avoid flushing for longer than a few minutes.
    1. If using 50 mL syringe, may need to re-fill. Leave needle + guard inserted in heart, but detach them from the syringe and refill the syringe.
13. Switch from saline to formaldehyde. Depending on which perfusion setup you have,
    1. Gravity:
        1. Clamp the saline hose shut.
        2. Take the needle + guard attachment off the saline hose and attach it to the formaldehyde hose. Secure tightly.
        3. Unclamp the formaldehyde hose.
    2. 50 mL syringe:
        1. Take the needle + guard attachment off the saline syringe and attach it to the formaldehyde syringe. Secure tightly.
        2. Apply pressure as normal.

3. Pump:
    1. Turn the pump off.
    2. Move the other end of the hose from the saline beaker to the PFA beaker.
    3. Turn the pump back on.

14. When specimen is well-fixed, stop fluid flow (clamp hose shut OR turn off pump OR stop applying pressure to syringe).
    1. Usually fixation time takes 10-15 minutes, and/or uses ~150 mL.
15. Remove needle with fluid source attached.
16. Remove any clamps/clips from subject and drain out chest cavity.
17. Decapitate subject and remove rat brain.
18. Put rat brain into a vial. Usually a 15 mL conical tube or a glass scintillation vial will fit the brain sufficiently.
19. Add same 4% PFA solution into the vial until the brain is more than submerged.
20. If you are not perfusing any more rats, clean up all equipment and waste.
    1. Drain blood + formaldehyde solutions into a dedicated hazardous waste container. Ensure that you have proper labeling of the container and plans for pickup by your hazardous waste collection service. Make sure your waste container is stored in secondary "spill" containment.
    2. Wash all tools used. Leave metal tools open and out to dry to prevent rust.
21. Post-fix brain at 4 degrees C (fridge, not freezer) **no longer than seven days**; overfixation causes masking or degradation of antigens.
    1. Three days should be a good balance of maintaining antigenecity while making the tissue sufficiently sturdy for processing.
    2. However, fixation for multiple weeks won't be a deal-breaker. There will just be slowly diminishing antigenicity as fixation duration goes on. But tissue that has been fixed for a year will pose significant issues for IHC labeling.
    3. Post-fix should be done in the fridge. Fixation is sped up by temperature, to a point.
22. After post-fix is complete, transfer your specimen to an appropriate solution either for storage or for sectioning. See the next section for solutions to use if you plan to section the brain within a week after fixation.
    1. If you plan to store the brain, or isolated sections, for a long time, I recommend storing samples in cryoprotectant (what I more often refer to as antifreeze) and keeping them in a -20°C freezer, OR in PBS with 0.05% sodium azide at ~4°C. Both seem to keep the tissue usable for years in my experience, and there is specifically literature support showing the preservation of antigenicity after 10+ years in cryoprotectant freezer storage ("Just cool it!" article: https://doi.org/10.1016/j.peptides.2004.02.004). Recipes for both solutions can be found in the Recipes appendix.
    2. If you are assessing anything with oxidative enzymes, do not use the PBS + azide approach for storage.
    3. One key thing to remember if storing in antifreeze: a brain requires time to let the cryoprotectant soak all the way to the center. Keep a brain in cryoprotectant solution in the fridge for a few days until it sinks to the bottom of its vial, then move it to the freezer.

III. Histological processing and staining [HPS]

A. Separating desired tissue [SEPR]

After the brain has been fixed, you'll need to determine how it will be parceled into smaller, stainable samples. Further, you'll need to figure out a preparation solution and/or a storage solution for that parcellation.

Generally, fixed tissue is sectioned (or sliced, as some people may say) into flat, thin sections. These sections could be relatively thick (1 mm), but such thick sections can't really be deeply stained nor visualized properly… setting aside the ways that tissue clearing methods work. For free-floating IHC staining, 40 um is the thickness that is often recommended, as antibodies seem to have issues penetrating deeper than 20 um on each surface without substantial assistance. Thus it begs the question: how will we section the brain?

There are three common choices for sectioning fixed tissue: 1. paraffin embedding and sectioning with a rotary microtome, 2. Freezing the brain and sectioning via cryostat, or 3. Keeping the brain as-is and sectioning via vibratome. Technically there is an offshoot to #2: a brain can be kept frozen in an open air environment, rather than in a temperature-controlled chamber, by surrounding the brain with dry ice. Then, a freezing sliding microtome (cryotome) cuts through the tissue. The blade can be kept at room temperature such that it cuts more easily and the cut section immediately warms to room temperature, making it more flexible and easier to handle.

There are pros and cons to each method, so no method is perfect for IHC or for fixed tissue in general. I have no experience with paraffin embedding & sectioning, so that information will unfortunately be omitted from this document. However, I have the following advice in regards to using sectioning methods that require freezing the brain.

Frozen brain sectioning:

1. Prep: Soak brains that are bound for sectioning in a 25% sucrose in PBS solution for 2-3 days.
    1. This step is necessary to prevent jagged ice crystal formation that will rupture even fixed cells and distort tissue morphology.
    2. If you are moving tissue out of a cryoprotectant solution (again, I call this antifreeze), such as one containing glycerol & ethylene glycol, this will prevent the freezing that is required for sectioning. I recommend doing a sucrose soak for one day, then replacing the solution with fresh sucrose solution and soaking for an additional day. This should wash out the antifreeze while replacing it with sucrose solution.
2. Using a razor blade - and a brain matrix if possible – divide the brain into smaller chunks. This step produces flat surfaces on the brain chunks where they can properly sit on the sectioning device. I prefer to separate the brain into at least four chunks – first by hemisecting, then by dividing the hemispheres into a front half and a back half. But, you can choose you own configuration.
3. How you freeze the brain depends on the sectioning apparatus.
    1. If using a cryostat,
        1. Acquire a cryostat pedestal/chuck that has been chilled to freezing temperature. Place the brain on this chuck; it will stick due to the bottom surface freezing onto it. Surround the base with embedding gel. Obtain dry ice that you've mashed into a powder, and place the chuck onto some of this powder. Cover it with more dry ice powder.
        2. Use the quick freeze bar on a cryostat. In this case, the chuck can be room temperature. Attach the brain chunk to the chuck, surround the base with embedding gel, and place the chuck onto the freeze bar inside the cryostat. Activate the freeze bar.
    2. If using a cryotome that has a chuck with a cup surrounding it,
        1. Place dry ice in the cup. Dripple water in the chuck in the center. Once the water is frozen, shave the resulting ice a bit with the cryotome blade by sectioning it normally. When a wide flat surface of ice is available, place the brain chunk onto it. Wait for it to stick from the base freezing, then cover carefully in powderized dry ice.
        2. Freeze brain using chilled hexane OR 2-methylbutane. Chill the hexane by placing in a beaker and surrounding beaker with dry ice. A few minutes later, submerge the brain in the fluid. It will bubble until the freezing process is

complete. This method freezes extremely quickly to avoid ice crystal formation. Sucrose post-fixation step can still be used if desired. I have had issues with this method in regards to timing, however. If the freezing solution gets too cold, the brain may fracture. If it is not cold enough, the brain will take too long to freeze and may deform. Regardless, the next step is to pre-chill the cryotome chuck by filling it with powderized dry ice. The frozen brain chunk can be placed onto the chuck and its base should be surrounded with embedding gel.

4. Section brain into slices that are between 15 and 40 micrometers thick. Stick with one measurement throughout an experiment as best as possible! Sections that are 30-40 um thick are just thick enough to be easy to work with, but not so thick as to prevent antibody penetration.
    1. Since I image my stains at low magnification, I am less concerned with sections being too thick and having planes that are out of focus. As such, I opt for 50 um for easier section handling, with the caveat that the middle 10-20 um may have lackluster labeling.
5. Long term storage: After you have acquired your sections, they can be migrated into a wellplate or vial and stored in cryoprotectant (again, antifreeze) at -20 °C, or in PBS with 0.05% sodium azide at +4°C. Can be stored for years, maybe decades in either state.

Vibratome brain sectioning

1. Brains need not be frozen in this case. However, I find that keeping brain chunks in cryoprotectant (antifreeze) prior to vibratome sectioning has yielded more consistent sections.
2. Prep 2% agarose gel in advance. 4% agarose can also work if brain chunks are particularly wobbly. Turn it back into liquid using either a hotplate or a microwave.
3. Apply a small, soft, but flat platform onto your vibratome chuck. I have used rolled-up masking tape or double-sided sticky foam pads (used for mounting posters onto walls).
    1. Although many folks apply the brain chunk straight to a vibratome's chuck, I opt not to do this because all vibratomes are configured to avoid cutting into their own chuck. So, even after bringing the chuck to the maximum height on the vibratome, there will be a >1 mm gap between the chuck's surface and the vibratome blade. That means that you are not able to section the last 1 mm of tissue that is still on the chuck. So, I elevate the brain, but I use softer materials that the vibratome can cut through without strain or much resistance if I decide to take every section straight to the bottom of the brain chunk.
4. Place the brain chunk, flat surface down, into a flexible plastic mold. If you don't have these on hand, you can get silicone ice cube or confectionary molds to do this.
5. Pour in the liquid agarose.
6. Transfer to a -20 °C freezer and keep it there for 5 min. Try to keep the mold and brain chunk level during the transfer.
7. Remove the mold from the freezer, then pop the agarose + brain from the mold.
8. Examine the side of this agarose block that has the flat side of the brain. Is all of this side of the brain still exposed? If not, gently scrape aside the thin agarose layer that is covering the brain just on that surface. Do not disrupt the rest of the agarose surround the brain chunk on other sides.
9. Apply a thin layer of superglue to your soft platform on the vibratome chuck.
10. Place the agarose block onto this superglue-coated platform, with the exposed side of the brain on the superglue.
11. Very gently apply downward pressure for a moment, then wait a few minutes for the glue to react and stick.
12. Set up vibratome and proceed with sectioning. Store sections as suggested in the end of the cryostat/cryotome protocol above.

B. Pre-antibody preparations [PRAP]

Prior to running your tissue through an IHC protocol, it is prudent to see if the following preparation are needed. In this section as well as in the following section, I recommend the use of an orbital rotary shaker to keep the sections stirring in their respective solutions. Rocking shakers, or rockers, are less desirable as they tend to make the tissue stick to the well sides, risking the tissue to be unusable.

1. Removal of cryoprotectant, formaldehyde, azide, or other residues: Rinse tissue in PBS 3 times, 5 minutes each (3x5).
2. Prevent nonspecific binding/staining (regarding non-antibody substances):
    1. **Block endogenous biotin:** Biotin is expressed moderately in some hindbrain areas and, after antigen retrieval, by oligodendrocytes in all brain regions. Such circumstances may require a wash with 0.05% avidin/streptavidin (which bind to ans sequester bioin) and then 0.005% biotin before using a biotinylated antibody.
        1. Commercially available avidin and SA are expensive, so you can use the cheap alternative homebrew "biotin blocking solution" described in the Reagents section (Appendix E). This is essentially egg white. Afterward, block any free avidin with the avidin blocking solution described in the Reagents section which is skim milk or milk powder.. Use distilled water in wash steps prior to and after using egg white. Alternatively, the blocking kits from Vector Labs may work well at moderate additional expense, but I have not used them.
        Sources:
        https://www.ihcworld.com/_technical_tips/biotin_tips.htm ;
        Miller, Rodney & Kubier, Patty. (1997). Blocking of Endogenous Avidin-Binding Activity in Immunohistochemistry: The Use of Egg Whites. Applied Immunohistochemistry & Molecular Morphology - APPL IMMUNOHISTOCHEM MOL MORP. 5. 63-66. 10.1097/00022744-199703000-00010.
        Miller, Rodney & Kubier, Patty & Reynolds, Brenda & Henry, Terry & Turnbow, Holly. (1999). Blocking of Endogenous Avidin-Binding Activity in Immunohistochemistry: The Use of Skim Milk as an Economical and Effective Substitute for Commercial Biotin Solutions. Applied Immunohistochemistry & Molecular Morphology. 7. 63-65.
    2. **Endogenous peroxidase** can cause false DAB, AEC, or tyramide staining. If using a peroxidase-based step (such as HRP conjugates) and you wish to stop endogenous peroxidase activity, be sure to incubate brain slices in a blocking buffer for 15 minutes. A blocking buffer may be A. 1% H_2O_2 in distilled water, B. 0.5% H_2O_2 in methanol, C. 0.1% sodium azide, or D. 0.02 N HCl (most effective).
        1. I prefer to incubate tissue in PBS with 0.05% sodium azide for 10 minutes, then in 1% H_2O_2 (in water or PBS) for 10 min, then rinsing off the residues of both steps with multiple exchanges of PBS. I do the azide step first, as otherwise doing the H_2O_2 step first will cause gas release from the tissue that makes the solution frothy and harder to work with. The H_2O_2 step still seems necessary to fully neutralize peroxidase activity that persists in the brain's vasculature despite exposure to azide (as evidenced by non-specific, squiggly-looking staining I've seen in tissues that have undergone tyramide amplification but I had forgotten to expose to 1% H_2O_2 beforehand).
        2. Do this peroxidase quenching step before adding any antibody conjugated with HRP or other peroxidase components. This step can be done at any time prior to introduction of HRP.
    3. **Neutralize or exhaust free aldehyde residues in the tissue**. Fixed tissue may still have reactive aldehydes that will grab antibodies and attach them to the wrong sites. Some suggest using sodium borohydride solution (see the Autofluorescence Reduction section) to neutralize these. Other folks use a much less hazardous alternative by trying to exhaust the fixing capacity of these residues through exposure to protein-containing solutions, such as glycine or bovine serum albumin.
        1. I don't normally do this, as other procedures I do (especially the inclusion of normal serum, see below) seem to be sufficient.
3. Reduce non-specific antibody binding: Incubate tissue for 30 minutes using PBS that has >2% normal serum added.

1. This step is used to block the secondary antibody from nonspecific binding (to irrelevant protein targets) and reduces background noise in the final image.
2. The normal serum that you use here, as well as what you use in the primary antibody solution, should be of the same animal as the secondary antibody. Example: if your secondary is donkey anti-rabbit, the normal serum should be donkey (and definitely NOT rabbit). Do not rinse after this step!
3. Although I often skip this step since I incorporate normal serum into my primary antibody solution, there may be some benefit in the normal serum getting "snagged" by non-specific attractants in the tissue first, before the primary antibody gets access to that same tissue.

C. Antibody staining of tissue [STN]

NOTE: Think twice before throwing away your used antibody solutions. See Part II, Section 1 about when they might be re-usable.

1. Expose tissue to the primary antibody solution overnight at room temperature.
    1. This duration and temperature may seem wild to some folks. The traditional route is to do this step at +4°C overnight. In previous decades, there were concerns and issues of non-specific antibody binding if the staining temperature + duration were too high. The thinking is that the specific antibody-antigen reaction will occur and finish first, but the non-specific "antibody gets caught on stuff" reaction is more slowly yet continually progressing in the background. I've found that for most antibodies I've used, and especially with the staining amplification methods I employ, background staining isn't really that much of an issue in my experience. Also, an overnight room temperature incubation is roughly equivalent to a 3-day fridge-temp incubation, which some protocols call for. *To my knowledge*, there isn't anything special about lower temperatures that favors specific antibody binding over non-specific antibody binding. Also, using a shaker at room temp on a countertop is much less logistically complicated than setting one up inside of a fridge, or a cold room.
2. Rinse in PBS at least 3x, 5 min each.
3. Expose to secondary antibody solution for 2-3 hours at room temp. Use same shaker.
4. Rinse in PBS at least 3x, 5 min each. If no further staining, skip to D. Mounting.
    1. If using avidin or streptavidin conjugates: immerse tissue in a solution of conjugate at a 1:300 dilution in PBS with 0.2% Triton X-100 for 90 minutes at room temp and use shaker. Rinse afterwards as normal; including Triton in the rinse is recommended to help dislodge non-specific binding of fluorophores.
    2. If using DAB labeling: incubate in 0.05% DAB in PBS + 0.015% H_2O_2, for 3-15 minutes. No shaker required, but you can agitate the dish lightly by hand. Rinse immediately to stop reaction 2 times for 15 seconds each in fresh PBS each time. Discard both rinse solutions as hazardous waste.
        1. Certain metals can be added to intensify the DAB reaction product into a darker color. Adding salts of nickel, cobalt, or copper (final conc. 0.05%) will do this. (Source: Hsu SM, Soban E. Color modification of diaminobenzidine (DAB) precipitation by metallic ions and its application for double immunohistochemistry. J Histochem Cytochem. 1982 Oct;30(10):1079-82. doi: 10.1177/30.10.6182185. PMID: 6182185.)
        2. AEC staining can be done in the same way, but acetate buffer will need to be used rather than PBS.
    3. If using tyramide amplification, immerse tissue in a solution of tyramide conjugate at a 1:300 dilution in PBS with 0.2% Triton X-100 and 0.003% H2O2 for 5-20 minutes at room temp and use shaker. Rinse afterwards as normal; including Triton in the rinse is recommended to help dislodge non-specific binding of fluorophores.

D. Mounting tissue sections onto slides [MNT]

I recommend viewing videos online about how to apply tissue onto slides using a dish for floating the tissue and a paintbrush. However, some pointers I'll note here are:

- Use horsehair paintbrushes, or otherwise soft flexible fiber for the bristles. Many synthetic fiber types are too rigid and are likely to tear through the tissue.
- If you plan to do any fluid-using procedures to the tissue after it is on a slide, I strongly recommend using either charged slides or gelatin-coated slides. Charged slides can be somewhat expensive but don't come with any residue. Gelatinized slides can be made in-house from regular slides, but they do leave behind some residue. Either way, these types of slides will force the tissue to stay on the slide as it is moved from liquid to liquid.
- Use solution that has some salt in it, but not too much. Typically, isotonic saline (0.9% NaCl in water) works fine. I don't recommend 0.1 M phosphate buffer. The concern is that if a solution is too salty, or if the salts that are left behind after evaporation are too large, this will leave a lot of salt residue on the slide and the sections.
- Choose how you will dry sections. Some people use slide warmers, but heat is problematic for some sensitive fluorophores and if you are using gelatinized slides. You can use a fan instead, but there is the risk of depositing dust on the sections if the environment is not kept very clean. The safest but slowest way is to lay them flat on a tray, then place a rigid covering over that tray which does not touch the slides and has substantial open space for air passage.

Making gelatinized slides in-house

Required materials

- Plain glass microscopy slides (not charged - those have "+" marks on one edge)
    - Note: Using a zig-zag pattern, each coplin jar can fit nine slides.
- 100 mL hot water - can be distilled, though this is not required. Or, a hot plate or microwave to warm water. If lacking a microwave or hotplate, getting hot water from a coffee shop should work.
- Store-bought plain gelatin (typically the Knox brand)
    - Although traditionally "subbed" slides are coated in chromium potassium sulfate ("chrom-alum") and gelatin, this chemical should be avoided as chromium is a toxic heavy metal. Laboratory gelatin is applied with this chromium compound as a 0.5% solution; the lab grade gelatin also has a higher gelling metric (~300 "bloom"). Considering these details, I've tried substituting it with higher concentrations (now 3%, previously did 2%) of store-bought unflavored gelatin (allegedly ~150 bloom) without any chromium.
- Two glass or plastic coplin jars

Procedure

1. Make sure microscope slides are clean. If not, use ethanol/rubbing alcohol on a paper towel to remove grease and dust. Let dry briefly.
2. Obtain 100 mL hot distilled water (but not boiling), then add 3g Gelatin to it (makes 3% solution).
    a. Note that 3 g gelatin is roughly 1.5 teaspoons.
3. Mix with a stirrer until there are no floating chunks and the solution is a transparent, slightly amber color. You may need to mash the chunks with the stirrer to get them to dissolve.
4. Pour the solution into the coplin jars loaded with the clean slides. Make sure that no slides are stacked flat against each other (lest they get stuck), though zig-zag arrangement is fine.
5. Keep slides in these jars for 30 minutes
    a. Time selected rather arbitrarily. Shorter times may suffice, needs testing.
6. After removal, place tilted up (but as little as possible) on the edge of a raised surface and let dry overnight. Try to protect from dust.

a. Placing them flat on the lunchtray will cause them to stick - not recommended!
b. I recommend covering them with an additional lunchtray or box to minimize dust fall. Make sure to arrange the trays perpendicular to allow air flow and evaporation to occur.

7. The slides should now be ready to have sections mounted onto them. After mounting the sections, do not use a slide warmer to expedite their drying - this will melt the gelatin! Just wait for these slides to dry again overnight before any staining procedures.

Recommendations for best outcomes with gelatinized slides

- Do not use any detergents on these slides, as most detergents will dislodge the tissue from the gelatin.
- Keep an eye on how well the tissue sticks to the slides. One option to increase adherence is to incubate the slides + tissue in formaldehyde. This will cross-link the gelatin to the tissue.

E. Coverslipping [CVP]

See the Mounting Media section in Part 2 about your choices for mounting/coverslipping media. Regardless of the media however, I recommend the following tips.

- Apply media in small droplets on top of brain sections.
- Ensure that the media does not autofluoresce or quench fluorophores.
- If you are using a medium that does not dry & solidify, use coverslips that have slightly less of a width than the slides you use. This is not to be confused with thickness, although it should be relatively thin.
- Once lowered, push on coverslip lightly and dry off excess media coming out the edges with a kimwipe.
- Nail polish can be used as a sealant for non-drying media. This creates a barrier to keep the aqueous mounting media within the coverslip-slide sandwich. Use nail polish remover to remove polish if you need to re-coverslip. Alternatively, less hazardous glues can work, as long as the medium is not water based. So, elmer's glue works well with mineral oil medium. Hot glue may work with water-based ones if the glue is chilled quickly on contact with the slide (but I haven't tried this!).

F. Disposal of wastes and reagents [DSPO]

Non-hazardous (simple rinse in the sink):

- Any uncontaminated buffered saline,
- expired normal serums,
- uncontaminated glycerol,
- expired biotin solution
- expired avidin and streptavidin solutions in low concentrations,
- diluted hydrogen peroxide (must be less than 10%),
- Most tracers in low concentrations: PHA-L, fluororuby/gold/emerald, biotinylated dextran amines (BDAs)
- Tyramide conjugates
- Diluted alcohols
- Cresyl violet solutions (<1%)

Hazardous (EH&S / OSHA pickup) –

- sodium azide residues
    - Note: I'm investigating how this could be neutralized with nitrous (not nitric) acid. Will update later.
- Any DAB-containing solutions
    - In a similar manner with azide, there's a way to neutralize DAB (And it's not bleach! That just makes precipitates soluble but not neutralized!), but I need to do more research. This is especially complicated if those neutralizing agents are not compatible with the metal compounds used to enhance DAB…
- All formaldehyde solutions,
- Any solution with ethylene glycol or polyvinylpyrridonole-40 (used in cryoprotectant solutions)
- 2-methylbutane
- Hexane
- Triton X-100 residues
    - This one I'm actually unsure about. Triton X-100 is definitely hazardous to the environment at higher concentrations (10%), but at the amounts used in IHC (0.2%), it may be the equivalent of using dish soap if not
- Cholera toxin subunit B, a tracer
    - Even though in theory it is non-hazardous, it has potential to have native whole cholera toxin.
- All solutions containing heavy metals, such as copper, cobalt, nickel, iron, and others. This includes the distilled water that is contaminated from rinsing these compounds off of tissue.
    - If a DAB solution gets neutralized but still has these heavy metals, it is still hazardous waste!

Part 2: Specifics about IHC

I. Antibody recycling [RRR]

Note: I wrote this part so very long ago – probably ~2014 – but the same pressures still remain on labs today.

As of the writing of this document, funding in science labs across the country is low and researchers are always attempting to reduce costs without sacrificing quality of the work. As this guide is set up for free-floating IHC, antibody solutions can be easily scavenged and re-used. But how does one decide whether or not to continue using the same solution, again and again? Several important factors can determine this: age of antibody solution, temperature at which it is maintained, number of uses on tissue, volume of antibody solution, and concentration of the epitope in tissue samples stained.

The first two concerns, age and maintenance temperature, can be maximized by being mindful of the solution's storage when not in use. Solutions should always be stored in the fridge and never frozen/thawed (unless you are diluting an aliquot). Keeping any solution at room temperature instead of at 4 degrees Celsius will reduce its shelf life, either by encouraging growth of microbes that degrade the active ingredients, or by allowing chemical reactions that also degrade these active ingredients to happen on a faster time scale. However, microbes can also form at fridge temperatures. Inclusion of a preservative in the solution (usually 0.05% sodium azide **SEE SECTION BELOW**) should stave off growth of most things unless instruments used to transport the solution are dirty. In short, storage at 4 degrees C and with a preservative allows for a longer shelf life. Despite these, when is a solution too old? Assuming it has been used at most only once, I've witnessed primary and secondary antibodies give effective staining after 6 months from dilution date. Some may last longer, but most solutions should be discarded after 9 months unless you have the time to re-test them. However, the other factors mentioned previously also play a role in an antibody solution's longevity.

An important concept to remember is that the antibody solution can eventually be “used up”, or depleted of its antibody titer. One major factor that determines this depletion is the concentration of the epitope in the tissue being stained. If it is a rare epitope (orexin for example), the antibody solution can likely be re-used several as the tissue will not pull too much of the antibody out of the solution. For more ubiquitous epitopes such as AMPA receptors, continuous re-use is not recommended. As AMPA receptors are everywhere in the brain, their high concentration will attract an equal or greater concentration of antibody out of the solution. Additionally, you should consider whether certain targets appear to decrease in number when re-using an antibody. One example I’ve encountered is from staining for Delta FosB, a transcription factor. Re-using the antibody seems to decrease the number of stained nuclei that can be seen. Since that influences cell counts, it will then influence data, and that’s a bad thing. So, for situations involving quantification, I advise against re-use of antibody solutions.

Some of the “titer depletion” issue can be offset by having a greater solution volume to tissue ratio. In other words, soaking one 50 um thick brain slice in 30 mL of an anti-AMPA receptor solution will not significantly reduce the antibody concentration in that solution. However, soaking the same brain slice in only 1 mL of the same solution may result in the solution giving substantially reduced staining upon a second use. In contrast, using an antibody against a rare epitope like orexin allows for many re-uses; 1 mL of anti-orexin antibody, as long as it is not too dilute, can likely be used to stain 5 different brain slices without noticing a drop in staining quality. Thus, the last important factor of antibody recycling, the previous number of uses, interacts with the volume of the solution and the amount of epitope in the tissue.

Despite the above guidelines, there is no general rule for when an antibody solution should be discarded. Use you best judgment. If you recall having better, clearer stains on the previous batch of

tissue compared to the current batch and you are re-using the antibody solution, it may be best to replace it. Remember not to compromise the quality of your work, and subsequently your time and effort, just to save some small amount of money on re-stocking an antibody.

II. Sodium Azide (TOXIC) [AZD]

Azide is used either as a preservative in antibody solutions (at a 0.05% concentration), or to stop peroxidase activity. It will prevent growth of microbes and fungi in your solutions by halting their metabolism. One thing to watch out for is that it "breaks" most peroxidases, if you are looking for those. But if you actually want to block endogenous peroxidase activity (to prevent interaction with reagents in the staining process), this is one way to go about it.

Be careful with this chemical! It binds irreversibly to hemoglobin, making red blood cells unusable. Poisoning from this chemical is similar to exposure to traces of cyanide or carbon monoxide – rapid breathing, dizziness, nausea, coughing, suddenly pale or blue skin, or other symptoms onset quickly if a significant enough amount is inhaled, ingested, or absorbed. If any of these symptoms appear shortly after handling the substance, do not hesitate to contact the appropriate health services. Set aside small amounts of powder for your future use under a fume hood with full protection – lab coat, gloves, eyewear, dust mask.

See the section at the end of Part 1 about disposal of wastes and reagents. Generally sodium azide-containing solutions should be disposed of as hazardous waste, unless a chemical method is devised to neutralize it. I know that the amount used in IHC preparations is quite low, typically around 0.05%, and then it is often further diluted when mixed with nonhazardous rinse buffer waste. However, there are two concerns aside from personal health involved with its disposal: 1. It will kill off or suppress necessary bacteria in waste water treatment plants, and 2. It could still combine with lead or copper in piping to form salts that can explode when impacted, even in small quantities.

Further reading:

- How Dangerous Is Too Dangerous? A Perspective on Azide Chemistry. Daniel S. Treitler and Simon Leung. *The Journal of Organic Chemistry* **2022** *87* (17), 11293-11295. DOI: 10.1021/acs.joc.2c01402)
- Facts About Sodium Azide (CDC): https://emergency.cdc.gov/agent/sodiumazide/basics/facts.asp

III. Antibody and conjugate handling in general [HDL]

- pH factor: Higher pH makes antibody binding more specific. This effect is a double-edged sword; reduces noise, but can weaken signal. However, keeping the pH around 7.4 is standard practice and there isn't much reason to deviate from that.
- Storage: If in powder form, reconstitute antibody/chemical in a volume of distilled water equal in number to the powder's gram amount (example: add 500 uL water to 500 ug antibody). Aliquot this solution into amounts no larger than 200 uL; try to split into several small aliquots to prevent waste. If you need more you can de-frost more than one. However, if you had just created one large aliquot from all the antibody solution and you do not use it, it isn't great to re-freeze and then thaw again. Re-freezing tends to denature proteins, so every time they go through freeze-thaw cycles, they decrease in efficacy in unpredictable ways. I recommend aliquot sizes ranging from 5 to 50 uL, depending on your intended working dilutions. Put into plastic vials and freeze at -80 degrees C, though -20 degrees C can suffice if you lack an ultra-cold freezer.
    - Some chemicals are not soluble in water, so heed the vendor's product sheet for what the appropriate solvent should be.
    - If you are re-constituting powders of enzymes such as HRP, store at -4 degrees C using a sterile cryoprotectant. For example, for a 1 mg amount of an HRP-conjugated antibody,

I mix 500 uL of distilled water with 500 uL of glycerol, then I dissolve the powder in this. Do not allow to freeze – the cryoprotectant should prevent freezing at the advised temperature. Do not add azide!

- Dilutions: Most antibodies can be diluted into a working solution consisting of PBS with normal serum, Triton X-100, and sodium azide unless stated otherwise. The normal serum acts as a stabilizer, as anything that degrades proteins will have to attack the normal serum and will do so less often to the antibodies of interest. Azide should be used to prevent microbial growth that would also degrade the antibodies. Incusion of Triton X-100 is not required, but I used it in my staining often, so it is a component of my antibody diluent. Store solutions at 4 degrees C. Re-check binding ability after 6 months.
    - As noted earlier, HRP-conjugated antibodies should never be diluted with solutions containing azide. Worth nothing is that normal serum also contains some azide, so such antibodies should also not include normal serum!

IV. Fixation [FXA]

The effects of fixatives vary due to their pH. An acidic fixative dilates blood vessels and penetrates tissue more quickly for a more complete perfusion, but does not fix so well. A basic fixative fixes very well, but needs more time to penetrate as it constricts blood vessels and does not diffuse well through tissue. Although some labs used an acid-base switchover mid-perfusion, I find that this is not so necessary. A neutral-ish pH fixative (typically pH 7.4) will work fine for most applications, especially if delivered via transcardial perfusion. But, experiment when troubleshooting, and try to follow protocols that were successful in published literature. Worth mentioning is that some researchers have used this acid-base switch in protocols to enact quick but thorough and robust fixation; one is intentionally called SWITCH (Web resource: http://chunglabresources.com/sw1
Reference: Evan Murray, Jae Hun Cho, Daniel Goodwin, Taeyun Ku, Justin Swaney, Sung-Yon Kim, Heejin Choi, Jeong-Yoon Park, Austin Hubbert, Meg McCue, Young-Gyun Park, Sara Vassallo, Naveed Bakh, Matthew Frosch,, Van J. Wedeen, H. Sebastian Seung, and Kwanghun Chung. Simple, scalable proteomic imaging for high-dimensional profiling of intact systems, **Cell**, Dec 3:163(6): 1500-14. doi: 10.1016/j.cell.2015.11.025. PubMed PMID: 26638076). More info can be found in the Part 3, Tissue Clearing section.

Antibodies can cross cell membranes in fixed tissue. However, if the fixation process masks your antigen, the antibodies may not bind. Note any antibody manufacturer recommendations about fixatives to use or not to use. Generally, overfixation tends to mask antigens, but underfixation will make the brain slices unmanageable and too delicate, so a balance must be struck. The best procedure utilizes a transcardial buffered PFA perfusion lasting 15 minutes minimum, longer if the subject is not rigid, and an overnight post-fixation of the isolated brain in a vial with double the volume of PFA compared to the volume of the brain. Despite the post-fix immersion of a brain in PFA, the perfusion process is still more effective in fixing the brain as it infiltrates and diffuses PFA through the brain from the inside out. Conversely, immersion in PFA fixes from the outside inward, and does so slowly – fixation of the outer layers of tissue limits movement of the PFA deeper into the tissue.

Flushing fixative at the start of the perfusion may cause coaggulation/clotting of blood in the brain. Thus, a saline flush typically precedes the fixation flush. The saline flush lasts 10-30 seconds and uses a buffered saline (such as PBS or isotonic saline) or Ringer's solution. The solution should have a pH at 7.4 and should be roughly body temperature. These factors ensure thorough flushing of the vasculature. The amount of saline used should be 15% of the subject's body weight for whole body perfusions and 5% for upper body only. Substances can be added to the perfusate to enhance vasodilation and prevent clotting. Vasodilators such as papaverine, lidocaine HCl, or sodium nitrite (not nitrate!) can be added to the saline. Lidocaine should be dissolved 50 mg/mL in 100% ethanol, then this stock solution should be added to the saline to dilute the lidocaine to 1 mg/mL (0.1%); it can also be added to the fixative solution in the same concentration. Sodium nitrite should be diluted to 1% in

the saline solution. Heparin can be added as an anti-clotting agent; add 10 units per mL of saline. Note that heparin comes typically as 180+ units per mg. The perfusate/fixative solution should also be in physiological range and body temperature to prevent vasoconstriction, though some argue on using different pH values. See Appendix E for solution recipes. If fixation decreases antibody binding, antigen retrieval may be needed. See Part 3, Antigen Retrieval.

V. Osmolarity [OZM]

Background:

Osmolarity is defined as the number of osmoles of solute per unit volume of solvent. Osmoles are the number of moles that contribute to the "osmotic pressure" of a solution, or its pressure to force water/solvent into or out of tissue. Osmolarity can change since volume changes due to temperature, but for our purposes this is mostly inconsequential.

Different substances can result in differing amounts of osmoles even if they are in the same amount of mole. One example involves comparison of substances which dissociate into ions (usually salts) versus substances that do not dissociate. Let's say we have 20 moles of NaCl and 20 moles of glucose. In solution, NaCl dissociates into Na+ and Cl-, so the number of moles of "salt pieces" is double the number of what the original salt was. In other words, if a salt dissociates into two ions, the amount of osmoles is double the amount of moles of the salt. For substances that don't dissociate in solution, the number of moles equals the number of osmoles. Thus, 20 moles of NaCl is 40 osmoles, whereas 20 moles of glucose is only 20 osmoles.

Why does any of this matter? Maintaining the shape and integrity of cells in an IHC preparation is important. Distorted morphology can result from mismatches of osmolarity, and such distortions affect analysis of the tissue. Hypertonic, or high osmolar, solutions will sap out the water from inside cells and cause them to shrivel. Water is attracted to areas in which solutes (salts) are most concentrated. Hypotonic, or low osmolar, solutions force water into cells since there are more salts in the cell compared to outside. This causes cells to swell and potentially burst. Isotonic solutions involve the osmolarity of the solution and the inside of a cell being roughly equal, this maintaining the cell's shape.

Applying this knowledge in IHC procedures:

Fixation: As tissue and cells become "stuck" in the shape they were immediately prior to or during fixation, it is important to avoid distorting that shape. For instance, hypertonic fixative solutions will cause cells to be stuck in a shriveled state. When you add buffer or other substances to fixative solutions, keep in mind that their purpose is not only to balance pH but also osmolarity.
Below are some quick conversions to keep in mind when adding salts/buffers to fixatives.
The ideal solution osmolarity range is 300-400 mOsm (milliosmoles) per liter. This matches the internal environment of most mammalian cells.
0.1 M NaPB (no added NaCl) @ pH 7.2, RT should yield ~400 mOsm
0.154 M (0.9%) NaCl → 308 mOsm
0.1 M (~0.6%) NaCl → 200 mOsm
0.18 M (~6%) sucrose → 200 mOsm
1.3 M (4%) formaldehyde → 1333 mOsm
Note: Osmolarity of PFA suggested to not matter because it penetrates cells and equilibrates inside and outside of the cell membranes. This is not true of other fixatives, though.

Incubation with antibodies: Osmolarity of antibody-containing solutions may not matter as much as that of fixative solutions. Indeed, fixed tissue will maintain shape far better than live tissue when confronting solutions of different osmolarity. But osmolarity could still may have an impact on

antibody binding. Hypertonic solutions that cause cells to shrivel may prevent access of antibodies to intracellular antigens. This issue likely occurs because of the "pores" formed by fixation collapsing shut. Thus, it would stand to reason that a hypotonic solution should be used to force open such pores. This may work, but one possible downside is the escape of intracellular antigens that are not completely fixed in place. Thus, antigens may get washed out in subsequent solutions unless the tissue is well-fixed. It may be best to just stick with an isotonic solution or at the most a mildly hypotonic solution during antibody incubations.

VI. Primary antibodies [1ST]

Primary antibodies should be selected carefully. Select a common species, such as rabbit, mouse, or goat. This way, it is easier to find a secondary antibody to bind against the primary (see secondary antibodies below). Also, when selecting your antibody from a vendor, check for any associated literature they may link to in which that specific antibody product has been used in prior studies. The more studies that use that specific antibody, the more popular and (hopefully) effective it may be.

Do you select **Monoclonal or Polyclonal**?

- Monoclonal antibodies (from "one clone") are all produced from the same cell line – typically a "hybridoma" - and all select the exact same epitope ('part' or 'site') of the same antigen with the same affinity.
    - Pro: They are highly specific.
    - Cons: These are harder to make, and tend to be more expensive. They may be too specific and have less overall binding, depending on the accessibility of that specific epitope. Also, if target epitope of antigen is damaged or masked, less binding occurs.
- Polyclonal antibodies are produced from a variety of cells. They are created from infusing an antigen into an animal, then taking some of their blood and isolating the immune fraction of that blood. A polyclonal antibody mixture may select for different epitopes on the same antigen, and with different affinities.
    - Pros: Easier to "manufacture", and thus are cheaper than most monoclonal antibodies. This type is more likely to bind to the target of interest.
    - Cons: Higher batch to batch variability. Some nonspecific binding. The non-specific binding may not be a concern if the target is a specific segment of your epitope that is only found in that protein within your tissue. Run a BLASTp search to check for related proteins that may be other potential targets of your polyclonal antibodies.

VII. Secondary antibodies and conjugates [2ND]

Select a species different from the primary, and check to be sure the antibody is against the primary. Secondary antibodies are usually something like "goat anti-mouse" - an antibody raised in a goat that seeks out any mouse antibodies. Secondary antibodies are almost always conjugated to some sort of reporter, enzyme, or easily-bound target. So, some examples of secondary antibodies for purchase might be "AlexaFluor 488 goat anti-mouse" (has a green fluorophore), "HRP goat anti-mouse" (has an enzyme that activates & deposits stains), or "biotinylated goat anti-rabbit" (has a tag that avidin/streptavidin can bind to specifically).

For multiple target staining, I advise picking a consistent host for the secondary antibodies, with multiple options directed at different primary antibodies. For example: Let's say I was staining for orexin and for GAD67. I have antibodies against these targets: goat anti-orexin, and mouse anti-GAD67. I can then use multiple secondary antibodies with different labels: AlexaFluor 488

donkey anti-goat, and Cy3 donkey anti-mouse. It is important to pick labels that do not result in overlap; don't pick two secondary antibodies that both have green tags, for example.

IIX. Deciding whether to employ a tertiary – Amplifying signal to noise ratio [3RD]

See figures at the end of this section for diagrammatic examples

Some combinations of primary and secondary antibodies may stain the epitope, but not to an appreciable/acceptable degree. There is a limit to how many secondary antibodies can bind to the primary, and this limits the signal-to-noise ratio regardless of extending incubation times or increasing antibody concentrations in solutions. To further amplify the signal, one can employ a tertiary step. There are a number of options that are detailed below.

- **3° antibody:** Use of a tertiary antibody may be easiest. This is an antibody that binds to the secondary antibody and not the primary. One example: a primary goat anti-orexin antibody can be bound with a secondary rabbit anti-goat antibody, which then can be bound by an Alexa647-conjugated donkey anti-rabbit antibody. This technique may be effective, but a tertiary antibody suffers the pitfalls that staining with any secondary may encounter – non-specific staining, being careful not to use two secondaries (attached to two separate primaries) in the same species, and so on.
- **PAP (Peroxidase Anti-Peroxidase):** Horseradish peroxidase is typically an antibody conjugate used for enzymatic staining. However, it can also be an immunologic target. A PAP complex consists of several of the same antibodies (let's say rabbit IgGs) against several peroxidase molecules. As IgGs have two binding sites, each IgG can bind to two peroxidase molecules. Then, the peroxidase molecule could be bound at multiple sites as well, allowing multiple separate IgG molecules to bind to it. The result is an interconnected chain of peroxidases and the IgGs against them. How it works in a protocol:
    1. 1° antibody (rabbit) binds to antigen
    2. 2° antibody (goat) binds to 1° antibody with one binding domain, while the second domain remains open.
    3. PAP complex with rabbit IgGs is made and put into solution.
    4. The open binding domain on the 2° antibody attracts and binds to one of the rabbit IgGs in the PAP complex.
    5. All the peroxidases in the PAP complex react with DAB to stain a focused area.

    Advantages: Complex reacts with DAB in a quicker and more focal manner compared to only a 2° antibody labeled with one peroxidase molecule, stain is intense and the signal-to-noise ratio increases substantially, user could dilute the primary antibody further to reduce background staining, can swap DAB with fluorophore-conjugated tyramide (described in TSA below).

    Disadvantages: Complex may be too large to fit into tight intracellular spaces, requires endogenous peroxidases to be inactivated first, and that Fab fragments of IgGs cannot be used for the 2° antibody. These issues can be resolved by using this method only for cell surface antigens and/or adding some detergent to permeabilize membranes, employing an endogenous peroxidase blocking step (see Regents section in Appendix E), and only using whole IgGs for the 2° antibody.

    Note: a variant of this complex can be used for fluorescence. Instead of peroxidase, a fluorophore can be used, and the IgGs could be directed against this fluorophore instead. But, as this is not reacted further, the signal would not be as intense as the DAB reaction product.

    (Ref: Bratthauer, G.L. (1994). The Peroxidase-Antiperoxidase (PAP) Method. In: Javois, L.C. (eds) Immunocytochemical Methods and Protocols. Methods in Molecular Bilogy, vol 34. Humana Press. https://doi.org/10.1385/0-89603285-X:165)

- **Dextran & Enzyme Polymer:** This method uses a polymer, made of dextrans (sugar) to link several reporters/enzymes and a few 2° antibodies together as a long chain. One vendor, Dako, has the product Envision, though other vendors may have their own versions under different names. How it works in a protocol:
    1. 1° antibody binds to antigen.
    2. Polymer binds to 1°.
    3. Sections are mounted for fluorescence analysis, or enzymatic polymers are reacted with a chromagen.

    Advantages: Easy to use, takes little time compared to other amplification methods, substantially amplifies signal especially for enzymatic reactions. *May* not require neutralization of endogenous peroxidases as the peroxidase signal is so heavily amplified at the antigen site.

    Disadvantages: Polymer may have difficulty fitting into intracellular spaces unless aided with detergents, 2° antibodies in polymer must be highly specific for the 1° antibodies or else much background staining will occur.

    (Ref: Werther K, Normark M, Hansen BF, Nielsen HJ. Immunoglobulin and Enzyme-conjugated Dextran Polymers Enhance u-PAR Staining Intensity of Carcinoma Cells in Peripheral Blood Smears. Journal of Histochemistry & Cytochemistry. 1999;47(7):959-963. doi:10.1177/002215549904700713)
- **Enzyme Polymer:** Similar to the dextran & enzyme polymer, a polymer is attached to a secondary antibody to amplify the signal. However, as the dextran "backbone" can be particularly bulky, some vendors instead directly conjugate enzymes/reporter molecules together in their own mini-polymer, condensing the size substantially. Vector Labs has one called ImmPRESS. One end of this polymer is attached to a single 2° antibody. How it works in a protocol:
    1. 1° antibody binds to antigen.
    2. 2° antibody with attached polymer binds to 1°.
    3. Sections are mounted for fluorescence analysis, or enzymatic polymers are reacted with a chromagen.

    Advantages: Easy to use, adds less time to staining compared to other amplification methods, amplifies stain substantially, and can fit into intracellular spaces better than PAP, ABC, or Dextran & Enzyme Polymer. Attached secondary antibody may encounter less steric hindrance than dextran version, since this one does not feature a large space-consuming chain, therefore the 2° antibody should bind to the 1° antibody more effectively.

    Disadvantages: Does not amplify as much as the dextran variant or certain other methods, may still have trouble fitting into particularly small intracellular spaces, requires highly specific 2° antibody to avoid background staining increase.

    (Ref: Ramos-Vara JA, Miller MA. Comparison of two polymer-based immunohistochemical detection systems: ENVISION+ and ImmPRESS. J Microsc. 2006 Nov;224(Pt 2):135-9. doi: 10.1111/j.1365-2818.2006.01679.x. PMID: 17204059.)
- **ABC (Avidin-Biotin Complex):** Biotin is a vitamin used in some amplification procedures, and is found in some tissues (see Part 1, Section III, Subsection B). Certain molecules bind to biotin with extremely strong bonds and sequester it; these include the egg white protein avidin and the bacterial protein streptavidin. Both of these compounds have 4 binding sites for biotin. The ABC method takes advantage of this fact. First, a reporter molecule (fluorophore or peroxidase) is biotinylated, meaning it is biotin-conjugated. Then, several of these conjugates are put into solution with avidin. The result is a network composed of avidin-biotin-reporter-biotin-avidin links. This complex has similarities in layout to the PAP, except that more binding sites are available (4 in avidin vs. 2 on IgGs), and multiple biotin molecules can be conjugated to one reporter molecule. Ideally, the biotin conjugate should be attached to the reporter with some sort of "spacer", usually a chain of carbon atoms that causes the biotin to "stick out" from the

reporter molecule and allows better access and less steric hindrance of avidin binding to the biotin. How it works in a protocol:

1. 1° antibody (rabbit; red) binds to antigen
2. Biotinylated (B) 2° antibody (goat; grey) binds to 1° antibody.
3. ABC complex with desired reporter (blue) is made and put into solution.
4. A free avidin (purple) binding site within the ABC complex binds to biotin on the 2° antibody.
5. Fluorophore is visualized or DAB reaction is carried out.

Advantages: avoiding the nonspecific binding effects seen from 3° antibodies or PAP method, increases in binding sites lead to increased signal-to-noise ratio, allows 1° to be diluted further.

Disadvantages: ABC size may prevent access to antigens (similar to PAP), blocking endogenous biotin may be required, and avidin may bind to compounds other than biotin. In regard to the third issue, avidin may have minor affinity to lectins such as PHA-L. This is a possible problem if using a PHA-L tracer that you are not using the avidin to stain against. A solution to this issue is by using an ABC complex where streptavidin is substituted for avidin. Streptavidin has equal affinity to biotin but negligible affinity to most other substances we use in brain tissue IHC. Alternatively, unwanted binding of avidin to endogenous lectins can be hindered by adding 0.2 M alpha methyl-D-mannoside to the ABC solution. Yet another solution is to get a deglycosylated version of avidin, which lacks the full avidin's lectin affinity (ref: Haugland RP, Bhalgat MK. Preparation of avidin conjugates. Methods Mol Biol. 2008;418:1-12. doi: 10.1007/978-1-59745-579-4_1. PMID: 18287645.)

One other major issue is problematic for researchers trying to stain multiple antigens - the ABC technique can only be used once in a tissue sample. In other words, two different antigens cannot be identified with two ABCs containing different reporter molecules, as the avidin in either ABC will not discriminate between the two types of biotinylated secondary antibodies as long as binding sites are available. This may result in the appearance of substantial overlap of the two different ABCs. I have tried to block the first biotinylated antibody with various avidin-biotin solution types, combinations, concentrations, and incubation times with little success.

(Ref: Bratthauer GL. The avidin-biotin complex (ABC) method and other avidin-biotin binding methods. Methods Mol Biol. 2010;588:257-70. doi: 10.1007/978-1-59745-324-0_26. PMID: 20012837.)

- **Labeled Streptavidin-Biotin (LSAB):** A secondary that is biotinylated can be identified using a streptavidin conjugate. This method is a simplified version of the ABC method, and only features reporter-conjugate streptavidin molecules binding directly to biotin on the 2° antibody without being chained to each other. How it works in a protocol:

1. 1° antibody binds to antigen.
2. Biotinylated 2° antibody binds to 1°.
3. Streptavidin conjugate binds to 2°.
4. Tissue mounted for fluorescence analysis, or tissue reacted with DAB if streptavidin is HRP-conjugated (as in figure).

Advantages: Penetrates tissue better than ABC or PAP complexes, economical if you have multiple streptavidin-reporter conjugates, useful if user only needs a slight (2x-5x) boost in the signal-to-noise ratio, shorter reaction times and avoidance of non-specific binding issues of IgGs in PAP or 3° antibody methods, does not have same affinity to lectins that avidin does.

Disadvantages: Does not amplify signal as much as ABC method, endogenous biotin must still be blocked if antigen retrieval is used or if staining the hindbrain, cannot stain two different biotinylated 2° antibodies with different streptavidin conjugates (will cross-react).

(Ref: Shi ZR, Itzkowitz SH, Kim YS. A comparison of three immunoperoxidase techniques for antigen detection in colorectal carcinoma tissues. J Histochem Cytochem. 1988 Mar;36(3):317-22. doi: 10.1177/36.3.3278057. PMID: 3278057.)

- **Tyramide signal amplification (TSA):** Certain kits market themselves as "signal amplification" tools using chemical interactions to amplify a signal. A well-known one is tyramide/tyramine signal amplification (TSA), a.k.a. Catalyzed amplification reaction detection (CARD) or catalyzed signal amplification (CSA). This method uses a secondary antibody conjugated to horseradish peroxidase. Using hydrogen peroxide as a catalyst, it chemically alters tyramide so that it binds to any lysine residues near the secondary antibody. If the tyramide is conjugated to a fluorophore, there is a dramatic signal increase. If the tyramide is conjugate to biotin, and an SA conjugate is used, the signal is amplified rather ridiculously. How it works in a protocol:
    1. 1° antibody binds to antigen.
    2. HRP-conjugated 2° antibody binds to 1°.
    3. Fluorophore or biotin-conjugated tyramide is reacted with tissue with H_2O_2.
    4. Mount & analyze, or label with streptavidin conjugate for biotinylated tyramide.

    Advantages: This technique, based on a reaction that progresses steadily with time, can amplify your signal-to-noise ratio as much as you will let it (depends on reaction time length, though it does seem to cap out after 30 min). Tyramide conjugates, depending on conjugate size, can easily access intracellular spaces compared with other amplification methods. In a multiple labeling application, can be used multiple times. Example:

    Stain #1: rabbit anti-cfos → HRP-donkey anti-rabbit → fluorescein-tyramide

    Between stains, neutralize first HRP from reacting any further by applying a peroxidase blocking solution that has azide. You can follow up with a 1% H2O2 solution to ensure neutralization. Then, be sure to apply three PBS rinses (5 min on shaker) afterward.

    Stain #2: goat anti-GAD67 → HRP donkey anti-goat → Cy3-tyramide

    Disadvantages: the kits and related supplies can be expensive, the kits are created for a specific protocol that can be hard to modify due to little information given about the product (for proprietary reasons), there is some loss of resolution on the subcellular level since the tyramides are binding near but not necessarily on the epitope.

    However, these issues can be addressed: 1. you can make the necessary reagents in-house for ¼ the price as of this writing, 2. making reagents in-house allows for complete control of concentrations and solution constituents, and 3. the resolution loss is not apparent unless one is analyzing tissue on the intracellular level.

    See Appendix C for more details on making tyramide conjugates and using them.

    (My preferred ref: Hopman AH, Ramaekers FC, Speel EJ. Rapid synthesis of biotin-, digoxigenin-, trinitrophenyl-, and fluorochrome-labeled tyramides and their application for In situ hybridization using CARD amplification. J Histochem Cytochem. 1998 Jun;46(6):771-7. doi: 10.1177/002215549804600611. PMID: 9603790.)

- **Enzyme-Labeled Fluorescence (ELF):** This process uses a different enzyme, alkaline phosphatase (AP), to convert a specific chemical substrate called 2-(5'-chloro-2-phosphoryloxyphenyl)-6 -chloro-4(3H)-quinazolinone (a.k.a. ELF-97) into an insoluble and extremely bright fluorescent product that deposits near the enzyme's location. This method shares some properties with both the DAB and TSA reactions. Like DAB, it creates an insoluble reaction product. Like both methods, the reaction progresses quickly over time. However, it does not require H_2O_2. Further, since it is a phosphatase, all solutions must avoid the use of phosphates. Thus, instead of using potassium or sodium phosphates to create buffers, the use of Trizma acid and base to create buffers is recommended. Otherwise, the phosphatase will react non-specifically to the phosphate buffer and cause much background labeling. The reaction product is a unique fluorophore – it is excited by UV/violet lasers (360 nm is ideal) and emits in the green spectrum (emission max at 530 nm). This large Stokes shift avoids the brain tissue autofluorescence resulting from UV lasers, as that autofluorescence

typically emits as cyan-colored. In other words, with the right emission filters, autofluorescence may not be an issue. How this works in a protocol:

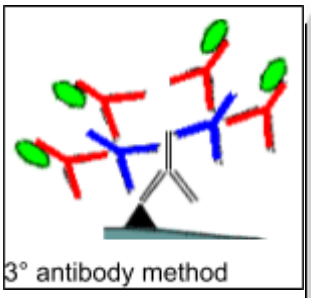
A. 1° antibody binds to antigen.
B. Biotinylated 2° antibody binds to 1°.
C. AP-conjugated streptavidin binds to 2°.
D. ELF-97 added to tissue and reacted for specific time.

Advantages: VERY intense amplification, autofluorescence easily surpassed and autofluorescence reduction treatments may not be needed, short reaction time added to protocol, labeling is stable for years even at room temp.

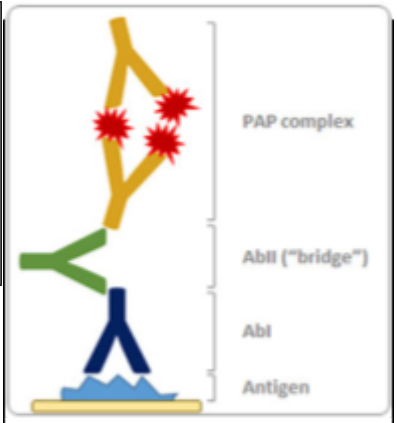
Disadvantages: Easily overamplifies most stains so it may be better suited for in situ hybridization, protocol must omit phosphates, subcellular resolution may be compromised, need specialized excitation/emission filters on microscope, expensive, only available in one color.

(Ref: Larison KD, BreMiller R, Wells KS, Clements I, Haugland RP. Use of a new fluorogenic phosphatase substrate in immunohistochemical applications. J Histochem Cytochem. 1995 Jan;43(1):77-83. doi: 10.1177/43.1.7822768. PMID: 7822768.)

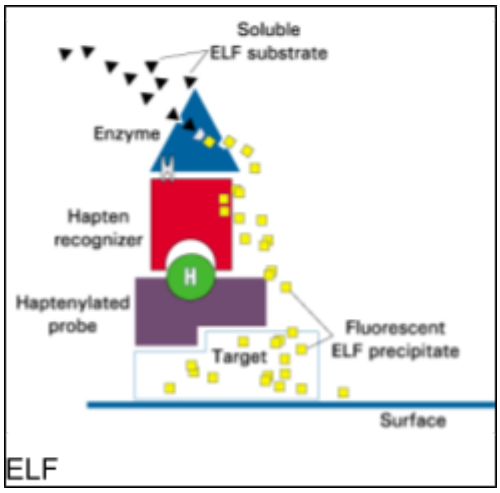
Figures of Amplification Techniques (converted to PNG files in July 2023)



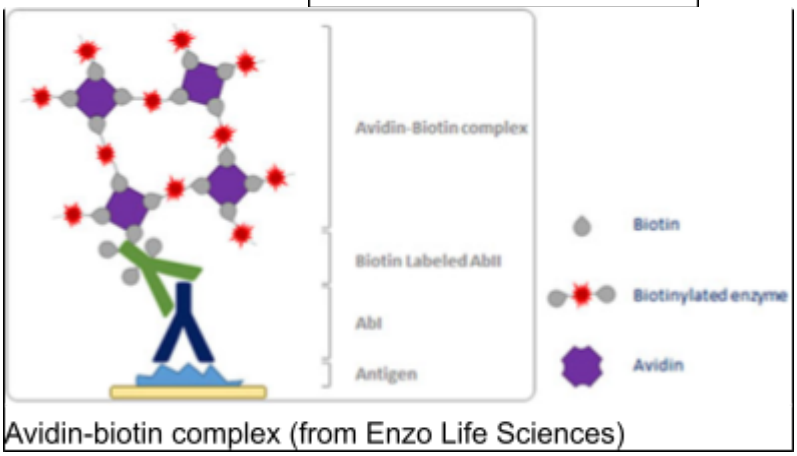
3° antibody method



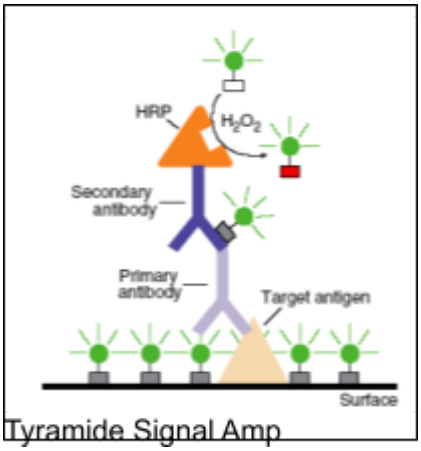
Peroxidase anti-peroxidase (from Enzo Life Sciences)



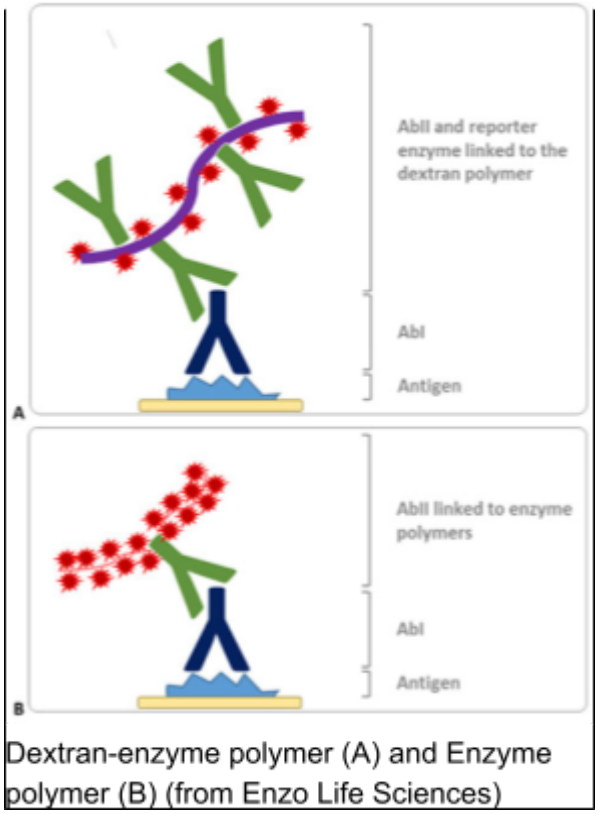
ELF



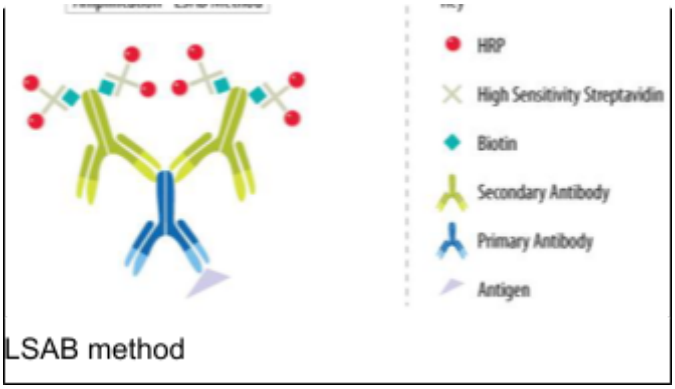
Avidin-biotin complex (from Enzo Life Sciences)



Tyramide Signal Amp



Dextran-enzyme polymer (A) and Enzyme polymer (B) (from Enzo Life Sciences)

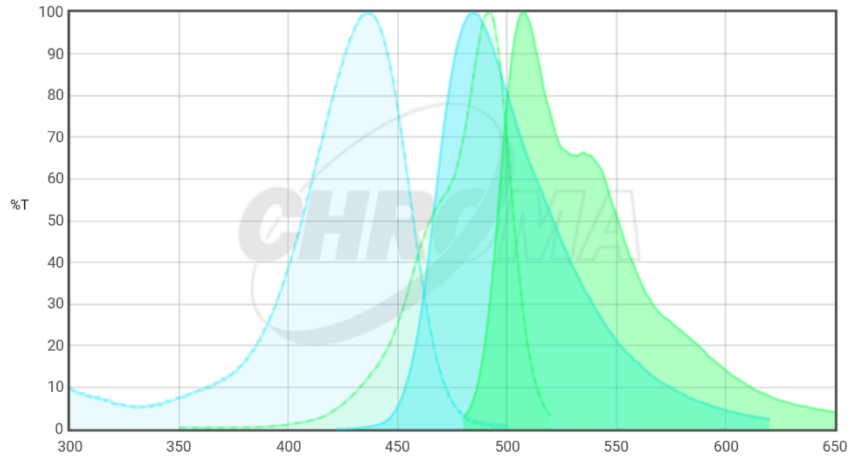


LSAB method

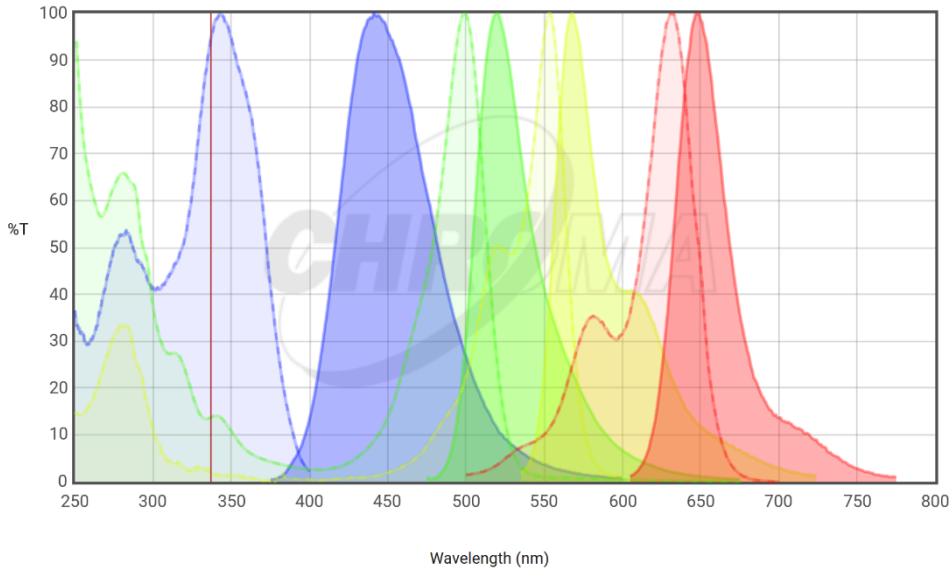
IX. Multiple labeling [MTLB]

There is a common desire to stain multiple targets within the same tissue section, especially if researchers are interested in co-localization. This *can* be done for chromogenic staining, but there are limitations to how well things can be seen. For example, labeling orexin with DAB results in some brown cells, labeling GAD67 with DAB + nickel will result in black cells. But, an observer may have a hard time determining when there is overlap if the black staining fully obscures the brown staining. So, fluorescence staining is mainly used for co-localization studies.

When selecting multiple fluorophores, ensure that their excitation and emission spectra are separate enough from each other! Although it's intuitive that you wouldn't want to use two green fluorophores for different targets, there are less-expected overlaps. One example would be the use of a green fluorophore and then a cyan fluorophore. In that case, it would be very possible for the cyan fluorophore to still be minorly to moderately visible in the microscope channel used for viewing green fluorophores. (Chart produced by widget on Chroma.com)



Generally, I've found that blue, green, red, and deep red fluorphores do not have much overlap in their excitation & emission spectra. This chart shows excitation and emission spectra for AlexaFluor 350, 488, 555, and 633. (Chart produced by widget on Chroma.com)



Also important to using multiple labels is to make sure that the lasers or excitation and emission filters on your fluorescence microscope are appropriate for viewing the fluorophores. On regular epifluorescence microscopes, the excitation light used for stimulating fluorescence comes from an arc light. As this light is white, filters isolate a specific range of wavelengths (bandpass excitation filters) to excite a fluorophore. A typical bandpass excitation filter for AlexaFluor 488 might allow through light of wavelengths 460 nm to 500 nm. This *may* present a problem if you have another fluorophore whose excitation spectrum has a significant part of the curve going through that range. As seen in one of the figures above, a cyan fluorophore could still be excited by that light range, even if minorly. Some researchers opt to use lasers as an excitation source. Lasers usually put out a specific wavelength with little range/deviation from that number, so they are less likely to cross-excite similar fluorophores. However, the power output usually has to be larger in order to stimulate adequate fluorescence from the tissue.

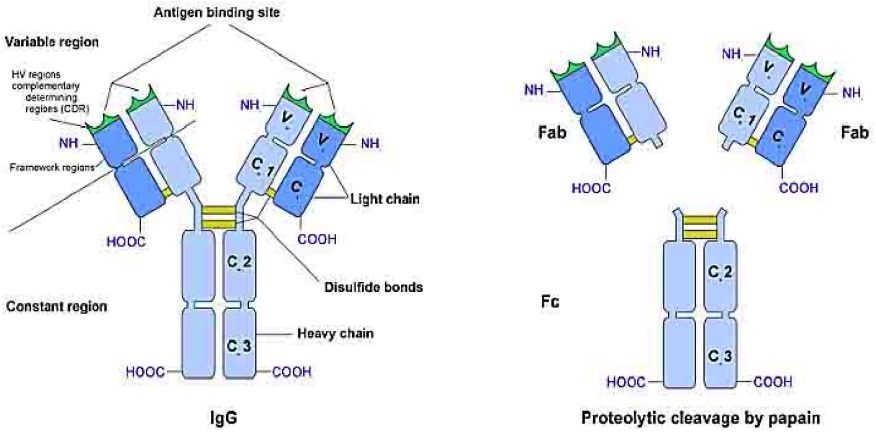
So we have an idea of which colors to pick and which colors to not pick when it comes to multiple fluorescent labels. But how might an IHC protocol like that actually work? I tend to mix multiple primaries together in the same solution, unless there are reasons to keep them separate (plans to re-use one but not the other, for instance). So a hypothetical primary cocktail I might make would be mouse anti-cFos, rabbit anti-DeltaFosB, and goat anti-EGR1. This would be diluted in 1x PBS with 2.5% normal donkey serum, 0.2% Triton, and 0.05% sodium azide. By combining these three antibodies together, we can do the primary staining step for all three in one overnight session, rather than having to do three sequential 16-hour sessions. If I were to use secondary antibodies that all had fluorescent labeled conjugated to them, such a cocktail might be: AlexaFluor 350 donkey anti-mouse, DyLight488 donkey anti-rabbit, and Cy3 donkey anti-goat. These too would be diluted in 1x PBS with 2.5% normal donkey serum, 0.2% Triton, and 0.05% sodium azide. Combining these together also decreases staining time, from six hours to two hours.

If you wish to perform multiple rounds of tyramide signal amplification, see the previous section on that. Specifically though, you cannot combine multiple HRP-conjugated secondary antibodies in the same solution and still expect the different-colored tyramides to "figure it out"; erroneous cross labeling is guaranteed. Instead, the secondaries would have to be sequential. Take the hypothetical from the previous paragraph. The primary incubation is finished, and we want to perform the TSA method for all three targets. The simplified protocol would look something like: HRP donkey anti-mouse, rinse, AlexaFluor 350-tyramide, neutralize first HRP with azide, rinse, HRP donkey anti-rabbit, rinse, DyLight 488-tyramide, neutralize second HRP with azide, rinse, HRP donkey anti-goat, rinse, and Cy3-tyramide. This certainly extends the time commitment of the protocol a lot, but the trade-off for me is worth it in how much each stain is amplified and how much less tweaking I would have to do with camera exposure & gain on the microscope and then brightness & contrast in image editing software.

Here is one more important consideration: make sure you acquire secondary antibodies that are highly cross adsorbed, even if you are not combining them in the same solutions. Secondary antibodies that are not screened in this way by the vendor can minorly to moderately bind to the wrong primaries! I have experienced this a few times, where a donkey anti-goat antibody still cross-labeled a rabbit primary antibody. When a vendor does cross-adsorbtion screening, they will usually tell you which species their secondary antibody should not react to. It may say "min react to mouse, rabbit, chicken, hamster". Be careful in reading those lists to make sure the animal you don't want to target is part of that list.

What if I am forced to buy two primaries from the same host?:
Well, no one is forcing you to do anything. But, in the case of such an inconvenience, multiple labeling is still possible, albeit more difficult. You can no longer stain with the two/three primaries mixed together, and additional blocking steps **MUST** be employed.

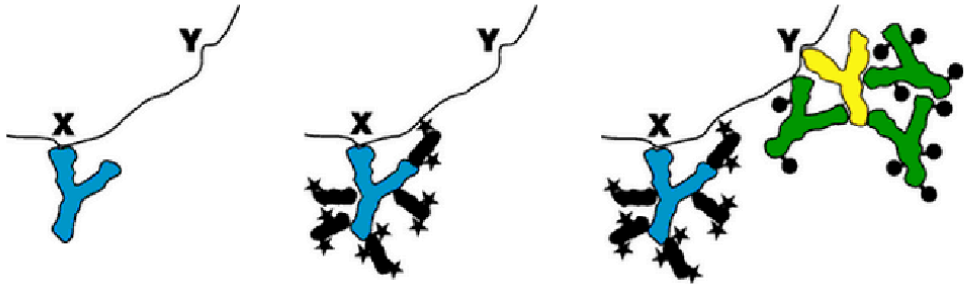
Whole IgG antibodies can be cleaved into antibody fragments called monovalent F(ab) fragments. These lack the recognition site (the F(c) fragment) and the second antigen binding site.



This prevents a secondary antibody from attracting a primary away from its target while it is in solution. A simple protocol goes as follows:

Key:

- Rabbit anti-Antigen X
- Rabbit anti-Antigen Y
- Fab fragment Goat anti-Rabbit IgG (H+L)
- Goat anti-Rabbit IgG (H+L)
- DyLight 488
- Rhodamine Red-X



1. Incubate with the first primary antibody, in this example rabbit anti-antigen X. Wash.
2. Incubate with excess Probe I-Fab antibody against the host species of the primary antibody, in this example DyLight 488-Fab fragment goat anti-rabbit IgG (H+L). Wash.
3. Incubate with the second primary antibody, rabbit anti-antigen Y, followed by Probe II-conjugated divalent secondary antibody, In this example Rhodamine Red-X-goat anti-rabbit IgG (H+L). Wash.

Caution: This protocol is subject to the following potential problems: 1.) Example A may require a high concentration of conjugated Fab to achieve effective blocking of the first primary antibody. If this results in unacceptable background, try a lower concentraton of the conjugated Fab followed by further blocking with unconjugated Fab. 2.) If aggregates of conjugated Fab are present and they bind to the first primary antibody, they may act as divalent or polyvalent molecules and capture some of the second primary antibody. This would result in apparent detection of overlapping antigens, even though the antigens may not be overlapping.

There are other ways to do multiple stains in the same primaries. This image was taken from www.jacksonimmuno.com, and more protocols can be found there. More information can also be found in Appendix B in this document. Be sure to consider how all possible cross-staining issues are blocked in your multiple staining run.

X. Mounting Media [MMD]

Mounting media, also known as coverslipping media, is often necessary for observing specimens under a microscope. Such media infiltrate tissue to make it more see-through. This see-through (transparency/translucency) aspect is absolutely necessary for brightfield microscopy. It is less required for fluorescent microscopy, but dry tissue tends to reflect light back at the scope and thus will have higher background.

Various forms of mounting media can be made in lab or can be bought. Though many different types of mountants exist, many operate under similar principles. There are glycerol-based, aqueous hardening, and xylene-based mountants. There are also various glues and adhesives, some of which I am currently testing (July 2023). Below are pros and cons to each.

Reagents of interest:

1. Glycerol-based mountants (See Appendix E for a recipe)
   - Pros:
     - Ingredients to make in lab are inexpensive and easy to work with.
   - Cons:
     - Allegedly require an antifading agent additive to prevent fluorescent signal degradation, though this may vary depending on the fluorophore in the tissue.
     - Non-drying; requires nail polish to seal the coverslip edges and prevent leakage.
     - Slides should be stored at -20°C to preserve fluorescence long term.
     - Mountant may contribute some background fluorescence if not correctly formulated

2. Solidifying/Curing aqueous mountants
   - Pros:
     - Very easy to use
     - Commercial ones usually come with antifade agents
     - Can make rather easily in lab (PVA mountant)
   - Cons:
     - Commercial ones are somewhat expensive
     - Commercial ones sometimes have DAPI as an additive, which may cause diffuse/unwanted DAPI staining
     - Vectashield is incompatible with certain fluorophores (see Appendix D)
     - Antifade agents included in commercial ones can also be incompatible with certain fluorophores.

3. Petrochemical-based mountants (DPX, Permount)
   - Pros:
     - Robustly preserve fluorescence long term, even when slides are stored at room temperature
     - Cures somewhat quickly; might be solid enough in 1-2 days
     - Compatible with Nissl stains (see Part 3, Section VII)
     - Does not appear to introduce additional background fluorescence
   - Cons:
     - Requires brief tissue dehydration (ethanol) and clearing (xylene) procedures
     - Incompatible with certain fluorophores (see Appendix D)
     - Requires working with dangerous chemicals (ethanol, xylene) under fume hood
     - Mountants themselves contain petrochemicals, such as toluene (carcinogenic)

- Nitrile or neoprene gloves are required, as latex does not block the chemicals enough
- Compresses Z dimension of tissue
- Certain types (such as Permount) quench most fluorescent signals while others (DPX) do not.

Mounting Procedures

A. "Curing" glycerol-based mountants (ProLong Gold, VectaShield, PVA):

1. Mount sections to slides from diluted buffer.
2. Briefly air dry sections, preferably with a fan, to ensure they stick. Damp sections will drift when media is applied.
3. Apply a thin horizontal line of mounting media near the top of the slide. Alternatively, you can add one drop of medium over every section.
4. Lower the slide down at an angle to avoid trapping air bubbles.
5. Cures at room temperature overnight. Protect from light.
6. These reagents continually cure over several days, which gradually increase their refractive index. If this is not desired, halt curing (progresses due to oxygen) by sealing the edges of the slide with nail polish.

B. Petrochemical-based plastic mountants like DPX:

1. Mount sections using a 0.1% gelatin solution (no PB!).
2. Briefly air dry sections, preferably with a fan. Drying with gelatin ensures that sections stick to the slide when dehydrating and clearing.
3. Sections must be dehydrated with graded ethanols and cleared with xylene (adapt from a Nissl staining protocol).
4. Remove one slide from xylene. Do not allow to dry!
5. Apply a thin horizontal line of mounting media near the top of the slide.
6. Lower the slide down at an angle to avoid trapping air bubbles.
7. Repeat mounting media application process with next slide. Slides can sit in xylene for several minutes without issues.
8. Dries at room temperature overnight. Dry in fume hood! Protect from light.

C. Aqueous mountants (non-drying, non-curing) like glycerol-PBS mixes

1. Before mounting, ensure that you possess coverslips that are slightly less wide than the slide's width. This allows for easier application of sealant later on.
2. Mount sections to slides from dilute buffer.
3. Briefly air dry sections, preferably with a fan, to ensure they stick. Damp sections will drift when media is applied.
4. Apply a thin horizontal line of mounting media near the top of the slide, or otherwise apply one drop of medium over each section.
5. Lower the slide down at an angle to avoid trapping air bubbles.
6. Let slides sit flat overnight, protected from light.
7. Trapped air has likely surfaced to the top sides of the sections. Press lightly on the coverslip to force these bubbles outward.
8. If too much mountant has been displaced, add a drop to the edge of the coverslip. Capillary action will pull this drop underneath the coverslip.
9. Ensure that there is enough mounting media underneath the coverslip. Avoid air pockets at the edges.
10. Apply sealant to the edges of the coverslip (see "Notes about mountants", below). Take care not to paint over the sections.
11. Air dry sealant.

12. If there is excess sealant, remove with a razor blade or solvent.

Notes about mountants:

- Curing and non-aqueous mountants compress a sample along its Z axis. Thus, these mountant types are ideal for widefield microscopy. However, for maintaining 3D structure for confocal microscopic imaging, non-drying aqueous media should be used.
- It is unclear whether ingredients to certain nail polishes quench fluorescence. Instead of using nail polish to seal up slides containing aqueous mounting media, use sealants containing inert ingredients. Electrophysiology-compatible silicone sealants such as Kwik-Sil (from World Precision Instruments) can work well, though this product is quite expensive. A cheaper alternative is a 1:1:1 mixture of Vaseline, lanolin, and paraffin, called VALAP (North, 2006; J Cell Bio., 172(1):9), though this tends to be messy, chunky, and it fractures off the slide easily. I've experimented with Elmer's glue as a sealant when using mineral oil as a medium, but that will not work for water-based mountants.

De-coverslipping

Did your stains not turn out the way you wanted? Don't throw away those slides – it may be possible to de-stain, re-stain, or treat further! Solidifying/Curing mounting media can be dissolved. However, the issue is that if you did not use charged or gelatinized slides, the sections will likely fall off. As long as it isn't an issue if the sections are out of order, they can be picked up and run through staining protocols. Be advised that they will be a bit more fragile, depending on the mounting medium used.

A. Dissolving ProLong Gold or polyvinylalcohol (PVA)-based mounting media:
   Immerse slides in PBS at 37°C for 30+ minutes. Be sure to leave the sample in this warmed PBS after coverslip removal to extract any further mounting medium (information obtained from product sheet).

B. Dissolving Permount or DPX:
   As both of these mounting media are xylene-miscible, xylene will dissolve these media. Immerse slides in xylene until coverslips can detach. Keep tissue in xylene for a few additional minutes to remove excess mounting media.

C. Dissolving nail polish sealant and removing aqueous mountant:
   Nail polish can be removed with - you guessed it - nail polish remover. Saturate a kimwipe with nail polish remover and drape it onto the slide. Avoid immersing the slide in nail polish remover or acetone, as this may either quench fluorescence, fix tissue further and mask antigens, or both. After nail polish is removed. immerse slides in warmed PBS to slowly dissolve glycerol and allow for careful detachment of the coverslip.

XI. On peptide staining, colchicine, and mRNAs

At one point, I had become interested in IHC labeling peptides to help distinguish neuron chemotypes. However, I quickly found that peptide staining often was not somatic in nature. Instead, it was frequently in the axons or terminals, which made it virtually impossible to do cell counts for peptide-possessing neurons. I searched the literature for answers to this hurdle, and found some minor aides.

- One reference I came across noted how some opioids are more visible in IHC staining when the tissue is pre-treated with 1% H_2O_2 in 0.1M PB for 30 min. The proposed mechanism was that the oxidative reaction makes the peptides more recognizable, though I imagine this might be very particular to the antibody involved.

- The use of some detergents at low concentrations (0.02%), such as Triton X-100 (but not SDS!) , may improve fiber labeling for opioids. However, one study said that perikarya immunoreactivity decreases substantially, possibly due to diffusion out of the cell. Axon staining may be resistant to this effect as the peptides are captured in vesicles.
- Pepsin and other proteases may cleave some opioid precursor peptides into their smaller components.

Colchicine and its historic, yet ethically problematic, use to improve IHC staining of peptides:

Colchicine is a compound that depolymerizes tubulin and has variable effects on mRNA expression. The fact that it deconstructs cytoskeletal pathways has been utilized by neuroanatomists. In particular, tubulin in axons is most vulnerable to these effects, and without proper tubulin structure many peptides cannot be transported from the soma to terminals. Thus, intracerebral colchicine administration "improves" peptide staining by restricting these peptides to their originating somata.

The common mode of administration is injection into the cerebral ventricle(s). Doses vary; injections into the lateral ventricle have contained 200 ug in 25 uL, 100 ug in 10 uL, or 100 ug in 20 uL for rats, while 12 ug in 3 uL has been used for mice. If one uses a dose of 100 ug in 10 uL, this is 1 mg in 100 uL for dilution purposes. The most important issue with ventricular injection is that it severely impacts the subject's quality of life; ICV colchicine-injected rats experience pain, stress, lack of appetite, and likely disturbances in renal functions. According to the literature in which colchine was used in this fashion: typical incubation time for maximal accumulation of peptides into somata is 24-48 hours, yet these rats may not live for much longer with the above doses used. Thus, ventricular injections of colchicine should be avoided at all costs. Modern animal handling protocols are barred from doing this method.

In theory, site-specific colchicine injections may be less problematic, as they only affect the site of injection and not the rest of the brain. Small injections to the VMH produce effects similar to VMH lesions, but in a transient manner such that rats recover normal ingestive functions by two weeks post-injection (ref info needed). Thus, colchicine is a short-term lesioning agent. However, some neuron types may be particularly sensitive to colchicine, and cytotoxicity may result. Cholinergic neurons in the basal forebrain have been noted to be easily killed by colchicine administration.

Site-specific colchicine can be used to characterize peptides of afferents to the injection site. One study (ref info needed) featured colchicine injection into the BNST to determine peptide-containing afferents that innervate the BNST. In this study, a dose of 10 ug in 0.5 uL (for dilutions, this is 2 mg in 100 uL) was used. Spread is estimated to be 500-700 um for 0.5 uL, ~1 mm for 1 uL. Temporary lesion studies used dosages of 2ug in 0.1 uL (spreads ~0.15 mm^2 w/ fluorescein-conjugated colchicine) in the VMH, 4ug in 0.5 uL, or 16 ug in 2 uL for LH. The 24-48 hour survival time still applies for maximal somatic peptide accumulation.

In summary, central colchicine appears to vastly improve somatic peptide labeling. However, it is messy, disrupts cellular structure and function, can outright kill off certain cell types, and most importantly is extremely injurious to rats when administered ventricularly. Even when it is used in a brain site specific manner, the damage it can cause may not be fully reversible. My recommendation is to not bother with colchicine injections at all. Instead, use in situ hybridization (ISH). ISH works somewhat for localizing peptides to somata, as the mRNA used to make those peptides is concentrated in the somata and remains sparse in axons and terminals.

How does one do peptide mRNA ISH staining? I have limited experience with it, so I advise contacting authors of publications in which it is used.

XII. Miscellaneous Notes [MSC]

- PBS can be made with either sodium or potassium phosphates. They make no difference for staining of fixed tissue.
- Some protocols use phosphate buffer, other protocols use phosphate buffer saline. Both should have equal osmolarity. The difference for the saline version is that the phosphate concentration is $1/5^{th}$ to $1/10^{th}$, and this is offset by adding sodium chloride to add back in salt. Sodium chloride doesn't really affect pH much compared to other solution components used in IHC. There has been some discussion about the amount of saline influencing ionic strength and thus antibody binding, but I'm unsure that this is a significant enough effect to cause noticeable staining differences.
- Regular plastic, non-netted 24-well plates hold a maximum of 3 mL per well. One can stain with as little as 0.5 ml per well in these. Increase by 0.5 mL for each additional brain section per well; 3 brain sections should be incubated in a little over 0.75 mL solution.
- Regular plastic, non-netted 12-well plates hold a maximum of 9 mL per well. These well plates are more appropriate for wide tissue sections (even including sagittal or horizontal cuts of the whole rat brain) or otherwise a larger space to track the progress of enzymatic reactions.
- Conjugating labels or haptens onto antibodies:
    - Biotinylation:
        - MW of Biotin 3-sulfo-N-hydroxysuccinimide ester sodium salt = 443.43 g/mol
        - A 10 mM solution has 4.43g/L = 4.43mg/mL = 1mg in 226 uL. Can dissolve sulfo-NHS esters in pure water, DMSO, or DMF. I use DMF as that has worked best in the past, but organic solvents may denature some proteins such as antibodies.
        - An 8:1 molar ratio of biotin ester to Fab fragment antibody should be helpful.
        - mL of protein solution x protein concentration in mg per mL / MW of protein * molar excess of biotin ester = mmol biotin ester to add to protein solution
        - Example using cross-multiplication:
            - For 1mL of 2 mg/mL IgG (150,000 MW), ~27µL of 10mM biotin reagent will be added as follows:
            - 1 mL IgG x (2mg IgG/ 1mL IgG) x (1mmol IgG/ 150,000 mg IgG) x (20 mmol biotin / 1 mmol IgG) = 0.000266 mmol biotin ester
            - 0.000266 mmol biotin x (1,000,000 uL / L) x (L / 10 mmol) = 26.6 uL biotin reagent
    - *Fab fragments with Fc fragment still in solutions amounts to 3x the protein at 50,000 kDA each. Should use calculation with IgG size.
- Berridge Lab microscope specs (Jan 2017)
    - 5x: 1.285 x 1.289 um / pixel
    - 10x: 0.643 x 0.645 um / pixel
    - 20x: 0.322 x 0.323 um / pixel
-

Part 3: Pre- and Post-Treatments of Brain Tissue for Better IHC Staining

I. Antigen Retrieval Techniques [ARTQ]

During the fixation process, the cross-linking of proteins that makes the tissue rigid enough to use/section may also mask your epitope. It may do so either by acting as a cage around your target of interest or by changing the target protein's conformation. Both possibilities prevent an antibody's access to the binding site. Antigen retrieval is a term that describes techniques intended to unmask epitopes that are blocked/deformed by fixation. The two main types that are referred to as "antigen retrieval" in the literature are: Heat-Induced Epitope Retrieval (HIER) and Proteolytic-Induced Epitope Retrieval (PIER). However, other methods exist that are technically antigen retrieval as well, but are usually not referred to as such.

I want to share my thoughts on the use of these various techniques. Antigen retrieval assumes that the tissue preservation process (formaldehyde exposure or other things) masks the epitope, and that these techniques will reverse that masking by undoing what the preservation process does. In some cases (HIER methods especially) these ideas are accurate. The consequence is that the tissue you use will be much weaker, warped, fragile, distorted, etc. after you apply these methods. Similarly, by using some of these methods, you are de-fixing the tissue and thus making it more vulnerable to degradation. Another caveat is that even if one method of retrieval is shown to work on a specific tissue prep and with a specific antibody, another retrieval method may not work for that same pair. It gives a bit of uncertainty of how exactly the fixation is being reversed, and how much reversal is truly required to make the tissue "stainable".

After my experiences trying most of these methods out, I now try to avoid performing any antigen retrieval unless I find that it is absolutely necessary for allowing an antibody to work on the target tissue - it makes the sections way too difficult to manage or partly ruins their analysis under the microscope. I would much rather control the fixation of tissue by limiting the fixative incubation duration (example: no more than 7 days in 4% formaldehyde) and/or using staining amplification methods to offset the meager antibody labeling in certain tissues/preps. Nonetheless, I've collected info on the various methods that I have seen, in case any are helpful. I find the PIER ones to be the easiest to control, and they seem to be relatively compatible with free-floating sections.

A. Heat-induced retrieval [HINR]

Somewhat fast and hot protocol:

1. Make or retrieve AR solution (see reagents). Can use citrate, citraconic, Tris-EDTA, or EDTA buffers.
2. Heat AR solution in glass to a stable 90 °C (95 for citraconic).
3. Retrieve sections that will undergo HIER.
4. Rinse sections in distilled water for 5 min.
5. Rinse sections in AR solution for 10 min at room temp.
6. Incubate sections in AR solution at 90 °C for 20-30 minutes (45 min for citraconic).
    - Note: can put sections directly from room temp to hot temp, rather than waiting for water bath to slowly reach desired temp.
7. Cool in fresh room-temp AR solution for 10 min.
8. Rinse 10 min with PBS.

Slower and warm protocol

1. Make or retrieve AR solution (see reagents). Can use citrate, Tris-EDTA, or EDTA buffers.
2. Heat AR solution in glass to a stable 60 °C.
3. Retrieve sections that will undergo HIER.
4. Rinse sections in distilled water for 5 min.
5. Rinse sections in AR solution for 10 min.
6. Incubate sections in AR solution at 60 degrees C for overnight
7. Cool in fresh room-temp AR solution for 10 min.
8. Rinse 10 min with PBS.

Considerations:

- Requires moderate heat source. Water bath is highly recommended. If lacking a water bath, use the hotplate for high temp, incubator for less hot temps.
- To create a makeshift covered heating chamber on a hotplate, set to hotplate setting 2. The setup I've used is a large glass petri dish on the hotplate with the buffer filling it ¾ of volume. I avoid having sections directly over the center of the hotplate, as the temperature will be hotter there than in the periphery. Use old glass microscopy slides as spacers between the basin of the petri dish and the bottom of the net-well containing the sections.
- If the epitope is incompatible with heat (some fluorophores like Fluorogold) or an acidic environment, do not use this method!
- Solution can be very hot! Wear protection, do not splash solution!
- AR solution can be stored in fridge. Can be reused. Retains efficacy for about 3 months.
- Citraconic method is allegedly best for tissue that has been overfixed for way too long – up to 7 years, in the case of human brain tissue. However, the pure powder is poisonous and corrosive, so it poses greater risk than other AR methods.

Refs:

https://www.ihcworld.com/_protocols/epitope_retrieval/citrate_buffer.htm
Kanai K, Nunoya T, Shibuya K, Nakamura T, Tajima M. Variations in effectiveness of antigen retrieval pretreatments for diagnostic immunohistochemistry. Res Vet Sci. 1998 Jan-Feb;64(1):57-61. doi: 10.1016/s0034-5288(98)90116-3. PMID: 9557807.
https://www.ihcworld.com/_protocols/epitope_retrieval/citraconic_anhydride.htm
Namimatsu S, Ghazizadeh M, Sugisaki Y. Reversing the effects of formalin fixation with citraconic anhydride and heat: a universal antigen retrieval method. J Histochem Cytochem. 2005 Jan;53(1):3-11. doi: 10.1177/002215540505300102. PMID: 15637333.

B. Protease-induced retrieval [PINR]

Protocol:

1. Pour solution into shallow container.
2. Warm retrieval solution to 37-42 °C. This can be done on a slide warmer; hot plates may get too hot too easily. An incubator may be ideal in this case.
3. Retrieve sections that will undergo PIER.
4. Rinse sections in distilled water for 10 min to wash off PBS.
5. Transfer into retrieval solution for 10 min.
6. Rinse sections in distilled water for 5 minutes, then in KPBS for another 10 min.

Considerations:

- Requires low heat source. A slide warmer brings it to the ideal temperature if using a glass petri dish or plastic square “Falcon” dish.
- Tissue digestion will be slower if solution deviates from the optimal pH stated in the recipe.
- If the epitope is VERY sensitive to heat or an acidic environment, do not use this method! It should be noted that I've achieved vastly improved staining of fluorogold with this PIER method; the tracer's original fluorescence remains largely unaffected, except that the emitted color changes to whitish-yellow.

- Solution is dangerous - corrosive! Wear protection! Do not splash solution! Do not pour down drain unless neutralized!
- Enzyme solution can be stored in fridge. Can be reused. Retains efficacy for about 6 months.

Refs:
https://www.ihcworld.com/_protocols/epitope_retrieval/pepsin.htm
Hazelbag HM, van den Broek LJ, van Dorst EB, Offerhaus GJ, Fleuren GJ, Hogendoorn PC. Immunostaining of chain-specific keratins on formalin-fixed, paraffin-embedded tissues: a comparison of various antigen retrieval systems using microwave heating and proteolytic pre-treatments. J Histochem Cytochem. 1995 Apr;43(4):429-37. doi: 10.1177/43.4.7534785. PMID: 7534785.

C. Strong Acid Exposure [SAEX]

Method 1 – Hydrochloric acid

(https://www.ihcworld.com/_protocols/epitope_retrieval/hcl.htm)

1. Prepare the solution by adding 20 ml of 10N (concentrated) HCl to 80 ml of distilled water. Be sure to mix the solution well; the pH should be in the range of 0.5-1.0
2. Incubate sections using the 2N hydrochloric acid solution for 10 to 20 minutes at room temperature. A range of times (suggestion 10, 15, and 20 minutes) incubation time should be tested to determine an optimal antigen retrieval time.
3. Rinse sections in two changes of a buffered solution, such as PBS or TBS, in order to neutralize acidity.

Method 2 – Formic acid

(https://www.ihcworld.com/_protocols/epitope_retrieval/formic_acid.htm)

1. Create a formic acid solution (10% in Distilled Water, pH1.6-2.0) by mixing 10 mL of 99% Formic acid with 90 mL distilled water. Mix well and pH should be around 1.6-2.0. Store at room temperature.
2. Incubate sections with formic acid solution for 10-20 minutes (optimal incubation time should be determined by user).
3. Rinse sections in PBS.

D. Freezing Tissue [FRZE]

Can also be used to flash-freeze tissue for long-term storage.

Background:

Though there are a wide variety of approaches to tissue preparation, many neuroscientists perform the process as perfuse → post-fix → sucrose → freeze → section. However, others freeze the tissue immediately after decapitation and section it as-is, unfixed. This can be done by immersion of the rapidly-removed brain into dry ice-chilled 2-methyl butate (aka isopentane) or hexane; this process flash-freezes the brain.

One issue that occurs any time tissue is frozen is ice crystal formation. Water expands when frozen, and the speed at which it is frozen determines the structure of the ice crystals. Consider that cells are fragile containers filled with water. Slow freezing results in large jagged pointed crystals that easily rupture most of the cell membrane and intracellular compartments, whereas flash-freezing causes ice crystals to be smaller and more cube-like, thus rupturing less of the membrane. A cryoprotecting substance such as sucrose can be incubated with the tissue prior to freezing, so that the cells absorb this sucrose solution and the resulting ice crystals are either not formed or otherwise form in rounded shapes. In the cases of fixed tissue where morphology preservation is key, steeping tissue in sucrose prior to freezing is a must.

But what if you *want* to rupture membranes? Using the traditional perfuse-fix-sucrose-freeze approach certainly keeps the tissue structure as preserved as possible. This procedure also preserves intracellular compartments, which retain their antigens within. These preserved membranes and

compartments can prevent the quick and easy access of antibodies in solution to the antigen of interest, especially if it is within the nucleus. Although formaldehyde fixation tends to permeabilize the cell membrane, it seems to do this only to a limited degree for the nucleus. If an investigator wants to stain for c-fos, a nuclear-localized antigen, some have found difficulty in getting the stain to penetrate in a reasonable amount of staining time. If the investigator is unconcerned with morphology and only wants to stain for c-fos in order to count activated neurons, it may be best to permeabilize the nuclei (and cells in general) by freezing the tissue without cryoprotection. This may be the crux behind the "Fast Fos" protocol recommended in one article (Sundquist & Nisenbaum, J. Neurosci. Methods, 2005, Vol. 141, pp 9-20).

Protocol:

1. Pour 2-methylbutane or hexane into a small (~50 mL) stainless steel beaker.
2. Put beaker into dry ice.
3. Add dry ice to the liquid in beaker. Continue until bubbling stops. Temperature will be around -80 °C.
4. Create a plastic ladle or some sort of device to lower a rat brain into this liquid and be able to lift it out. A ladle crafted from tin foil with holes poked through the spoon may work as well.
5. Fatally anesthetize and perfuse animal briefly with saline as usual; perfusion may be skipped if you are unconcerned with clearing out blood from the brain tissue.
6. Decapitate animal and extract brain as quickly as possible.
7. Using ladle, dip brain in liquid for ~25 seconds.
8. Remove and store at -80 °C until ready for sectioning.
9. Section tissue and simultaneously thaw-mount sections onto charged microscopy slides
10. Dry sections on slides.
11. Immerse in PFA solution for 10 minutes for 20 um sections, 20 min for 40 um sections.
12. Rinse, and stain whole slides.

E. Antigen "Straightening" with SDS [ASDS]

Sodium dodecyl sulfate (aka sodium lauryl sulfate) is used to denature large proteins and "flatten" their shape for better antigen binding. In some situations, SDS has been used on tissue and may improve binding of antibodies that were originally intended only for western blot use. One example is pSTAT3 staining by the Munzberg lab – I have personally used their protocol with notable success. The protocol varies between papers:

- Methanol with H2O2 added, then 0.3% glycine, then 0.1% SDS; no timings mentioned (Zhang et al., 2011)
- 1% H2O2, then 0.03% SDS; no timings mentioned (Perello & Raingo, 2013)
- 1% NaOH and 1% H2O2 in H2O for 20 min, then 0.3% glycine for 10 min, and then 0.03% sodium dodecyl sulfate for 10 min. (Munzberg et al., 2003)
- 1% NaOH, then 1% H2O2 in distilled water for 20 minutes at room temperature, rinsed with PBS 3 times for 10 minutes each, incubated with 0.3% glycine in PBS for 10 minutes, rinsed with PBS 3 times for 10 minutes each, incubated with 0.03% sodium dodecyl sulfate (SDS) in PBS for 10 minutes (Cui et al., 2012)
- An additional general protocol calls for 1% SDS in 0.01 M PBS (pH 7.4) treatment for 5 min. (I'm not sure where I obtained this particular one from…)

In reviewing these above protocols, there are various reagents involved. However, I'm not convinced that all are essential for the core goal - to alter the antigens in the tissue so that a "western blot-preffering" antibody can be used for IHC. For instance, I suspect that the methanol and NaOH treatments are meant as peroxidase inactivators, especially since they are combined with H2O2. Peroxidase inactivation is not necessary unless

using DAB, AEC, or tyramide staining protocols, and if you use those labeling methods, you may already have endogenous peroxidase inactivation steps in your regular protocols anyway. The purpose of glycine seems unclear to me; it may be there to deal with neutralizing free aldehyde residues from the formaldehyde fixation process, but it seems non-essential.

Parsing past the various other elements of the above protocols, the commonality is the use of SDS in concentrations ranging from as low as 0.03% up to as high as 1%. Incubation times are not very long, ranging just 5-10 min. It will be up to you, the experimenter, to determine which exact parameters are ideal. But if you are not sure how to compose a tissue pretreatment step and are trying to figure out how make it, consider the following:

- Concentrations of 4% to 8% SDS are used for the tissue clearing procedures in CLARITY and other things. These strong concentrations are applied to whole brains rather than sections, and the whole brains have more stabilization than just fixation with formaldehyde (extra scaffolding with hydrogel impregnation, fixation with glutaraldehyde, etc.). My point in bringing this up is that normal *whole* fixed tissue can be deteriorated by these concentrations of SDS because the fats are essentially sucked out, and some small percentage of the proteins as well. So, I imagine a 1% SDS concentration may still have these deleterious effects on the whole organs even if normally fixed. Now consider that you'll instead apply SDS to *tissue sections* rather than tissue blocks or whole organs. Thus, I think 1% SDS will typically be too strong of a concentration for all but the most well-fixed and thick tissue sections.
- Given that last bullet point, what's the ideal concentration if not 1%? Well, we know the lower end of what was done by Munzberg et al. in multiple publications was 0.03% SDS. This may be a bit dilute if you're just trying to see if you can make an antibody work that hasn't worked so far on tissue. So, perhaps try 0.1% SDS.
- The solution to dilute SDS into could be a few options. At less than 1%, SDS should dissolve into regular PBS without *too* much trouble. Still, do expect that straight SDS powder or pellets will not dissolve immediately; it will take time and stirring, though heat shouldn't be required. I've seen it dissolved more effectively in pH 8 borate buffer. Or, distilled water might be a simpler alternative.
- It's probably fine to keep incubation time to 10 min. I feel that 5 min may be short, but your experience may vary. But be aware that even at lower concentrations of SDS, such as 0.03%, tissue will slowly be destabilized if incubated in the SDS for long periods of time (30+ min).
- In your tissue pretreatment procedure, make sure to add adequate rinsing steps after incubation with SDS. SDS prevents antibody binding to targets, and it can also inhibit enzymes such as HRP.

OK, you've read to the end of this section just looking for a protocol so you don't have to do guesswork. My recommendation for formaldehyde-fixed, 50 um free floating sections is:

1. Rinse off previous step from tissue with PBS, multiple exchanges.
2. Incubate tissue in 0.1% SDS in PBS for 10 min.
3. Rinse off SDS with at least 3 rounds of PBS rinses, 5 min each.

Feel free to modify this as needed. Add this SDS procedure to the beginning of your staining protocol, before applying the primary antibody (or if you do normal serum "preblock", then do this SDS stuff before that). I don't think the SDS treatment will interact negatively with steps that block endogenous peroxidase - if anything, SDS can serve as another way to do exactly that. Beyond these, I am not sure how this SDS procedure may interact with other tissue pre-treatments (other types of antigen retrieval, deparaffinization, avidin blocking of endogenous biotin, etc.), so you will have to try it out.

II. Detergents [DTER]

Detergents and/or surfactants are used in IHC for specific purposes. In many cases, their addition into a protocol can serve as another means to retrieve antigens. However, there are many different detergents with different properties. This section will only briefly summarize useful facts about commonly-used detergents. If more specific information is required, seek out a detergents manual online – some IHC reagent vendors such as Dako or Pierce provide such manuals. This page may be particularly helpful:

http://www.labome.com/method/Detergents-Triton-X-100-Tween-20-and-More.html

Reasons for using detergents:

- Create holes in cellular and/or intracellular membranes, allowing antibodies and other substances to get through
- Prevents aggregation of proteins, such as antibodies, in solutions
- Some detergents change the 3D structure of proteins and/or solubilize them
- Reduces molecular friction; useful for allowing antibodies to get into compartments faster or for unbound conjugates to get scrubbed out of tissue faster

Common IHC detergents

Triton X-100 – One of the most common IHC detergents. Permeabilizes membranes somewhat irreversibly. Sometimes added to antibody solutions, but usually only added to the initial normal serum blocking solution. Too much of this detergent (or exposing tissue to it for too long) may cause poorly-fixed proteins to migrate outside of their typical intracellular compartments. May also oversolubulize the cell membrane, liberating membrane-bound proteins; avoid using for such proteins which may include neurotransmitter receptors. Use at 0.1-0.3%.

Tween-20 – A milder alternative to Triton X-100 that is also widely used. Does not solubilize proteins as much as Triton, useful in scrubbing out unbound substances. Does not have similar issues with oversolubilizing membranes. Used at 0.05-0.5%.

Saponin or Digitonin – Permeabilizes membranes, but does so reversibly. Operates by removing cholesterol from membranes. Once removed from solution, membranes typically re-form. Does not work on membranes that lack cholesterol, which include mitochondria and nucleus. Used at 0.1-0.5%.

SDS – Short for sodium dodecyl sulfate and also known as sodium lauryl sulfate. Does not easily form detergent "micelles" (tiny detergent spheres). As such, SDS can work its way into the complex structure of proteins and straighten them out, potentially solubilizing them. May be helpful for antibodies that have only been confirmed in Western Blots, which also use SDS. One target that I've used this approach for is phosphorylated STAT3 (for leptin signaling), though that case may have been relevant just for that particular antibody. Will precipitate out of solution at temperatures below RT and in the presence of potassium salts. Use as directed by specific protocols; can range from 0.1-2%.

CHAPS and CHAPSO – Detergents that solubilize the cell membrane but not the nuclear envelope. CHAPSO is more soluble in solution. Used at 2%.

Urea – Acts somewhat like a surfactant, though not strictly a detergent. Solubilizes proteins effectively. Used at 5% in a pH 9.5 buffer; may need to heat as done with antigen retrieval.

III. De-staining and re-staining tissue sections [DSRS]

One may want to de and re stain particularly valuable and rare sections. This section gives some idea of how to do that.

1. De-coverslip Curing mountants (ProLong Gold, PVA) by soaking slides in 0.1 M PB at 37°C for a few hours. Carefully remove coverslip.
   OR
   De-coverslip Petrochemical-based mountants (DPX, permount) in xylene for 1 hour, remove coverslip, re-hydrating sections in water and rinse in PB/PBS.

2. Incubate slides in “stripping buffer” (25 mM glycine HCl with 10% SDS, acidified to pH 2 with HCl) for 30 minutes at 50°C with gentle shaking.
    - This denatures/dissociates antibodies and some fluorophores, but does not unbind tyramide conjugates or certain other enzymatic precipitates (DAB, AP). AlexaFluor dyes (488 in particular) bound to tissue with tyramide are resistant to the technique. Ref: Pirici et al., 2009 J Histochem & Cytochem.
    - The use of SDS will typically dislodge sections from slides. As long as the sections are not too frail, they can go through the staining process again in a free-floating manner.

Worth noting is that some tissue-clearing methods (see next section) utilize varying amounts of SDS to strip antibodies from the tissue. In general, the protocols may range in temperature from 37 to 50 degrees C, and use concentrations of SDS ranging from 4% to 8% SDS.

IV. Tissue Clearing (Clarity, CUBIC, etc.) [TCLE]

Overview:
This section will probably be a work in progress forever. 😛
The methods are continually updated, perfected, or something else newer and better is developed. However, accumulating info here may help provide ideas of what can and cannot be done for creating cleared tissue chunks.

A. CUBIC

Considered a less harsh tissue clearing method than CLARITY while still allowing some antibody penetration ability and not needing acrylamide. Derived from:
http://www.sciencedirect.com/science/article/pii/S0092867414004188
Etsuo A. Susaki, Kazuki Tainaka, Dimitri Perrin, Fumiaki Kishino, Takehiro Tawara, Tomonobu M. Watanabe, Chihiro Yokoyama, Hirotaka Onoe, Megumi Eguchi, Shun Yamaguchi, Takaya Abe, Hiroshi Kiyonari, Yoshihiro Shimizu, Atsushi Miyawaki, Hideo Yokota, Hiroki R. Ueda. Whole-Brain Imaging with Single-Cell Resolution Using Chemical Cocktails and Computational Analysis. Cell, Volume 157, Issue 3, 2014, Pages 726-739, https://doi.org/10.1016/j.cell.2014.03.042.

Protocol:
1. Perfuse and post-fix animals using 4% PFA as usual.
2. Post-fix for 2 days @ 4°C.
3. Rinse off brain with PB or PBS.
4. Cut into coronal slabs using brain matrix or vibratome.
5. Incubate in one of the following; shake for all steps:
    - For samples that do not contain endogenous (mostly protein-based) fluorescence, Reagent #1 for 3-6 days @ 37°C Exchange solution after 3 days, continue until white matter is substantially cleared. Use shaking incubator.
    - For samples that contain weak endogenous fluorescence, incubate in 50% Reagent #1A (diluted with distilled water) for 6 hours @ RT, 100% Reagent #1A for 2 days @ RT, exchange solution, incubate @ 37°C for 3-5 days or until white matter is substantially cleared.
6. Rinse off brain with PB or PBS for 1 day @ RT (adding azide).
7. If immunostaining, read on. If not, skip to step 8.
    A. Rinse brain more; a few hours at room temp should suffice.
    B. Incubate in 1° solution for 3 days @ RT. Will require 5x concentration that would be used for 40-50 um sections.
    C. Rinse for 6 hours, exchanging every two hours.

D. Incubate in 2° (regular concentration seems to work) for 2-3 days @ RT. Can consider doing @ 4°C for longer to preserve fluorescence.
E. Rinse for 6 hours, exchanging every two hours.
F. (Optional) If amplifying with TSA, CLARITY protocols suggest reacting for 3-5 hours. Rinse afterward.
G. (Optional) Counterstain with dye (NeuroTrace, DAPI, or diluted cresyl violet) for 1 day @ RT.

8. Incubate in one of the following:
   - For samples that do <u>not</u> contain endogenous fluorescence, reagent #2 for 2 days @ RT.
   - For samples that contain weak endogenous fluorescence, incubate in 50% reagent #2 (diluted with PB/PBS) for 1 day @ RT, 100% reagent #2 for 1-2 days @ RT,
9. Image sections; keep immersed in reagent #2.
10. Rinse sections in PB/PBS for 6 hours (exchange 2x), then put into antifreeze.
11. Store in fridge, then move to freezer after 1 day for long term storage.

<u>Excerpts from original CUBIC Protocol text:</u>

"ScaleCUBIC-1 (reagent 1) was prepared as a mixture of 25 wt% urea (Nacalai Tesque Inc., 35904-45, Japan), 25 wt% N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine (Tokyo Chemical Industry CO., LTD., T0781, Japan), and 15 wt% polyethylene glycol mono-p-isooctylphenyl ether/Triton X-100 (Nacalai Tesque Inc., 25987-85, Japan). ScaleCUBIC-2 (reagent 2) was prepared as a mixture of 50 wt% sucrose (Nacalai Tesque Inc., 30403-55, Japan), 25 wt% urea, 10 wt% 2,20 ,20 '-nitrilotriethanol (Wako Pure Chemical Industries Ltd., 145-05605, Japan), and 0.1% (v/v) Triton X-100. For whole-brain clearing, each fixed brain was immersed in 10 g of reagent 1 at 37C with gentle shaking for 3 days, after which the solution was exchanged and the sample immersed in the same volume of fresh reagent 1 for an additional 3–4 days. The treated brain was washed with PBS several times at room temperature while gently shaking, immersed in 20% (w/v) sucrose in PBS, degassed, and immersed in reagent 2 (10 g per brain) for 3–7 days. After imaging, the sample was again washed with PBS, immersed in 20% (w/v) sucrose in PBS, and stored in O.C.T. compound at –80C.

"Some technical tips regarding reagent 1 preparation are as follows: 1) N,N,N',N'-tetrakis(2-hydroxypropyl)ethylenediamine is a highly viscous liquid and can be used as an 80 wt% working solution. 2) The quality of Triton X-100 product seems critical for preserving fluorescent signals and we highly recommend the product indicated above. When the same chemical from other vendors is used, the quenching effect on recombinant fluorescent proteins should be checked.

"Both reagent 1 and 2 were prepared just prior to use. When mixed, a hot stirrer should be used except during the addition of Triton X-100. Because water evaporation will make it difficult for the highly concentrated chemicals to dissolve, the weight should be monitored frequently for the addition of evaporated water during the mixing step. After confirming that all the mixed chemicals are dissolved, the reagents are cooled to room temperature, and finally Triton X-100 is added. Both CUBIC reagents should be degassed before use."

<u>3D Immunostaining of CUBIC Samples:</u> "A fixed brain block was treated with reagent 1 for 3 to 6 days, washed with PBS, immersed in 20% (w/v) sucrose in PBS, and frozen in O.C.T. compound at –80°C overnight. The frozen sample was then thawed, washed with PBS, and subjected to immunostaining with the primary antibodies in 750 µl of 0.1% (v/v) Triton X-100, 0.5% (w/v) bovine serum albumin, 0.01% (w/v) sodium azide in PBS for 3 days at 37°C with rotation. The stained samples were then washed with 10 ml of 0.1% (v/v) Triton X-100 in PBS several times at 37°C with rotation and then stained with the secondary antibodies in 750 µl of 0.1% (v/v) Triton X-100, 0.1% (w/v) bovine serum albumin, 0.01% (w/v) sodium azide in PBS for 3 days at 37°C with rotation. The

stained samples were then washed with 10 ml of 0.1% (v/v) Triton X-100 in PBS several times at 37°C with rotation, immersed in 20% (w/v) sucrose in PBS, degassed, and immersed in reagent 2 for 24 to 36 hr. Note that longer treatment with reagent 2 may cause nonspecific signals in the void structures such as vessels and ventricles. ”

B. SWITCH - fast fixation and antibody penetration

Web resource: http://chunglabresources.com/sw1
Reference: Evan Murray, Jae Hun Cho, Daniel Goodwin, Taeyun Ku, Justin Swaney, Sung-Yon Kim, Heejin Choi, Jeong-Yoon Park, Austin Hubbert, Meg McCue, Young-Gyun Park, Sara Vassallo, Naveed Bakh, Matthew Frosch,, Van J. Wedeen, H. Sebastian Seung, and Kwanghun Chung. Simple, scalable proteomic imaging for high-dimensional profiling of intact systems, **Cell**, Dec 3:163(6): 1500-14. doi: 10.1016/j.cell.2015.11.025. PubMed PMID: 26638076.

This procedure utilizes SDS as a vector to cause fixatives and antibodies to penetrate deeply into tissue, and then pH shifts or washes to “activate” them to act on said tissue. The SWITCH method of fixation can actually be performed on tissue that is already fixed! Further, the fixation makes the tissue clear-able as well as able to be de-stained and re-stained. Thus, a tissue chunk can go through multiple rounds of IHC labeling.

In case one’s antibodies cannot penetrate deeply into tissue, the SWITCH technique should help. The Chung lab found that elution of antibodies in 0.5 mM SDS in PBS prevents antibody binding but facilitates fast diffusion & infiltration into tissue. Brain samples must first be equilibrated to this solution, then they can be migrated to the same solution but with antibodies added. Then the samples and solution are incubated at 37°C for 12 hours. The sample is transferred to PBS**T** without antibodies and incubated at 37°C for 12 hours.

A 0.5 mM SDS solution has ~144.2 mg per liter, which is a 0.01442% solution. This is immensely less concentrated than the SDS concentrations used in tissue clearing in CLARITY protocols. Addition of SDS is best made from a stock solution however; perhaps a 1% stock should be made, then a minute amount could be pipetted into the antibody solution final volume.

HRP conjugates cannot be used in solutions containing SDS; research indicates that SDS deactivates the enzyme somehow even at millimolar concentrations. But if using SWITCH approach for HRP conjugates, a stabilization agent may be necessary. A patent (US 4782023 A) describes using a 2% dilution of ~6000 MW polyethylene glycol and a 0.2% dilution of a calcium salt (calcium chloride prefered). However, the tests were run after maintaining HRP samples at 37°C for 7 days. Addition of these reagents may not be necessary for just the 1 day of reaction.

There was no mention of using normal donkey serum or BSA in antibody dilutions, but these should be added to protect antibodies from protein-degrading events that may occur in the solutions.

C. CLARITY

Reference: Guo Z, Zheng Y, Zhang Y. CLARITY techniques based tissue clearing: types and differences. Folia Morphol (Warsz). 2022;81(1):1-12. doi: 10.5603/FM.a2021.0012. Epub 2021 Feb 12. PMID: 33577077.

Although this is considered the foundational tissue clearing approach, I haven’t included info on this nor its variants (PACT, PARS). I’ve tried some of this family of techniques, but I’ve had limited

success with tissue becoming porous enough to allow antibodies in. SWITCH seems superior in theory, but I have not yet tested it.

D. SHIELD

Web resource: http://chunglabresources.com/sh1
Reference: Young-Gyun Park, Chang Ho Sohn, Ritchie Chen, Margaret McCue, Gabrielle T. Drummond, Taeyun Ku, Dae Hee Yun, Nicholas B. Evans, Hayeon Caitlyn Oak, Wendy Trieu, Heejin Choi, Xin Jin, Varoth Lilascharoen, Ji Wang, Matthias C. Truttmann, Helena W. Qi, Hidde L. Ploegh, Todd R. Golub, Shih-Chi Chen, Matthew P. Frosch, Heather J. Kulik, Byung Kook Lim, Kwanghun Chung. Protection of tissue physicochemical properties using polyfunctional crosslinkers, *Nature Biotechnology*, doi:10.1038/nbt.4281

This technique has had my attention from when it was first showcased at a neuroscience conference. It provides the same advantages of the SWITCH method developed by the same lab, but it also has compatibility with a broader range of antibodies compared to SWITCH-fixed tissues, and it probably has lower autofluorescence issues since glutaraldehyde is not involved. Unfortunately, the main epoxy ingredient, GE-38, seems to not be available for purchase within the U.S. due to some restrictions.

Info on GE-38:
Polyglycerol 3-polyglycidyl ether, CAS #: 118549-88-5
Allegedly not approved for sale in the US. May be able to get samples...
Some links refer to GE500, aka 1,2,3-Propanetriol, homopolymer, 2-oxiranylmethyl ether
Original supplier was CVC Thermoset Specialties, but this was bought out by Huntsman (one of the links below)
Possible sources include:
https://azelisamericascase.com/product/erisys-ge-38/
https://adhesives.specialchem.com/product/a-huntsman-araldite-dy-s

Alternative suggested on Chung lab page's discussion was GE-36 (Propoxylated Glycerin Triglycidyl Ether, CAS ???; or possibly GLYCEROL PROPOXYLATE TRIGLYCIDYL ETHER, CAS 37237-76-6). But posts were asking about this rather than asserting that it works.
Vendor: https://azelisamericascase.com/product/erisys-ge-36/

Paper implies (not stated, but I deduced from the data shown) that TGE (EX-313, aka glycerol triglycidyl ether) might have similar albeit less robust protection of epitopes compared to GE-38.
CAS #: 13236-02-7
Vendors for TGE:
https://crosslinkers.evonik.com/en/products/Epodil
https://www.silverfernchemical.com/products/glycerol-triglycidyl-ether/

V. Autofluorescence Reduction [AFR]

Autofluorescence occurs when the tissue gives off its own fluorescence, in response to high-intensity light, despite not yet being stained. Certain proteins or substances in the brain may cause this to occur, or fixation may induce this effect. In PFA-fixed brains, autofluorescence can occur at most wavelengths. Ultraviolet/Violet light induces cyan-ish background, blue light induces olive-brown backgrounds, and green light induces hazy red background. Generally, red light induces little to no background. Here are some techniques to neutralize these types of autofluorescence.
Refs:
https://www.ihcworld.com/autofluorescence.htm

Autofluorescence: Causes and Cures, from Toronto Western Research Institute
https://www.researchgate.net/file.PostFileLoader.html?id=535ae654d039b11f4d8b45f9&assetKey=AS%3A273519282196488%401442223486569

A. Sodium borohydride pre-treatment

Intended to reduce reactive byproducts of fixation.

1. Rinse sections with distilled water for 10 min
2. Incubate sections in 0.1-0.5% $NaBH_4$ in distilled water for 30-15 min (depends on fragility of sections and epitopes; I recommend 0.3% for 15 min on standard-fixed 50 um sections).
3. Rinse with distilled water for at least 10 min or until bubbles disappear

Considerations:

- Borohydride is explosive and reactive!
- Perform any procedures with plastic containers and not metal.
- Perform treatment under hood, as gasses released are flammable!

B. Copper sulfate post-treatment

This protocol assumes that you will perform the treatment on the sections after they have been mounted onto slides. But, for ease, this could probably be modified so that sections remain free-floating during this treatment.

1. Make or retrieve copper sulfate solution: 10 mM CuSO4 in 50 mM ammonium acetate at pH 5.0.
2. Mount sections and air dry them onto charged or gelatinized microscopy slides. Dry slides enough so that the sections stick and do not appear damp.
3. Dip in distilled water for 10 min (removes dried buffer salts)
4. Dip in copper sulfate solution for 10 min.
5. Dip in distilled water for another 10 min (removes excess copper sulfate)
6. Blot dry, coverslip with appropriate mounting media.

Considerations:

- Copper sulfate is poisonous! Dispose any solutions containing or contaminated with copper sulfate as hazardous waste!
- Reduces lipofuscin granules and general autofluorescence.
- This method seems to induce nuclear staining that is excited by 405 nm laser and emitted as orange. This may only be an issue if attempting to image Fluorogold directly using a longpass filter. Can occur when not rinsing adequately with distilled water before mounting. Photobleaching tissue appears to exacerbate this issue.
- Although CuSO4 may dim Fluorogold emission brightness and that of other fluorophores, the benefit of reduced autofluorescence outweighs this effect. If anything, the fluorophores are more visible with rather than without treatment.

C. Sudan Black B post-treatment

1. Make or prepare 0.1% Sudan Black B in 70% ethanol
2. Mount sections and air dry them on charged microscopy slides. Do not underdry or overdry.
3. Dip in distilled water for 10 min (removes buffer salts).
4. Dip in SBB solution for 10 min.
5. Dip in distilled water for another 10 min.

6. Blot dry, coverslip with appropriate mounting media.

Considerations:

- Reduces lipofuscin granules and general autofluorescence.
- May increase background in the deep red (AlexaFluor633 and 647) range. May not be an issue if you have strong staining in the deep red spectrum.
- This treatment allegedly dims Fluorogold emission brightness; I did notice such a reduction. This may occur for other fluorophores as well, but the benefit of reduced autofluorescence *usually* outweighs this effect.
- SBB tends to stain most things, so don't splash it around. Stains can be washed out relatively well with 100% ethanol.
- Incompatible with certain types of mounting media.
- This approach may darken the sections, so hopefully this does not obscure fluorescence.
- This approach also exposes sections to ethanol. If you are using fluorophores or stains that are ethanol-soluble or sensitive, avoid using this method.

VI. Photoconversion of Fluorophores into Chromagens [PFCH]

Sources:

Lübke J. Photoconversion of diaminobenzidine with different fluorescent neuronal markers into a light and electron microscopic dense reaction product. Microsc Res Tech. 1993 Jan 1;24(1):2-14. doi: 10.1002/jemt.1070240103. PMID: 7679591.

Dobson Katharine L., Howe Carmel L., Nishimura Yuri, Marra Vincenzo. Dedicated Setup for the Photoconversion of Fluorescent Dyes for Functional Electron Microscopy. Frontiers in Cellular Neuroscience. Vol 13. 2019. DOI=10.3389/fncel.2019.00312 https://www.frontiersin.org/articles/10.3389/fncel.2019.00312/full

When fluorescent dyes are excited by an energy source, they sometimes release photons of a different wavelength back. However, other reactions may occur from this high-energy light bombardment. Excited electrons from the dye can be transferred to nearby oxygen molecules. Normally, oxygen is in a stable and low-reactive state (called triplet state). With the energy transferred from the excited dye, oxygen becomes much more reactive and toxic (called singlet state). Though this form of oxygen can oxidize living tissue, it can also oxidize DAB into an insoluble, electron-dense reaction product. In short, excited dyes in the presence of DAB and oxygen convert from a fluorescent probe to a chromagen. Note that this process does not require an enzyme such as HRP, nor does it require another oxidizing compound such as H_2O_2. The DAB reaction product in this case can be imaged both via light microscopy and by electron microscopy, as it is electron-dense.

Dyes and tracers that have been tested and confirmed to photoconvert include: eGFP, YFP, DAPI, Fast Blue, Propidium Iodide, Ethidium Bromide, Lucifer Yellow, Nuclear Yellow, Diamidino Yellow, Texas Red, DiI, DiO, DiA, BODIPY FL C5-ceramide, Fluorogold, Fluororuby and rhodamine derivatives, Fluoroemerald, Fluoroscein, Eosin, and likely many others not listed here. If you plan to photoconvert a dye not on this list, be sure to investigate first whether it has been utilized for photoconversion of DAB in published work. Some dyes (Fast Blue, Nuclear Yellow, & Diamidino Yellow) may not convert at their ideal fluorescence excitation wavelength but instead at longer wavelengths such as 530 nm.

The method of inducing photoconversion of DAB varies between studies, and there are a variety of techniques to improve the staining signal. Factors that increase the DAB product signal include: bombardment of sample with light that is closest to the wavelength of the excitation maximum of the fluorophore, performing reaction in an oxygen-rich environment, pre-incubating with DAB in darkness, performing reaction on ice (to prevent tissue heating from the light), darkening the reaction product with the addition of metals, and adding certain compounds to decrease non-specific reactions. Such compounds are aminotriazole for inhibiting peroxisomal catalases, or excess H_2O_2 or sodium azide to inhibit endogenous peroxidases in general. Without these additives, background “punctate”

staining may occur, particular in peroxisomes on the intracellular level. Additives/Post-treatments that change the color or intensify the stain include osmium tetraoxide, potassium ferrocyanide, nickel chloride, or cobalt chloride (all of these are toxic!).

In regard to the equipment used in photoconversion, either a 100W lamp or a microscope's mercury arc lamp should suffice in quick reaction. Sections may be easiest to react if they are floating. If using a microscope, there will be limited space for this. Create a basin on the slide by forming a perimeter wall of nail polish. Be sure not to use gelatinized or charged slides, as any dirt in the solution will stick to the slides. Coverslipping before/during this process should be avoided, as coverslips can absorb/reflect excitation light somewhat and may partially hinder oxygen access to the reaction sites.

Considering these factors, here is a potential protocol for photoconverting fluorophore-containing tissue:

1. Rinse sections 3x with PBS.
2. Pre-treat tissue with 1% H_2O_2 in PBS for 15 min OR 0.5% azide in PBS for 15 min OR 20 mM aminotriazole + 0.001% H_2O_2 in PBS for 1 hr.
3. Pre-incubate with freshly-made cold 0.2-5% DAB (in Tris-buffered saline) for 15-60 min in darkness, with incubation time depending on section thickness (40-200 um typical in literature). Pre-incubation allows for DAB to penetrate tissue without reacting to it.
4. React with new batch of fresh cold DAB solution under light source for 15-60 min, depending on light source power and the fluorescent dye being converted. Perform on ice or in cold conditions to prevent tissue heating. Add metal compound such as NiCl to cause DAB precipitate to be a different color. NOTE: reaction may be enhanced by reacting in a closed chamber with controlled oxygen flow.
5. Change DAB solution every 10-15 minutes.
6. Coverslip, or enhance with metallic substance and coverslip. For osmium tetraoxide, use at 1% in PBS for 30-60 min. Incubation concentrations and times for OsO_4 may vary – seek specific protocols online if using this method.

VII: Counterstain considerations [CSCS]

A. Nissl Staining, Brightfield

The Nissl stain labels RNA, which is particularly prolific in neurons compared to other cells in the brain. Where there are clumps of RNA, those are referred to as Nissl bodies. A common Nissl-staining substance is cresyl violet. Used at a 0.1% solution in acidified water (or acetate buffer), it can add a dark purple stain that distinguishes between gray matter and white matter in the brain.

I have made modifications to the traditional Nissl stain protocol in order to lower hazards and expand accessibility. Various protocols for traditional Nissl staining can be found online, but all of them seem to rely on using carcinogenic & toxic ingredients at some point in the process. I have modified the defatting step to use alcohol, and I have removed the use of xylene and Permount at the end of the protocol in favor of quick drying and applying mineral oil. Other coverslipping media may work, but they cannot be water-based, as that will leech the cresyl violet out of the sections. Thus, PVA medium does not work and glycerol-based media do not work. The medium should be hydrophobic. Certain fast-drying glues may work; I will be testing this in the future.

The protocol below is my modification of the one from the Viapiano lab's document found online, as well as using some details from a protocol on NeuroscienceCourses.com

(http://neurosciencecourses.com/uploads/3/4/4/9/34494139/_creysl_violet_stain_neurosciencecourses.pdf) .

Sections should be dried onto gelatinized slides before commencing this protocol. Label slides with pencil, not pen - ethanol will dissolve ink.

Protocol:

1. Make 0.1% cresyl violet solution.
    1. In a 50 mL conical tube, add 0.05 g (50 mg) cresyl violet acetate powder, 47.5 mL ddH2O, and 2.5 mL distilled white vinegar (assuming it is 5% acetic acid).
    2. Mix overnight (may dissolve passively over longer period without aggitation).
    3. Can filter before use, though I found this unnecessary. There will be undissolved sediment in bottom of container, but this can be ignored or decanted.
2. Set up eight coplin jars or staining basins with the reagents below. Immerse the slides containing your sections into the reagents below for the specified lengths of time.
    1. Diluted ethanols can be made as needed. 70% = 15 mL distilled water, 35 mL 100% ethanol.
    2. Isopropanol can be substituted for ethanol, even midway through the staining process, as long as the concentration is equal to that of ethanol for the respective step.
3. 100% EtOH, **10 min (defats sections for CV penetration)**
4. 70% EtOH, **5 min (begins to rehydrate sections)**
5. dH2O, **3 min (rehydrates sections)**
6. cresyl violet, **10 min (stains)**
7. dH2O, **5 min (removes excess CV)**
8. dH2O, **5 min (continues removing excess CV)**
9. 70% EtOH, **3 min (dehydrates sections to prep for next step)**
10. 100% EtOH, **variable; try 30 sec but keep checking. Can take up to 10 min. (Differentiates staining such that white matter is de-stained but gray matter remains stained.)**
11. Let slides dry on a tray. They should be ready in about 5 min.
12. Coverslip with a hydrophobic medium - mineral oil is recommended. AVOID AIR BUBBLES!
    1. Try not to make a mess with excess mineral oil.
13. If using a coverslipping medium that is non-drying, seal with either glue or nail polish.

B. Nissl Staining, Fluorescent

NOTE: This particular protocol is experimental. I have gotten it to work but need to confirm that the stain works consistently. Will update after summer 2023.

Pros:

- Cheap
- Easy

Cons:

- Only one color option
- May not label somata - depends on how the stain and differentiation are executed (must observe pics from brains treated with triton of varying concentrations)
- Must mount in oil to prevent leeching out of stain into media

C. DNA- and RNA-binding dyes

Questions I haven't had time to investigate:
Longevity of working solution?
Reusability of already-used working solution?

DAPI is fine for a counterstain, but not great. It fails to adequately delineate minor brain region boundaries the way that Nissl staining or cholinesterase staining do. For more info, see Appendix F.

Acridine orange may be useful. However, it may have multiple excitation & emission spectra depending on whether it is bound to DNA, RNA, or unbound. It causes whole cells to fluoresce in the green emission spectrum, but it also binds to lysosomes and emits in the red spectrum.

The NeuroTrace dye family is a series of RNA-binding dyes, made of proprietary substances. I have tried the Green, Red, and Deep Red varieties. The Blue variety did not have excitation & emission spectra compatible with the microscope filters I use. I had issues trying to get Red to work. Green worked and sometimes labeled somata in addition to Nissl bodies (but see Pros & Cons below). Deep Red looked more like traditional Nissl staining patterns.
Pros:

- Easy
- Green version (usually) labels somata and full perikarya.
- Compared to some pics of NeuN, the green version stains greater extent of soma.
- The Deep Red variant will avoid background fluorescence, due to tissue just not having autofluorescence in those bands.
- Again compared to NeuN, seems to avoid staining nucleus (might make a good combo with DAPI...)
    - Based on recent testing (Feb 2023), in some tissue the exact opposite is true!
- Does not seem to leech out into normal aqueous media

Cons:

- Inconsistency in how it labels Nissl vs somata. In Berridge lab, it was able to label somata. But in recent experience (early 2023), it predominantly labeled cell nuclei as evidenced by almost complete overlap with a DAPI co-stain. This may indicate degradation of the RNA in the fixed tissue. Unclear what is happening in these cases.
- Kinda pricey, but not as expensive as antibodies.

D. Using IHC on neuron-specific targets

Neurons happen to possess various markers that are abundant in them and not (or less so) in other cell types. As such, performing IHC to stain such markers presents another way to distinguish gray vs white matter, and to distinguish areas by cell density in a manner similar to, or better than, Nissl staining.

Pros:

- Can select multiple color options
- Cannot be washed out by PBS or aqueous mountants
- Seems to stain neuronal somata, but much more robustly stains neuronal nuclei

Cons:

- Expensive plus follow-up reagents add more expense
- Requires IHC then maybe TSA (and potentially click chemistry) to be bright enough

Perhaps combined with the click-chemistry approach (as per Antonov et al. 2019), any of the IHC-type stains can produce very bright yet high resolution counterstains.

General guide for neuronal markers: https://www.biolegend.com/en-us/neuron-markers

The following seem to stain most of the cytoskeleton. This might make the staining patterns "too busy" for a counterstain, at least on tissue sections that are thicker than 30 um.

- Neurofilament H (NF-H)
- Beta 3 tubulin (B3 tubulin)
- MAP2

Here are other targets that tend to target soma, cytosol, and/or nuclei of neurons.

- NeuN/FOX3: A.k.a. FOX3 (analog to FOX1 in C. Elegans). Binds to substance that is found in neuronal nuclei and to some degree in neuronal cytosol.
- HuC and/or HuD: A.k.a. Elav-like or Elavl (just the Hu part). Elavl3 = HuC, Elavl4 = HuD. Wikipedia notes that HuD is found only in neurons and is an RNA-binding protein. https://en.wikipedia.org/wiki/HuD_(protein)
- Gamma enolase: A.k.a. Neuron specific enolase (NSE) or enolase 2, otherwise expressed in cancers.
- Contactin-2 A.k.a. Cntn2. A neuronal membrane-associated protein.
- CYN-1 antibody: Created in late 90s. Authors claim it targets cytosolic neuron-specific epitope. What epitope that is has not been characterized. If it is targeting Cyn-1 and it doesn't just happen to share the name, then Cyn-1 is PPIA. That protein's expression is not restricted to the brain. It is ubiquitously expressed in the brain, but it is unclear if it is *not also* expressed in glial cells. Original paper: J Ericson, P Rashbass, A Schedl, S Brenner-Morton, A Kawakami, V van Heyningen, T.M Jessell, J Briscoe. Pax6 Controls Progenitor Cell Identity and Neuronal Fate in Response to Graded Shh Signaling. Cell, Volume 90, Issue 1, 1997, Pages 169-180. https://doi.org/10.1016/S0092-8674(00)80323-2. https://www.sciencedirect.com/science/article/pii/S0092867400803232?via%3Dihub

E. Cholinesterase staining, Brightfield

See Appendix F.

F. Cholinesterase staining, Fluorescent

Still being tested. No news on this in 2025.

VIII. Antibody Penetration Enhancement [APNT]

Various approaches can be utilized that allow antibodies and chemical conjugates to penetrate deeper into tissue, beyond the usual 40 um free-floating section limit. Below are general categories, within which are several options that can be considered.

<u>Use different fixatives</u>

A. *Zamboni's fixative*: 2% PFA with 0.2% picric acid in PB (pH 7.4)

However, Eldred et al (1982) report that addition of picric acid can produce nonspecific punctate staining and decreases antibody penetration.

B. *More acidic fixatives* may allow for better penetration. Basic fixatives are good at preserving ultrastructure but also impede antibody penetration.

Perform Antigen Retrieval

A. *Proteolytic methods*

A. *Proteinase K* treatment with 5ug/mL (0.25 units/mL) at 37°C in Tris-EDTA buffer, pH 8.0 for 5-10 minutes.
(Higher concentrations than this are not recommended for free-floating 40 um sections)

B. *Pepsin* treatment with 0.25 mg/mL at 37°C in 0.2 N HCl, pH 2.0 for 5-10 minutes
(Higher concentrations than this are not recommended for free-floating 40 um sections)

C. *Heat-induced methods*

1. *Citrate buffer*
2. *Basic buffer*

Increasing reaction variables

A. *Higher temperatures* for incubations and rinses.

1. 1° antibody at room temp for 4 hours
2. 1° antibody at 37°C for 2 hours

B. *Longer incubation times*. Instead of just incubating overnight at 4°C, try 48 hrs.

C. *Add microwave energy*. Microwaving also expedites the Western blot process. Application of 150 watt microwaving in 4 seconds on/off intervals over 15 min with 1° antibody and 5 min with 2° antibody was sufficient for clean western blot staining of actin (Liu and Toyokuni, 2009).
Applying vacuum during microwave processing aids the infiltration of fixatives and sucrose (for cryoprotection), although it is recommended to already have the tissue infiltrated by formaldehyde/methylene glycol solution beforehand.

Permeabilize the tissue with freezing

A. *Freeze-thaw cycles*. Medium to fast speed freezing forms cubic ice crystals that forcibly expand pores in the tissue.

1. 3x in dry ice or liquid nitrogen
2. Incubate sections in 30% sucrose for 3 hrs, then 3 freeze-thaw cycles with liquid nitrogen.

B. Zaborszky and Heimer (2014) suggest a variant of the above; incubate sections in a 10% sucrose solution in PB (0.1 M?), freeze solution with liquid nitrogen, and thaw to room temp. Perform 2-3 cycles.

1. 4x freeze-thaw cycle of 1hr -80°C, 1hr RT in absolute methanol
2. Keep sections in cryoprotectant (2% glycerol and 20% DMSO in PBS). then hang them in liquid nitrogen until frozen, then put them back into cryoprotectant. Repeat once.

Permeabilize the tissue with delipidation

A. *Detergents.* Triton X-100 at concentrations of 0.1 to 1% in incubation solutions. However, Piekut and Casey (1983) note that even in 80 um 4% PFA-fixed vibratome sections, inclusion of 0.4% Triton in 1° antibody solutions only aids in staining the outer 10 um of hypothalamic tissue. Allegedly, higher concentrations of Triton interfere with avidin-biotin binding (Zaborszky and Heimer, 2014).

B. *Ethanol treatments* to remove lipids from tissue

1. *Graded buffered ethanol sequence*: 10, 25, 40, 25, 10% ethanols in 0.1 M PB, 5-10 min each (Eldred et al., 1983).
2. *50% ethanol in distilled water for 30 min*. Used by Llewellyn-Smith and Minson (1992) to stain peptide-containing fibers in 50-70 um sections. Only worked if sections cut through fibers.

C. *DMSO treatments* to partially solvate tissue components.
    1. Add 1% DMSO to all rinses and incubation solutions.
    2. Soak tissue in 10% DMSO (in buffer) overnight, then rinse thoroughly.

D. *Osmotic shock.* Zaborszky and Heimer (2014) suggest that fixative is made in hypoosmotic buffer (0.03 M PB) in order to damage cell membranes. The caveat is that antigens may dislocate within the tissue.

Increase antibody infiltration speed

A. *Electrophoresis* during immunostaining. Liu and Kao (2009) fixed 120 um corneas in 4% PFA (and added 0.2% glutaraldehyde in some cases to anchor soluble cytoplasmic proteins). Then, the tissue and antibodies were embedded in a three-part agarose column, where agarose is dissolved in Tris-glycine buffer (TGB, pH 7.4) made from 25 mM Tris and 250 mM glycine-HCl. Agarose is liquified at 55°C. In the column, the first layer contains the tissue in liquified 1% agarose in TGB. After hardening, the second layer is made with antibodies dissolved in 0.5% agarose. The third layer contains 2% agarose in TGB. This and other columns are submerged in TGB in a gel electrophoresis apparatus and a 4-10 mA current is applied for 10-24 hrs. The columns are oriented based on the net charge of the antibody conjugates. 1° antibodies were conjugated to fluorophores in this study. Specimens were imaged with confocal microscopy. They used concentrations of antibodies similar to those used in section staining.

B. *Incubation under vacuum* (20 Hg). Creation of a low-pressure fluid environment likely causes faster movement of solutes in the solution and could expand pores formed by the fixation process.

Increase air/fluid pressure while staining:

- See notes from SfN 2018. Rumored to triple penetration depth at 2 atmospheres

Using Fab fragments

- Antibody penetration is a potential issue when staining thick sections, especially in light of recently-developed clearing techniques. An IgG molecule is large - ~150,000 Da - and thus it has issues fitting through tight spaces. Fab antibody fragments are a third of the size and are effective for quick, deep penetration into thicker tissue samples. Studies have shown that in 1 mm thick CLARITY-treated brain sections, regular IgG molecules take 6-7 days to fully penetrate the sample, whereas Fab fragments penetrate in 2 days. Thus, it is of much interest to fragment antibodies and label them in-house.
- Considerations:
    - Secondaries that are normally sold by vendors mainly target the Fc region of the primary antibody. This part is cleaved off in the fragmentation process, so normal secondaries will not bind to an Fab fragment. You must then instead select secondaries that specifically target Fab sequences.
    - If making your own Fab fragments, the fragmentation process introduces many new buffer components that should be removed (perhaps by desalting or protein concentration) prior to labeling with reactive labeling agents. Poor immunoreactivity of newly-generated Fab fragments is likely due to overdigestion by papain.

Multiplex Procedure:

In one study, an assortment of these techniques was used to stain whole hemispheres of a mouse brain (Gleave et al., 2013; http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0072039)

1. Perfusion-fix in 1% PFA

2. Post-fix in 1% PFA for 2 hours at RT
3. Separate hemispheres (or chunks ~4mm)
4. Wash with PBS
5. Dehydrate in graded methanol solutions (up to 100%) over one day
6. 4x -80°C freeze-thaw (1 hour for each freeze)
7. Rehydrate in graded methanol solutions to PBS over one day
8. Digestion with 10mg/mL Proteinase K for 5 minutes (Other labs use **much** less, about 20 ug/mL of Proteinase K at 37°C for 10 min. This was even too much for my free-floating 40 um sections, so for regular sections 5ug/mL may be best.)
9. Microwave antigen retrieval in citrate buffer
10. Rinse
11. 1° ab at 37°C for 48hrs with 5% DMSO and 0.01% Triton.
12. Rinse in similar solution for 48hrs; change solution at 24 hrs.
13. 2° ab in similar solution for 72 hrs
14. Similar long rinse
15. Clear tissue for 3D visualization

IX. Antifade use in mounting media [AFMM]

General resource:
https://bidc.ucsf.edu/sites/g/files/tkssra806/f/wysiwyg/Mountants_WCIF.pdf

Antifade agents serve to prevent the effects of photobleaching when tissue is under epifluorescent excitation light. Most antifade agents do this by acting as free radical scavengers / antioxidants, thereby stabilizing the excess energy that a fluorophore is bombarded with. There are various antifade compounds out there (see link above), but each has its pros and cons. A particular concern I've had is that antifade additives to in-house made mounting media seem to blunt the initial fluorescent emission of fluorophores. Couple this phenomenon with the fact that current-generation fluorophores – AlexaFluors, Cyanines (Cy), and various other proprietary ones – are made to be resistant to photobleaching, and it makes antifade agents seem somewhat unnecessary. That said, if you are using older generation fluorophores or you have special cases where it makes sense to use antifades, see the following info.

Assuming you are using a mountant that is just buffered glycerol (90% glycerol, 10% PBS), you could add 4% n-propyl gallate, 1% DABCO, or Para-phenylenediamine (PPD).

Para-phenylenediamine (PPD) allegedly does not work with the Cyanine dyes.

2',2-thiodiethanol (TDE)
Staudt T, Lang MC, Medda R, Engelhardt J, Hell SW. 2,2'-thiodiethanol: a new water soluble mounting medium for high resolution optical microscopy. Microsc Res Tech. 2007 Jan;70(1):1-9. doi: 10.1002/jemt.20396. PMID: 17131355.

Other resources
http://www.ihcworld.com/_protocols/histology/aqueous_mounting_medium.htm
http://www.ncbi.nlm.nih.gov/pubmed/3926864
http://www.ncbi.nlm.nih.gov/pubmed/2464041

Appendix A: Calculating Antibody Dilutions [AXA]

Antibodies can have a large range of effective concentrations, depending on how much target there is to bind in the tissue, effectiveness and specificity of the binding, and desired speed of binding. Typical working dilutions for primary antibodies range from 1:200 to 1:50,000, whereas secondary and tertiary solutions are typically used from 1:200 to 1:600. You must always determine what the best concentration is for *your own work*, even if someone recommends a certain dilution for your application. For instance, vendors typically give primary antibody dilution ranges of 1:50 to 1:500 for IHC, but this is almost always far too concentrated for effective staining. Determine the best dilution(s) empirically with a dilution curve test.

The CART Antibody as an Example:

Let's say you want an antibody against CART made in mouse (mouse anti-CART). You get the antibody, 100 ug in 100 uL, from the provider. What do we do with it? First, aliquot it into several smaller volumes (20 uL) and freeze any that you will not be using immediately.

When testing a new antibody, you need to use a dilution curve test to determine the most dilute and yet still effective concentration. Antibodies tend to be expensive, so if you can dilute them as much as possible while still maximizing your signal-to-noise ratio, your antibodies can go a long way. Also, higher dilutions almost always result in less background and more specificity. You can choose/estimate a dilution curve range based on the methods sections of literature that used the same stains or even the same antibodies from that vendor.

For a pure (1:1) stock of anti-CART in 20 uL, let's say you determined that you want dilutions of 1:200, 1:500, 1:1000, 1:2000, 1:5000, and 1:10000 based on information from other papers. Let's assume you will use one regular 24-well plastic incubation tray for your stains, and you decide to do two brain slices for each well (each one from a different subject, in case fixation differed). This would require about 1 mL per well. If we have 6 conditions, that means we will use 6 wells. These calculations assume you are using the same secondary labeling method at the same concentration for all of those six conditions.

Directions for a dilution curve:
A 1:200 dilution of the pure stock means you must dilute the stock amount with 200x its volume. This does not require the full antibody stock you received, but instead a fraction of it. We established that you only need 1 mL per dilution. However, let's increase this number to 2 mL of a 1:200 solution so that there is enough for the serial dilution. Considering the 200x, what then is 1/200 of 2?
2/200 = 0.01 mL = 10 uL = half of a 20 uL aliquot

- Dilute the 10 uL from the aliquot in 2 mL of antibody diluent (PBS + additives).
- Stir to mix, but don't shake as that would cause frothing/foaming.
- Put 1 mL of this 1:200 dilution into the 1:200 well
- Take the remaining 1 mL of the 1:200 solution and add 1.5 mL of antibody diluent to make a 1:500 solution.
- Put 1 mL of this 1:500 solution into the 1:500 well.
- Take the remaining 1.5 mL of 1:500 solution and add 1.5 mL antibody diluent to make a 1:1000 solution.
- Put 1 mL of this 1:1000 solution into the 1:1000 well.
- Take the remaining 2 mL of the 1:1000 solution and add 2 mL antibody diluent to make 1:2000.
- Put 1 mL of this 1:2000 solution into the 1:2000 well.
- Store 2 mL of the remaining 1:2000 solution in the fridge.
- Take the last 1 mL left of the 1:2000 solution and add 1.5 mL antibody diluent to make 1:5000.

- Put 1.5 mL of this 1:5000 solution into the 1:5000 well.
- Take the remaining 1 mL of 1:5000, dilute with 1 mL antibody diluent to make 1:10000.
- Put all 2 mL of this 1:10000 solution into the 1:10000 well.

Now you have a (relatively) complete dilution curve. On rare occasion, some less effective antibodies may only work at concentrations more condensed than 1:200 (perhaps 1:50), so be sure to try that if the staining is still not pronounced enough and the secondary antibodies are confirmed to be appropriately diluted. Also be mindful of whether the vendor has pre-diluted the antibody they sold you. Vendors often will have antibodies pre-diluted; quite an egregious practice, in my opinion. I've seen 200 ug / 1 mL, and this is a starting 1:5 dilution. Be sure to factor that into your dilution tests.

Appendix B: IHC Staining with Multiple Primaries from the Same Species [AXB]

I. Intro

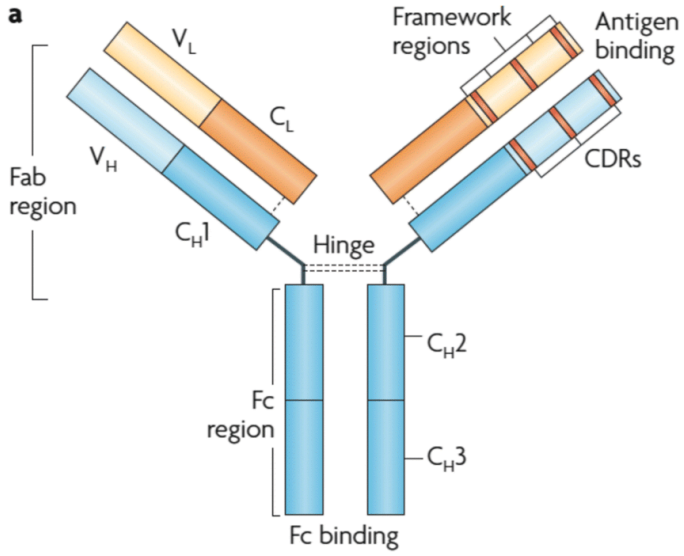
Generally, it is more difficult to perform a multi-labeling IHC protocol using antibodies that are derived from the same species host. If not done correctly, there is high risk that the targets will be cross labeled, such that they falsely appear to overlap, and may give false information on their distribution in the tissue. However, it is not necessarily an impossible task to do this multiple same-species primary staining. This approach may be necessary if a certain antibody is particularly rare, or otherwise alternatives in different species are prohibitively expensive.

There are a few different ways to perform this type of staining. The following info in the protocols below was obtained from Jackson ImmunoResearch's web site. I migrated and paraphrased the essential parts here in case the URL breaks in the future. But before reviewing the protocols, it is worth reviewing how Fab fragments would work in these scenarios.

II. Info on Antibody Fab Fragments

In case this info is not mentioned elsewhere in this handbook, I've added some background on Fab fragments.

A normal IgG antibody has a Y shape, containing one Fc fragment (the antibody identifier) and two Fab fragments (the antigen targeting parts). While a fully intact secondary antibody would have one Fab part bind to the antigen (the first primary antibody), the other Fab part will still be unoccupied and unbound. The consequence of this is that the second primary antibody will get snagged by these partially bound secondary antibodies.



Obtained from https://link.springer.com/article/10.1007/s12016-019-08739-8

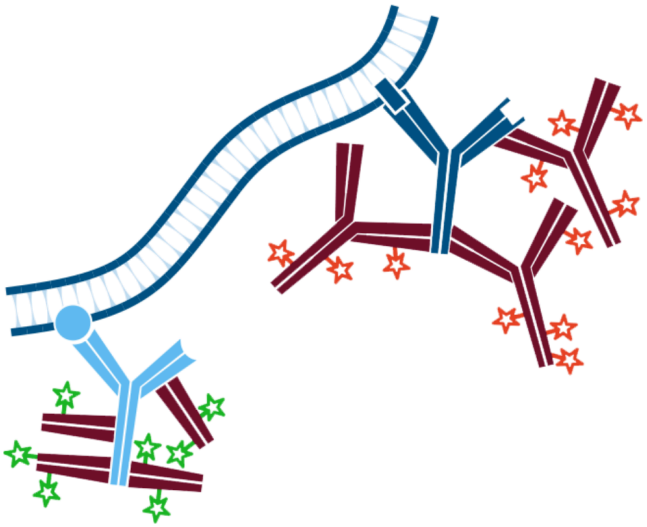
There are a few ways to navigate this issue. Some approaches utilize Fab fragments of antibodies, rather than their whole IgG version. Some antibody distributors provide Fab fragments that are conjugated to fluorophores, enzymes, or haptens. Although the selection is usually a bit more limited

(potentially due to the small size of the antibody fragment), it compensates for this by avoiding the cross-binding issues mentioned above.

III. Approach 1: Use of conjugated Fab fragments for labeling and blocking

https://www.jacksonimmuno.com/technical/products/protocols/double-labeling-same-species-primary/example-a

1. Perform pre-antibody blocking step by incubating sample in normal serum.
2. Incubate the sample with the first primary antibody.
3. Rinse.
4. Incubate with the first secondary antibody - specifically the Fab fragment form - with a label/conjugate.
    a. When all Fab fragments of a secondary antibody have bound to the first primary antibody, few to no unbound secondary antibody antigen binding sites will remain open. This prevents the second primary antibody from being "snagged" by unoccupied secondary antibody sites. It also coats the first primary antibody, such that it is bound by just the first secondary antibody and there is no room for the second secondary antibody.
    b. JacksonImmuno suggests using excess Fab fragment in the incubation. The exact amount was not specified and may need to be optimized by the user. My suggestion is to try double the concentration you would use for a normal intact IgG secondary antibody.
5. Rinse.
6. Incubate with second primary antibody.
7. Rinse.
8. Incubate with second secondary antibody with a different label/conjugate. This can be a regular whole IgG rather than an Fab fragment.
9. Rinse.

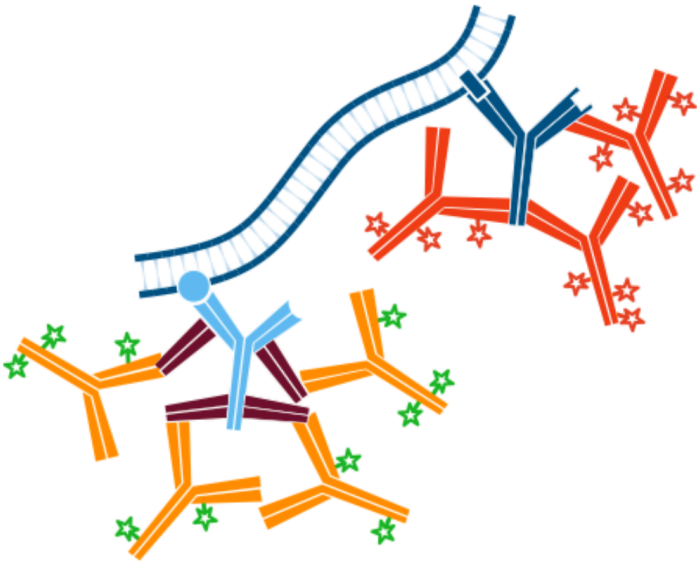


Picture from webpage linked above.

IV. Approach 2: "Convert" one of the primaries into a different species with an Fab fragment coating

I recommend this approach less than the previous one, due to it being more complicated and requiring more reagents. That said, it's possible it may better suit some folks' specific needs.
https://www.jacksonimmuno.com/technical/products/protocols/double-labeling-same-species-primary/example-b

1. Perform pre-antibody blocking step by incubating sample in normal serum.
2. Incubate the sample with the first primary antibody.
3. Rinse.
4. Incubate with the first secondary antibody - specifically the Fab fragment form - **without** a label/conjugate.
    a. This approach will coats the first primary antibody, such that it is bound by just the first secondary antibody and there is no room for the second secondary antibody. It will also provide an alternate outward-facing "appearance" to subsequent antibodies.
    b. JacksonImmuno suggests using excess Fab fragment in the incubation. The exact amount was not specified and may need to be optimized by the user. My suggestion is to try double the concentration you would use for a normal intact IgG secondary antibody.
5. Rinse.
6. Incubate with a tertiary antibody that is designed to target the unconjugated secondary antibody Fab fragments. The tertiary antibody can be normal intact IgGs, and should have a label/conjugate.
7. Rinse.
8. Incubate with the second primary antibody.
9. Rinse.
10. Incubate with second secondary antibody with a different label/conjugate. This can be a regular whole IgG rather than an Fab fragment. It must be from a different species that the first secondary antibody.
11. Rinse.

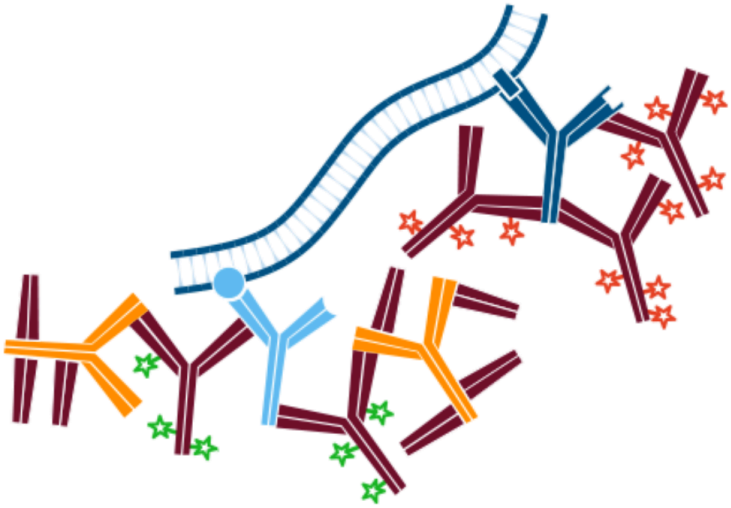


Picture from webpage linked above.

V. Approach 3: Performing two blocking steps to neutralize the first secondary antibody

This approach is the one I recommend the least. I suspect it will be more unpredictable in regards to some binding sites being left unoccupied either on the target antigen or on the targeting antibody. It also requires the most reagents. Despite this, the protocol is provided here. https://www.jacksonimmuno.com/technical/products/protocols/double-labeling-same-species-primary/example-c

1. Perform pre-antibody blocking step by incubating sample in normal serum.
2. Incubate the sample with the first primary antibody.
3. Rinse.
4. Incubate with the first secondary antibody - specifically the whole IgG form - with a label/conjugate.
5. Rinse.
6. Incubate with unconjugated antibody that is the same species as the primaries. The simplest and far less expensive way to do this is through incubation with the normal serum from that animal, rather than just using the IgGs isolated from the serum.
    a. This approach will occupy/block the unbound Fab branches of the currently-attached secondaries, thus preventing them from "snagging" the second primary antibody later.
    b. Although using normal serum from the primary antibody species is the simpler and more cost-effective way of doing this, Fc fragments could be used instead. Much like how Fab fragments can be separated from the IgG structure, so too can the identifier branch of the IgG - the Fc fragment. I'm not convinced using Fc fragments would yield noticeable benefit compared to normal serum.
7. Rinse.
8. Incubate with Fab fragments that target the primary species but are unconjugated.
    a. This should coat any remaining open antigen sites from the previous incubation step.
9. Rinse.
10. Incubate with the second primary antibody.
11. Rinse.
12. Incubate with the second secondary antibody, in whole IgG form and with a different label/conjugate.
13. Rinse.



Picture from webpage linked above.

Appendix C: Tyramide Signal Amplification Usage and Reagent Preparation [AXC]

I. Intro

I recorded two videos on this method that may help explain the TSA technique beyond what is documented here.
Presentation & Explanation: https://youtu.be/u_JswJUwzts
Demonstration: https://youtu.be/aKsMS5cwTvs

See Part 2, Section IIX for more info. TSA is a useful technique for dramatically boosting your IHC staining signal. For certain antibodies that have low affinity to their epitope, this technique can compensate for dim staining that typically occurs otherwise.

II. Mechanism of action

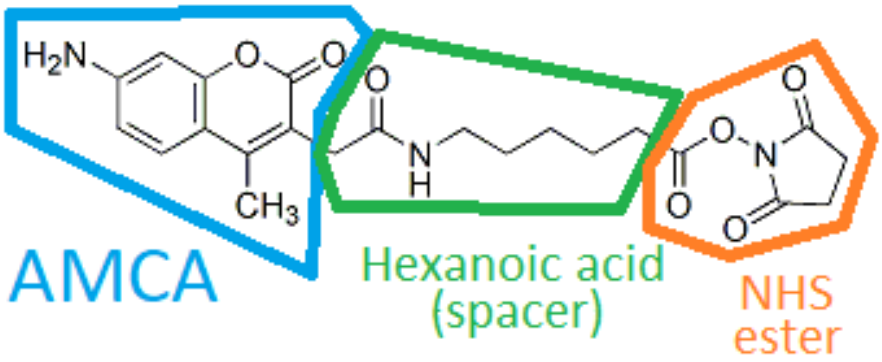
TSA is enzyme catalyzed, hence why it is also referred to as Catalyzed Amplification Reaction Detection (CARD). The technique works somewhat similarly to DAB staining. HRP, bound to the antigen of interest by an antibody, catalyzes the reaction that activates tyramides into their reactive form that binds to nearby lysine residues. H_2O_2 is used as an electron donor to fuel the reaction. Using this process, tyramides in solution continue to deposit onto tyrosine residues of proteins near the antigen as long as HRP, H_2O_2, and tyramide conjugates are active and present. Similar to DAB, there is an ideal range of time that the reaction must progress to reveal the antigen and not increase background staining. This varies based on antigen and antibody concentrations.

III. Synthesis of tyramide conjugates

Notes:

- Check the molecular weight of the compound on the product label to make sure it matches in the "Specific reagents" list! If it doesn't, the calculation must be re-done to determine the right amounts of tyramine stock and ethanol to add.
- DMSO may be an easier to use substitute for DMF, but I'd need to do some investigation first to see if it yields conjugates with the same efficacy.
    - Careful: TEA is **not** compatible with DMSO! It will decompose DMSO.
- We are used to seeing fluorescent dyes, such as Cy3 and AlexaFluor 488, already attached to other molecules such as antibodies. In order for those dyes to become attachable to a protein, they are typically modified to have an added "functional group". The commonly used functional to attach such dyes to tyramine is the NHS ester group. Another name for this is a succinimidyl ester.
- Although NHS-esters of "older" classes of fluorophores (i.e. fluorescein, rhodamine) are even cheaper, they *allegedly* lack the longevity and lower background staining of "newer" classes (AlexaFluor/Dylight 488, Cy3, etc). So, if possible, I recommend spending a smidge extra on those NHS-esters if possible. Another consideration is that some dyes, especially the older forms, can have somewhat high background staining. But this can be remedied by following the staining with a few exchanges of 0.2% Triton in PBS.

- I've had issues with Cyanine dye NHS esters successfully yielding tyramide conjugates. Despite those dyes being sometimes more robust than older alternatives, I now prefer to use the older options due to more successful outcomes - and cheaper costs.
- Although some dyes have just an NHS ester attached, others may actually use a spacer (a bridge of several atoms) between the ester and the main dye molecule. The diagram below (modified from Biotium) labels the different components of the blue dye with the hexanoic acid spacer, AMCA-X SE (a.k.a. 6-((7-Amino-4-methylcoumarin-3-acetyl)amino) hexanoic acid succinimidyl ester).



Important notes about reagents:

- Tyramine HCl:
  - The substance is tyramine HCl and not tyramide; it is called tyramide after it is conjugated to something.
  - I recommend using tyramine HCl that is either new from the manufacturer, has been in the fridge for no more than two years, or has been at -20 deg C for no more than five years. It's hard to say how quickly it will or will not degrade. But as it is relatively inexpensive, it is best to just get a fresh batch if you are unsure if an older batch you have is still sufficient quality.
- N,N-dimethylformamide (DMF)
  - Make sure you get the anhydrous type.
  - DMF must be extracted from its stock bottle using a needle and syringe. Do not remove the bottle cap! This stuff attracts water and moisture. That moisture can cause unintended hydrolysis of the dye esters you'll be using in later steps.
  - Keep track of the integrity of the bottle cap. If the cap and/or septum look significantly tarnished, or if there is a noticeable fishy smell from it, consider getting a new bottle. Dispose of the old bottle as hazardous waste.
- Alcohols
  - After the conjugation reaction, instructions typically state that the concentrated conjugate solution should be diluted with 100% ethanol. I have tried this with 100% isopropanol and had no issues. So, if getting 100% ethanol is a challenge due to potable alcohol regulations, isopropanol works just as well.
  - I don't use denatured 100% ethanol, as I do not know how the additives will react with the tyramide conjugates or dyes over multiple years in storage.

Tyramine stock solution:

- Dissolve 1 mg tyramine HCl in 100 uL of N,N-dimethylformamide (DMF).
    - Tyramine HCl molecular weight is 173 g/mol
        - 1 mg = 0.00578 mmol
    - DMF must be extracted from its stock bottle using a needle and syringe. Do not remove the bottle cap!
        - Since needles are imprecise within the 0.1 mL range, I recommend withdrawing a rough estimate of slightly more DMF than what is needed, ejecting the needle contents into an empty centrifuge tube, and then withdrawing from that tube with a micropipetter to add to another centrifuge tube containing the tyramine powder. Discard unused DMF as hazardous IHC waste.
    - Do this away from open flame, with proper chemical and fire protection and neoprene gloves. The chemical does not ignite spontaneously but sparks and flame should be avoided as one would with ethanol. Doing this in a fume hood would be ideal but probably not necessary, given the small amount.
    - Smaller amounts of tyramine could be used, but it is extremely hard to weigh less than 1 mg.
- Add 1 uL of triethylamine to this solution.
    - Caution: toxic, highly flammable, corrosive
    - The tyramine HCl stock solution has to have its pH raised, preferably by an organic base, to raise it to a final pH 7 to pH 8.
    - Per the Hopman reference: “The tyramine-HCl stock solution “B” was prepared by dissolving 10 mg/ml DMF (equivalent to 58 umol) to which a 1.25-fold equimolar amount of TEA (10 ul of 7.2 M stock solution; 72.5 umol) was added.”
    - The amount of triethylamine being added in ***my*** recipes is much less, as the total volume I use is 1/10th of what the Hopman recipe uses (I make 100 uL of the 10mg/mL tyramine stock solution, while they made 1 mL at the same concentration).

Dye ester stock solution:

- Dissolve 1 mg dye NHS ester form in 100 uL of DMF.
    - DMF must be extracted from its stock bottle using a needle and syringe. Do not remove the bottle cap!
        - In case you did not already do this for the tyramine stock solution: Since needles are imprecise within the 0.1 mL range, I recommend withdrawing a rough estimate of slightly more DMF than what is needed, ejecting the needle contents into an empty centrifuge tube, and then withdrawing from that tube with a pipetter to add to another centrifuge tube containing the dye ester powder. Discard unused DMF as hazardous IHC waste.
    - Do this away from open flame, with proper chemical and fire protection and neoprene gloves. The chemical does not ignite spontaneously but sparks and flame should be avoided as one would with ethanol. Doing this in a fume hood would be ideal but probably not necessary, given the small amount.

Synthesis procedure:
The amounts of stock solutions used in these procedures vary based on the desired conjugate. This is due to differing molecular weights of the conjugates, and the aim of maintaining a 1.1:1 equimolar ratio of dye ester versus tyramine. (Or, put differently, the ratio of moles tyramine to moles dye ester is 0.91:1.)
More can be read in an article by Hopman et al., 1998 (http://www.ncbi.nlm.nih.gov/pubmed/9603790).

There are a few consistent steps to do:

1. When adding tyramine stock to a dye ester stock, lightly mix (not with vortexer!), then let sit in darkness for 2 hours at room temp to allow reactions to occur.
2. Per one of my earlier notes, 100% ethanol could be replaced with 100% isopropanol.
3. If using ethanol, I don't know if you should use the denatured type - I'm not clear about how the additives in denatured alcohol may interact with this reaction.
4. After adding alcohol, mix gently (no vortexing!) and store @ -20°C. Mixture will remain liquid at normal freezing temperatures.

AMCA-tyr
(note there are multiple forms)

- AMCA NHS ester:
  - [chemical name: Succinimidyl-7-amino-4-methylcoumarin-3-acetate]
  - **CHECK** MW: one version was 330.29 g/mol, another version from Lumiprobe (#3606, in 2026) is 366.76 g/mol
  - Amount of tyramine stock to add:
    - To the MW 330.29 g/mol version: Add 48 uL of tyramine stock to the 100 uL of dye ester stock. Mix lightly and react in darkness for 2 hours at room temp. Add 952 uL of 100% ethanol, mix gently, store @ -20°C.
    - To the MW 366.76 g/mol version: Add 43 uL of tyramine stock to the 100 uL of dye ester stock. Mix lightly and react in darkness for 2 hours at room temp. Add 957 uL of 100% ethanol, mix gently, store @ -20°C.
      - Calculations: 1 mg = 0.00272 mmol dye. 0.00272 * 0.91 = 0.00248 mmol tyramine. 0.00248 * 173 g/mol (MW of tyramine) = 0.429 mg of tyramine to be added. Tyramine stock vial is 1 mg / 100 uL, so this would be ~43 uL.
- AMCA-X, SE:
  - [chemical name: 6-((7-Amino-4-methylcoumarin-3-acetyl)amino)hexanoic acid, succinimidyl ester]
  - MW 443.45 g/mol (using in Vieira lab as of 2022)
  - Add 35 uL of tyramine stock to 100 uL AMCA-X SE stock solution. Mix lightly and react in darkness for 2 hours at room temp. Add 964 uL of 100% ethanol, mix gently, store @ -20°C.
    - Calculations for double-checking if reagent doesn't work: 1 mg = 0.00226 mmol dye ester. 91% of this amount is 0.00205 mmol, which is how much tyramine should be added. 0.00205 * 173 g/mol (MW of tyramine) = 0.354 mg of tyramine to be added. Tyramine stock vial is 1 mg / 100 uL, so this would be ~35 uL.

Dylight 488-tyramide (DL488-tyr):

- MW = 1011 g/mol

- Add 17.2 uL of tyramine stock to 100 uL dye ester stock. Mix lightly and react in darkness for 2 hours at room temp. Add 983 uL of 100% ethanol, mix gently, store @ -20°C.

Fluorescein-tyramide (fluo-tyr):

- MW = 473.4 g/mol
- Chemical names:
    - 5/6-carboxyfluorescein succinimidyl ester
        - May be labeled as 5 or 6 as the bond to the benzene ring seems to be between positions 5 and 6. Literature suggests there are modest functional differences in regards to fluorescence (the excitation + emission maxima), but generally not enough to be meaningful for most setups.
        - 5-Carboxyfluorescein N-succinimidyl ester (MilliporeSigma, 2026)
    - FAM NHS ester, 6-isomer (Lumiprobe, 2026)
        - Do not get the diacetate version! It must be activated by intracellular enzymes. Per Lumiprobe: "This molecule is non-fluorescent until the acetate groups are cleaved by intracellular esterases resulting in the formation of a highly fluorescent fluorophore with emission in the green spectrum range (absorption maximum at ~492 nm, emission maximum at ~517 nm). Due to this characteristic, this product is used for tracking cells and flow cytometric evaluations and studying intracellular esterase."
- Add 34 uL of tyramine stock to 100 uL dye ester stock. Mix lightly and react in darkness for 2 hours at room temp. Add 966 uL of 100% ethanol, mix gently, store @ -20°C.

Rhodamine-tyramide (rho-tyr):

- Chemical name: 5(6)-Carboxytetramethylrhodamine N-succinimidyl ester
    - This one is sometimes referred to as "TAMRA".
- MW = 528 g/mol
    - Some vendors report slightly different molecular weights, such as 537 g/mol.
- Add 30 uL of tyramine stock solution to the 100 uL of rhodamine NHS ester stock. Mix lightly and react in darkness for 2 hours at room temp. Add 970 uL of 100% ethanol, mix gently, store @ -20°C.

Cy3 tyramide (Cy3-tyr)

- Chemical name: 3H-Indolium, 2-[3-(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)-1-propen-1-yl]-1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-3,3-dimethyl-, tetrafluoroborate
- MW = 641.5 g/mol (Lumiprobe, #21020)
- Combine 24.5 uL of tyramine stock with 100 uL dye stock. Mix lightly and react in darkness for 2 hours at room temp. Add 975.5 uL of 100% ethanol, mix gently, store @ -20°C.
    - Calculations: 1 mg = 0.00156 mmol dye. 0.00156 * 0.91 = 0.00142 mmol tyramine. 0.00142 * 173 g/mol (MW of tyramine) = 0.245 mg of tyramine to be added. Tyramine stock vial is 1 mg / 100 uL, so this would be ~24.5 uL.

Sulfo-Cy3 tyramide (SCY3-tyr)

- Chemical name: 3H-Indolium, 2-[3-(1,3-dihydro-1,3,3-trimethyl-5-sulfo-2H-indol-2-ylidene)-1-propen-1-yl]-1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-3,3-dimethyl-5-sulfo-, inner salt, sodium salt
- MW = 751.91 g/mol

- Calculations: 751.91 g/mol. 1 mg = 0.00133 mmol
  Need tyramine at 91% amount of what dye is at.
  0.00133 *.91 = 0.00121 mmol tyramine
  Tyramine HCl MW = 173 g/mol. 1 mg = 0.0058 mmol
  0.00121 / 0.0058 = 0.209 = 20.9%
  Assuming 1 mg in 100 uL for tyramine and 1mg / in 100uL for dye,
  Use 20.9% of that 100uL tyramine stock when combining with dye.
- Combine 21 uL (rounded up) of tyramine stock with 100 uL dye stock. Mix lightly and react in darkness for 2 hours at room temp. Add 979 uL of 100% ethanol, mix gently, store @ -20°C.

Cy5 tyramide (Cy5-tyr)

- Chemical name: 3H-Indolium, 2-[5-(1,3-dihydro-1,3,3-trimethyl-2H-indol-2-ylidene)-1,3-pentadien-1-yl]-1-[6-[(2,5-dioxo-1-pyrrolidinyl)oxy]-6-oxohexyl]-3,3-dimethyl-, tetrafluoroborate
- MW (from lumiprobe, cat #23050): 667.54 g/mol
- Add 23.5 uL of tyramine stock to 100 uL dye stock. Mix lightly and react in darkness for 2 hours at room temp. Add 976 uL of 100% ethanol, mix gently, store @ -20°C.
  - Calculations: 1 mg = 0.00150 mmol dye. 0.00150 * 0.91 = 0.00136 mmol tyramine. 0.00136 * 173 g/mol (MW of tyramine) = 0.236 mg of tyramine to be added. Tyramine stock vial is 1 mg / 100 uL, so this would be ~23.5 uL.

IV. Performing a TSA staining reaction

1. Perform and finish antibody staining, including incubation with an HRP conjugate.
2. Dilute the tyramide conjugate stock to 1:100 to 1:500 in PBS.
   1. No azide – it will ruin the HRP. Normal donkey serum is not necessary and may also contain azide. Triton may only be needed with thick tissues.
   2. I typically go with a 1:300 dilution.
3. Optional: Pre-incubate tissue in tyramide conjugate solution for 10 min.
   1. This pre-incubation allows it to soak into tissue before reaction is "activated" by H_2O_2. But, this step may not be needed for relatively thin tissue (<50 um).
   2. A similar process is done by some investigators that use DAB staining.
4. Add H_2O_2 to tyramide conjugate solution that is bathing the tissue. The H2O2 should be at a final concentration of 0.003%.
   1. If the tyramide solution is a volume of 10 mL, add 10 uL of 3% H_2O_2 stock to achieve the correct dilution.
   2. For reference, 3% is the concentration in the store-bought brown bottles.
5. Incubate tissue in this "activated" solution for 5-20 minutes.
   1. Determine the ideal incubation time through some initial testing.
   2. In my experience, the stain doesn't really progress much more beyond 30 min.
6. Rinse with PBS.
   1. I recommend having Triton X-100 at a concentration of 0.2% to help unstick dye that is not specifically bound to the target sites.
7. If using biotin tyramide, stain with streptavidin conjugate for 1-1.5 hours. If not staining further, mount onto slides.

V. Performing Two Sequential TSA stains

In Part 2 Section IX, I discussed the need to neutralize a prior round of HRP application before performing a second round.

1. Perform IHC normally. Stop after incubating with only one type of HRP conjugate.
    1. For example, if you have goat and rabbit primaries, follow with only HRP donkey anti-goat or anti rabbit, NOT both.
2. Rinse.
3. Perform TSA process with your first tyramide conjugate. Include pre-incubation step.
    1. Let's say this conjugate is AlexaFluor 350-tyramide.
4. Rinse
5. Incubate in PBS with 0.05% sodium azide for 10 min.
6. Rinse.
7. Incubate in 1% H2O2 for 10 min.
8. Rinse (be sure to do the full three 5 min PBS rinses).
9. Incubate in second HRP conjugate for standard length of time (whatever you normally do).
10. Rinse.
11. Perform TSA process with your second tyramide conjugate. Include pre-incubation step.
    1. Must be different color from tyramide conjugate #1.
    2. Let's say this conjugate is AlexaFluor 488-tyramide.
12. Rinse
13. Mount tissue on slides.

After this process, you should have two antigens brightly stained, with one stained blue and the other stained green.

VI. Additional Considerations

- Tyramide conjugate stock solutions remain effective for at least a year when stored at -20 °C. In my experience, they seem to last for five years, maybe longer.
- The synthesis reactions add the dye esters in a 1.1 : 1 mole ratio to tyramine HCl. See reference at bottom for reasoning of this specific ratio.
- Calculation example for a conjugate's synthesis:
    - Tyramine HCl MW is 173, so 1 mg = 0.0058 mmol. Fluorescein NHS ester MW is 473, so 1 mg = 0.0021 mmol.
    - The dye ester is always used in an amount of 1 mg per 100 uL DMF, and the full volume is used in the conjugation reaction.
    - Although tyramide is initially dissolved as 1 mg in 100 uL DMF, much less than that volume is used in the conjugation reaction.
    - The reverse of the molar ratio is 1 mol dye ester : 0.91 mol tyramine HCl.
    - An equimolar ratio would involve 0.0021 mmol of tyramine added to 0.0021 mmol fluorescein NHS ester, but we want less than equal: 0.0021 * 0.91 = 0.0019 mmol tyramine.
    - The amount of tyramine stock solution that contains this mass can be determined by first dividing this final desired molar amount from the full amount that is in the tyramine stock solution: 0.0019 / 0.0058 = 0.327 = 32.7%
    - So, we only want to use 32.7% of the volume of the tyramine stock solution when adding it to the dye ester stock solution.
    - Thus, we add 32.7 uL (or about 33 uL) of tyramine stock to 100 uL of the fluorescein NHS ester stock.

- If using biotin esters in your synthesis, purchase biotin esters that have a long spacer between the biotin segment and the ester segment. This allows for easier binding of avidin/streptavidin; shorter spacing between biotin and the ester part may cause too much steric hindrance for strepavidin conjugate binding. Refer to aforementioned Hopman paper for their biotin ester.
    - You will need a streptavidin conjugate to stain tissue-bound biotinylated tyramide. The resulting stain may be MUCH brighter than performing TSA with only a fluorophore-conjugated tyramide.
- Research whether certain esters from certain suppliers actually work for making tyramide conjugates. Avoid anything where people online have said that a particular dye ester failed to yield a viable tyramide conjugate.
- If the antigen is found in most or large regions of the brain, the tissue may visibly take on a tint of the color of the tyramide conjugate used (or the streptavidin conjugate solution if using biotinylated tyramide). Don't be surprised by this unexpected visible coloration of the tissue, but do be wary of the intense fluorescence that will likely be given off by these stained areas – have the gain on your camera/detector on the microscope turned way down.
- Tissue fluorescence penetration issues: Relatively ubiquitous antigens such as neurotransmitter receptors will result in an odd effect from the TSA reaction. The outer layers of the tissue, heavily peppered with your antigen of interest, may absorb/react with the tyramide conjugate and prevent it from flowing deeper into the tissue despite the existence of just as much antigen further inside. This results in the outer 5-10 um on each surface of the tissue being way overstained, while the deeper layers are comparatively understained. This effect not only occurs due to the abundance of the antigen, but also due to the short reaction time (~10 min) and limited quantity of tyramide conjugate in the solution. These three factors - antigen abundance, short reaction time, and tyramide conjugate dilution - prevent deep penetration of the tyramide conjugates. Therefore, try to avoid using TSA for ubiquitous antigens, or otherwise use much thinner sections (~20-25 um). A pre-incubation with the tyramide conjugate, in absence of H_2O_2, may also help.
- Some sources say that the unbound dye and tyramine molecules should be dialyzed out. I have not found the need to do this, though you should determine this on a case-by-case basis.
- As noted elsewhere, I treat sections with 0.05% sodium azide (in PBS) and then with 1% H_2O_2 before I start any IHC staining – even before primary antibody incubation. These steps will eliminate any endogenous peroxidase activity that would otherwise cause background staining. It should be done in the order of azide then H_2O_2, otherwise massive foaming will occur on brain sections.
- Regular NHS esters should be dissolved in DMF or DMSO to make stock solutions; allegedly they are poorly soluble in water. Sulfo-NHS esters are water soluble (best dissolved in pure water), but are also soluble in DMF or DMSO.
    - However, <u>triethylamine is incompatible with DMSO</u>! As for water-soluble variants, I am unsure which base is best to add.
- According to Lumiprobe, pH 8.3-8.5 is ideal for efficient labeling reaction. Other sources suggest that neutral pH is best, and pH above 8 incurs greater hydrolysis of the NHS ester (bad). Original Hopman protocol used TEA to raise pH of DMF, but they were not specific about the final pH value. They assume it is pH 7-8.
- The protein to be labeled should be dissolved in matching solvent. For instance, if ester is dissolved in DMF, so should the protein to be conjugated. If using water to dissolve ester, protein can be dissolved in 0.1 M phosphate buffer.
- A range of times are suggested for reactions. The times range from 30 minutes to 4 hours. Considering that the reaction is self-limiting (eventual ester hydrolysis), letting it react for long times may be best.

- Both the NHS ester stock and protein solution should not contain amines or amine-based buffers such as Tris or glycine. These will interfere with the labeling reaction. Recommended to desalt or buffer exchange if these are major constituents of your solutions.
- Tyramine is small and thus a 1.1:1 molar ratio of ester to tyramine is suggested - there is likely only one binding site on a given tyramine molecule. However, larger proteins have more potential labeling sites.
- Lumiprobe manuals suggest a minimum 8:1 molar ratio of biotin ester to antibodies or Fab fragments. In cases where conjugation is being done in aqueous media and the antibody concentration is low, Pierce manual suggests that up to a 50:1 (!) molar ratio should be used.
- More can be read in the CARD article by Hopman et al., 1998 (http://www.ncbi.nlm.nih.gov/pubmed/9603790). Note the two typos in their Table 1:
    - For biotin ester, Stock B tyramine solution should be used in 1/10th the volume that the table states - 29 uL instead of 289 uL.
    - The mass of dye ester used should be mg not ug.

Appendix D: Suspected Incompatibilities [AXD]

Overview:

As occasionally mentioned in this manual, certain procedures may be incompatible with others in regards to properly labeling and revealing the desired antigens. Below are some incompatibilities reported either in literature, personal communications, or personal experience.

Antigen x Tissue Treatment

- Heavy detergent use, fixation with alcohol or acetone, or treatment with ethanol will over-solubilize cell membranes, liberating membrane-bound antigens.
- Antigen retrieval, although helpful in revealing some antigens, may destroy or liberate other antigens
- Do not add azide to HRP-containing soluions! Peroxidases, especially HRP, can be permanently inactivated by tissue treatment with methanol, H2O2 (>0.01%), sodium azide, and HCl (or anything lowering pH below 2). Surprisingly, fixation and re-fixation with 4% formaldehyde does not substantially degrade peroxidase activity unless tissue are fixed for months.
- Pepsin antigen retrieval, being at low pH, may reversibly quench GFP signal. Do not treat GFP-containing brains with acidic solutions. This deactivates GFP's fluorescent signal. Allegedly this can be reversed by subsequent immersion in an alkaline solution.

Autofluorescence reduction treatment x Fluorophores/Fluorescence

- Sudan Black B-stained tissue should not be combined with VectaShield mounting media. Excessive background staining may result.
- Sudan Black B staining may cause increased background in the red to far red spectrum. Avoid use with red- and far red-emitting dyes.
- Sudan Black B staining will quench fluorogold and Fast Blue tracer fluorescence.
- Copper sulfate treatment (specifically the ammonium acetate) quenches GFP signal. Do not treat GFP-containing brains with acidic solutions. This deactivates GFP's fluorescent signal. Allegedly this can be reversed by subsequent immersion in an alkaline solution, but I did not have success with this yet.

Other treatments x Fluorophores/Fluorescence

- For some reason, Prolong antifade mounting media may stain tissues/cells blue despite the vendor's exclusion of DAPI into the mixture. This may have been a bad batch effect, but I'm putting it here as something to watch out for. Only use blue fluorophores if fluorescence is bright enough. Blue stains include DAPI, Dylight/AlexaFluor 350 and 405, and AMCA.
- Xylene clearing and ethanol dehydration of sections, as well as using xylene-based mountants, have been reported to quench AMCA and rhodamine.
- Heat-induced epitope retrieval will quickly degrade several fluorescent markers. These include Fluorogold, Fast Blue, and potentially Fluororuby. Even AlexaFluor dyes may be vulnerable. Do not use HIER on tissue that already contains fluorophores.
- Dilute cresyl violet stains, used in fluorescent Nissl staining, have complete spectral overlap with all red-emitting dyes and substantial overlap with far red-emitting dyes. Do not use these dyes in conjunction with this staining method, as they will not be discriminable under microscope.
- The same applies for acridine orange stains and green- or yellow-emitting dyes; too much spectral overlap.

Appendix E: Product information [AXE]

Order info (from Fisher unless otherwise specified). Catalog numbers were input Dec. 2011, so much of it *might* be out of date.

Potassium Phosphate Monobasic (Crystalline/Certified ACS), Fisher Chemical
Formula: KH2PO4
Cat. No.: P285-500

Potassium Phosphate Dibasic (Fine White Crystalline Powder), Fisher BioReagents
Formula: K2HPO4
Cat. No.: BP363-500

Potassium Hydroxide (Pellets/Certified ACS), Fisher Chemical
Formula: KOH
Cat. No.: P250-500

Sodium Borate Decahydrate ACS, EMD Chemicals > Poly Bottle; 500g
Formula: Na2B4O7·10H2O
MSX03551
EMD Chemicals
Cat No.:SX0355-1

Triton* X-100, Acros Organics
Polyethylene glycol mono [4-(1,1,3,3-tetramethylbutyl)phenyl] ether
Formula: C34H62O11
AC21568-2500
Acros Organics
Cat. No.: 215682500

Glycerol (Certified ACS), Fisher Chemical
Formula: Glycerin, 1,2,3-Propanetriol; C3H8O3
Cat. No.: G33-1

Ethylene Glycol (Certified), Fisher Chemical
Formula: 1,2-Ethanediol; C2H6O2
Cat. No.: E1784

Potassium bicarbonate
Formula: KHCO3
Cat. No.: P235-500

Appendix F: Reagent Recipes [AXF]

(It is very likely that I obtained many of these recipes from IHC World. In other cases, I did devise some myself, or heavily modified them from specific references.)

Acetate buffer

0.05 M, (pH 5.5)

- Notes:
    - Can be used for AEC staining.
- Recipe 1:
    - In 10 mL distilled water, add 68 mg of sodium acetate trihydrate (MW 136.1). Mix to dissolve and adjust pH to 5.5 using concentrated HCl or glacial acetic acid.
- Recipe 2:
    - If one does not possess sodium acetate, a combo of diluted glacial acetic acid and sodium hydroxide could be used instead. I used vinegar (5% acetic acid = about 0.87 M) diluted further in distilled water. In a 10 mL tube, add 545 uL distilled white vinegar, 425 uL of 1 M NaOH, and 9 mL distilled water. Shake well before use.
        - Rationale: We add 425 uL of NaOH solution as this immediately converts 425 umol of acetic acid to acetate ions. This leaves 75 umol acetic acid. According to the Henderson Hasslebach equation and the fact that acetic acid has pKa of 4.75, this should yield a pH of ~5.5 while still being 0.05 M “acetate”. See https://answers.yahoo.com/question/index?qid=20120309130228AAItgLb

Agarose, 2%

- Used for forming an embedding medium for vibratome sectioning of brain chunks.
- Simply dissolve 1 g low melting point agarose powder in 50 mL distilled water OR PBS. Use a stirring hotplate; heat and constant stirring must be applied, and the solution is only ready once fully transparent (excluding air bubbles)

AEC (3-amino-9-ethylcarbazole)

- Notes:
    - Carcinogen.
    - Stains tissue with red precipitate when reacted with HRP or other oxidizing substances.
    - Stain has less resolution than DAB staining.
    - Stain can be dissolved by alcohol, so it is not advisable to treat the tissue with any ethanol exposure nor to be coverslipped with a polyvinyl alcohol medium.
- Stock solution (1%)**:** Add 10 mg 3-amino-9-ethylcarbazole (AEC) to 1 mL of one of the following solvents: 100% ethanol, N,N-dimethylformamide (DMF), or dimethylsulfoxide (DMSO). Store in freezer - first two solvents will not freeze, DMSO will.
    - DMF is the most hazardous choice of the three solvents.
- Working solution (0.05%): Add 50 uL of AEC stock to 1 mL of 0.05 M acetate buffer. After a pre-incubation step (see step #9 below), add 5 uL of hydrogen peroxide to obtain a concentration of 0.015%. Mix well - it is now ready to incubate with sections.
    - Make right before use, dispose of as hazardous waste (carcinogen) after use. This substance appears to precipitate out in water a lot but still works to react with sections.
- For more information: https://ihcworld.com/2024/01/20/protocol-for-aec-peroxidase-substrate-solution-red/

Antibody diluent buffer (a.k.a. blocking buffer)

- Notes:
    - Per the name, used for diluting antibodies.
    - Also serves as a vector and protective solution for said antibodies; the Triton helps penetration into tissue, the azide helps prevent microbial growth in both the solution and the tissue, and the normal serum (usually goat or donkey - whatever animal your secondary antibodies are made from) is an obstacle for protein degradation that would normally attack the antibodies.
    - Triton concentrations used in staining range from 0.1-0.3%. Not adding any may prevent antibodies from reaching intracellular spaces. Too much ruins membranes and cytoarchitecture.
    - Also used for blocking antibody-caputing sites on the tissue that would otherwise cause non-specific binding of the primary antibody.
    - DO NOT USE THIS TO DILUTE ANTIBODIES OR REAGENTS THAT CONTAIN OR COME INTO CONTACT WITH HORSERADISH PEROXIDASE (HRP)! The azide that is added, as well as the trace amounts in the normal serum ingredient, will permanently disrupt HRP function. For diluting HRP-conjugated reagents, use only the PBS and Triton ingredients of the following recipe.
- Recipe:
    - Obtain 47.5 mL 1x PBS.
    - Add 1.25 mL of 1:1 normal serum stock (final concentration now 2.5 % by volume)
    - Add 1 mL of 10% Triton stock (final concentration now 0.2% by volume)
    - Add 0.25 mL of the 10% sodium azide stock (final concentration now 0.05% by volume).
    - pH should still be at an appropriate range (7.4) as long as the PBS you used was.

Antifreeze (a.k.a “Cryoprotectant”)

(Modified from IHC World Recipe)

- Version 1
    - Recipe, Makes 50 mL:
        - 5 mL of 0.2 M phosphate buffer
            - OR ~5 mL of 1x PBS
        - 15 mL ethylene glycol
        - 15 mL glycerol
        - 15 mL distilled water
        - Mix well, but avoid shaking to prevent foaming.
    - Notes:
        - Prevents freezing of tissue at -20 degrees C, allowing storage for years.
        - This solution is helpful for preservation of tissue for light and electron microscopy.
        - Although it could be re-used by filtering out brain bits, you may decide to avoid this in case the brains add contaminants (such as formaldehyde or azide).
        - Addition of a preservative such as azide or thymerosal is probably not necessary and may even be problematic for any endogenous enzyme activity you want to keep intact.
    - Additional info and calculations:

- The molar density (essentially the molarity of the pure chemicals) of the ingredients are calculated below:
    - Ethylene glycol: MW = 62.07, density = 1.113 g/cm^3 = 1113 g/L. 1113 / 62.07 = 17.93 mol/L
    - Glycerol: MW = 92.09, density = 1.261 g/cm^3 = 1261 g/L. 1261 / 92.09 = 13.7 mol/L
    - Omitting calculations for 0.2 M PB as the osmolarity is determined by its two constituents (see notes in the Phosphate buffer recipe below).
    - Also omitting calculations for 10x PBS as the osmolarity is determined by multiple constituents (see notes in the Phosphate buffer SALINE recipe below).
- The osmolarity that each then contributes is
    - Ethylene glycol: 1793 mOsm * (15/50) = 538 mOsm
    - Glycerol: 1370 mOsm * (15/50) = 411 mOsm
    - 0.2 M PB: 555 mOsm * (5/50) = 56 mOsm
        - Or if using 10x PBS, it is ~3400 mOsm. To reach similar osmolarity as 0.2 M PB, it has to be diluted to ~1/6th its concentration (specifically, 1/6.12)
    - Total osmolarity of antifreeze solution = 1005 mOsm

- **Version 2**
    - Recipe, makes 100 mL:
        - 50 mL of 0.1 M phosphate buffer
        - 30 g sucrose
        - 1 g polyvinylpyrrolidone (PVP-40)
        - 30 mL (or until @ 100 mL) of ethylene glycol
    - Notes:
        - Prevents freezing of tissue at -20 degrees C and allows storage for years.
            - But in my experience, the solution may separate out and some freezing may still occur. Generally the tissue should be protected during this freezing process, though.
        - Allegedly best for preservation of fluorescence in brain tissues, but I don't see why the PVP-40 specifically would impart that quality.
        - However, wikipedia on PVP says "It is also exceptionally good at absorbing polyphenols… Polyphenols are common in many plant tissues and can deactivate proteins if not removed."
        - Although it could be re-used by filtering out brain bits, you may decide to avoid this in case the brains add contaminants (such as formaldehyde or azide).
        - Addition of a preservative such as azide or thymerosal is probably not necessary and may even be problematic for any endogenous enzyme activity you want to keep intact.

Antigen retrieval solutions:

(Most of these were likely copied from IHC World protocols)

- Heated buffer reagents, all types (pick one):
  Store all in fridge, should last over 3 months.
    - Citraconic anhydride buffer (0.05%, pH 7.4): Add 50 mg Citraconic anhydride to 100 mL Distilled water. Mix to dissolve. Adjust pH to 7.4 – suggest using Na2CO3-NaHCO3 solutions.
    - 10 mM Citrate buffer

        - Option 1: 10 mM Sodium citrate: Add 0.93 g tri-sodium citrate dihydrate to 250 mL distilled water. pH to 6 with HCl
        - Option 2: 10 mM Citric acid: Add 0.48 g anhydrous citric acid in 250 mL distilled water. pH to 6 with NaOH.
    - Tris base buffered saline: Add 1.525 g Tris base to 250 mL distilled water. Add 2.2 g NaCl. pH to 9.
    - Tris Base Buffer: Add 0.3 g Tris base to 250 mL distilled water. pH to 10.
    - EDTA Buffer: Add 92.5 mg EDTA to 250 mL distilled water. pH to 8.

- HCl solution
    - *Note: Used at room temperature. Do not add detergent.*
    - Add 20 mL of 10 N (aka 10 M) HCl stock to 80 mL. (a 1:6 dilution). pH should be around 1.

- Enzymatic Reagents:
    - *Note: Store at 4°C. Should last about a month – test to make sure. Can be re-used.*
    - Pepsin solution (0.15%): Dilute concentrated HCl to a 0.01 N solution with distilled water until reaching a volume of 10 mL. Should be pH 1.5-2.0. Add 15 mg pepsin, stir well.
        - *Pepsin solution can be neutralized by raising to pH 9.5; it can now be disposed down the sink, as the pepsin has been permanently deactivated and the acidity has been neutralized.*
    - Trypsin stock solution (0.05% in distilled water): Dissolve $CaCl_2$ in 10 mL distilled water and adjust the pH to 7.8 with NaOH. Add 5 mg trypsin (Sigma Type II), mix to dissolve, store at -20ºC.
    - Pronase solution (0.05% in distilled water): Dissolve $CaCl_2$ in 10 mL distilled water and adjust the pH to 7.8 with NaOH. Add 5 mg pronase (Sigma Type II), mix to dissolve, store at -20ºC.
    - Protease solution (0.1% in distilled water): Protease 10 mg, Distilled water 10 mL. Mix to dissolve, Adjust to pH 7.8 using 1N NaOH, store at -20ºC. Note: Stock solution can be made by reconstituting powder in dH2O. Stock solutions are usually 20 mg/mL (2%), aliquoted and frozen at -20 °C.
        - Note: Tried this concentration on sections from brains that have been in formalin since 2006 (testing in 2019 for Vieira Lab Delta Fos B project, sections from brains A1, B1, & C1). The protease worked *too* well; sections were too digested. Recommend decreasing concentration to 0.025% - 1/4 of the original working solution concentration. The protease we used was Qiagen's #19155, containing 7.5 Anson units in 125 mg in the original product vial.
    - Proteinase K solution: (20 ug/ml or 0.6 units/ml): Add 0.4 mg to 20 mL Tris-EDTA Buffer @ pH8.0. Mix well, store for up to 6 month at 4 ºC..

Avidin Blocking Solution (0.2% biotin)

- Add 60 mg biotin to 30 mL PBS.
- Add 600 uL of the 10% Triton X-100 stock solution (dilutes to 0.2%).
- Store at 4 degrees C.
- Notes: Saturates any tissue-bound avidin/streptavidin, such as that introduced by the "Biotin Blocking Solution" below, with excess biotin. This prevents attraction of biotinylated antibodies away from their targets. Dry milk could be another alternative to this biotin step since it has much biotin, but it also may have some undesired bovine immunoglobulins that can interfere with staining. Biotin is inexpensive as of this writing.

Biotin Blocking Solution

- Mix 1 raw egg white (no yolk) in distilled water to a final volume of 100 mL.
- Strain out particulate matter.
- Add 2 mL of the 10% stock Triton X-100 (dilutes to 0.2%).
- Store at 4 degrees C. Dispose of after 1 week if no azide is added.

- Notes: Used to cover any free biotin either in the tissue (not much found in the brain) or from already-bound antibodies. Raw (uncooked) egg white has much avidin, and is much cheaper than isolated avidin or streptavidin. Use distilled water in wash steps prior to and after using egg white, as buffer salts cause it to precipitate out.

Blocking buffer

- See Antibody Dlient Buffer above.

Borate Buffers

- Type A: 0.1 M sodium tetraborate decahydrate (to mix with PFA): Add 38.14 g sodium tetraborate decahydrate (MW: 381.37) and 2 g NaOH into 1 L of distilled water. pH should be around 9.5. Store at room temp.
- Type B: 0.2 M boric acid (for Clarity clearing): Add 1.236 g boric acid (MW = 61.83) to 100 mL distilled water. Adjust pH to 8.5 using drops of liquid 1 M NaOH.

BUFFERS

- See separate entries for phosphate buffer, phosphate buffer saline, borate buffer (above), citrate buffer (below), and others.

Cholinesterase staining solution

(least hazardous version)

- Stock solution recipes
  - 0.1 M citrate buffer, 50 mL; pH ~5 based on ingredient ratios
    - 5 mL of 1 M citric acid (check cabinet, orange rack)
    - 8.3 mL of 1 M NaOH (check cabinet, orange rack)
    - Fill to 50 mL with distilled water.
  - 1 M Copper sulfate stock
    - Check if already made - may be in cabinet
    - MW of copper sulfate pentahydrate is 249.69,
    - 1 M = 249.7 g/L = ~250 mg/mL = 2.5 g in 10 mL
  - FIX, SHOULD BE 0.5 M —--- 1 M potassium ferricyanide stock [ALREADY MADE - FIND 15 mL TUBE WITH ORANGE SOLUTION IN CABINET]
    - Check if already made - may be in cabinet
    - MW is 329.24
    - 1 M = 329.2 g/L = 329.2 mg/mL = 3.9 g per 10 mL
  - 1 M ATChCl (acetylthiocholine chloride) stock
    - Ignores the ATChI (acetylthiocholine iodide) stock currently in the freezer.
    - Should already be made. Check freezer for 50 mL tube labeled ATChCl, and use one aliquot from that tube.
    - MW of ATChCl is 197.73
    - 1 M stock is 197.7 g/L = 197.7 mg/mL (or roughly 1 g in 5 mL)
- Reaction solution (based on recipe of Karnovsky and Roots, 1964), 10 mL:

        - ~9.66 mL of 0.1 M citrate buffer, pH 5.0,
        - 25 uL of 1 M CuSO4 (2.5 mM final conc),
        - 100 uL of 0.5 M potassium ferricyanide (5 mM final conc), and
        - 10 uL of 1 M acetylthiocholine chloride (1 mM final conc).
            - Re-freeze any remaining ATChCl in the aliquot; return to storage tube in freezer.
- Notes:
    - Reaction times vary based on freshness of tissue, and tissue type. For formaldehyde-fixed brain tissue, reaction rate is slow. Will stain sections quite darkly if left overnight, or at a minimum 8 hours.
        - However, can short-cut: The stain that coats the tissue is reactable with other stains. So, DAB or similar oxidation-sensitive compounds can add another, quicker staining "layer" onto tissue that is lightly stained with the cholinesterase working solution.
        - Incubate tissue in cholinesterase working solution for 30 min, then rinse tissue, then incubate in typical DAB staining solution (see elsewhere in this section) for another 10-20 min.
        - Important!: If using DAB staining to expedite/amplify cholinesterase stain, the tissue should have gone through endogenous peroxidase blocking steps before everything else! So, prior to applying cholinesterase stain solution, incubate tissue in distilled water with 0.05% sodium azide for 10 min, then in 1% H2O2 (in distilled water) for 10 min, and finally rinse off in distilled water multiple times in 5 min intervals.
    - Creates insoluble residue. Perform staining in dish/container that is dedicated just for this type of staining.
    - Solution is toxic due to use of heavy metals. Dispose of as hazardous waste.
    - Incompatible with most other stains due to how dark it becomes.

Citrate buffer

0.1 M, 50 mL, pH ~5

- 5 mL of 1 M citric acid
- 8.3 mL of 1 M NaOH
- Fill to 50 mL with distilled water.

Citrate-phosphate buffer

(0.16 M and 0.093 M respectively), pH 4, 24 mL

- 3.78 mL of 1 M citric acid
- 4.5 mL of 0.5 M Na2HPO4 (dibasic)
- 15.72 mL dH2O

Clarity Technique solutions:

- Hydrogel solutions (for Clarity method)
    - *Hydrogel for passive infiltration on whole brains:* Add 4g acrylamide and 0.25g VA-044 to 100 mL of 0.1 M PB. Store at 4°C for no longer than one month.

    - *Hydrogel for perfusions:* Add 40 g acrylamide and 2.5g VA-044 to 500 mL of ice-cold 0.2 M PB. Add 500 mL of 8% PFA. Keep solution cold to prevent premature hydrogel polymerization. Use same day as mixed.
- Clearing solutions:
    - Using 8% SDS: Add 8 g sodium dodecyl sulfate to 100 mL of 0.2 M borate buffer.
      OR
    - Using 4% SDS: Add 4 g sodium dodecyl sulfate to 100 mL of 0.2 M borate buffer.

- Refraction index matching solutions (for imaging Clarity specimens):
    - sRIMS solution (Yang et al., 2014 Cell): 70% sorbitol (w/v) (Sigma S1876) in 0.02 M phosphate buffer with 0.01% sodium azide, pH to 7.5 with NaOH. Clears depth to 1 mm in PACT-modified tissue.
    - RIMS solution (Yang et al., 2014 Cell): "40 g of Histodenz (Sigma D2158) in 0.02M phosphate buffer with 0.01% sodium azide for a total volume of 30 ml, pH to 7.5 with NaOH, which results in a final concentration of 88% Histodenz (w/v) with RI = 1.46 (used throughout this work unless otherwise noted). Estimated cost to produce is $3/ml while FocusClear is $36/ml. We note that the refractive index (RI) of RIMS may be adjusted to match the specific tissue/imaging system: it is expected that the RIMS RI may range from 1.38 (30% Histodenz w/v) to 1.48 (95% Histodenz w/v)."

Copper Sulfate solution

- Recipe
    - Add 0.772 g ammonium acetate to 200 mL distilled water (makes 50 mM).
    - pH to 5.0 with HCl.
    - Add 0.5 g $CuSO_4$.
    - pH to 5.0 with NaOH.

- Notes:
    - Store at room temperature.
    - Can be re-used, but be aware that residues of buffers on the tissue may affect this solution.
    - Used for autofluorescence reduction;
    - Lasts for years.
    - TOXIC; Dispose as hazardous waste.

Coverslipping reagents (a.k.a, mounting media)

- Aqueous (non-curing, must be sealed with wax or nail polish)
    - *Buffered Glycerol Type 1*
        - Note: Keeps at room temperature for years when 0.05% azide is added.
        - Make 0.4 M potassium bicarbonate by adding 4.005 g $KHCO_3$ to 100 mL distilled water.
        - Raise pH to 8.6 using potassium hydroxide (KOH).
        - Add 100 mL glycerol.
    - *Buffered Glycerol Type 2*
        - Mix 10 mL of 0.1 M NaPB (pH 7.4) with 90 mL glycerol.
        - Add anti-fade agent if necessary: either 100 mg of p-Phenylenediamine hydrochloride or 500 mg of n-propyl gallate.

- Curing (starts aqueous, solidifies)

- *Polyvinyl Alcohol (PVA) mountant*
    - Original 200 mL recipe. From Lovell lab?
    1. In a 200+ mL bottle, weigh 48g of glycerol.
    2. Add 19.2g of PolyVinylAlcohol (PVA), 87-90% hydrolyzed, 30k-70k MW, Sigma product #P8136.
        1. Other forms that are higher molecular weight will NOT dissolve! Unsure about the effects of hydrolysis level.
    3. Mix well by rolling or tilting bottle upside down until PVA is coated with glycerol.
    4. Add 48mL of distilled water.
    5. Mix for a few hours using a rotator/shaker/rocker at room temperature. Tube must turn completely upside down for proper mixing. Use slow setting.
    6. Add 88ml of 0.2M Tris-HCL at pH 8.0-8.5. Mix well by inverting tube. Tris-HCl buffer recipe below.
        1. Obtain 100 ml distilled water.
            1. But, check to see if you have sodium hydroxide pellets, or sodium hydroxide solution. If using sodium hydroxide solution to raise the pH, only start with 80 mL dH2O (rather than 100 mL) to make sure you don't go over-volume. That said, added water should not change the pH substantially.
        2. Add 3.2 g Trizma HCL
        3. Add 3-4 pellets Sodium Hydroxide.
        4. pH should be between 8.0-8.5, raise with more NaOH pellets if necessary.
        5. Store in fridge for up to 2 months.
    7. Warm to 50°C in shaking incubator (approximately 20- 30 minutes). Mix by inverting tubes every 5 minutes. Solution should turn from opaque to translucent or clear.
        1. If lacking heated incubator, warm in microwave until just before bubbling. Heat in microwave-safe container!
    8. Let stand for two hours to allow air bubbles to float to the top and disperse.
        1. Original protocol suggested dividing into falcon tubes and centrifuging at 5000g for 15 minutes to remove microscopic air bubbles. If left alone, those air bubbles expand as the medium dries on a slide - no bueno. Centrifugation can be done instead of letting the solution stand in the fridge if you cannot wait and you have access to a centrifuge.
    9. Remove any undisolved clumps.
    10. Divide into 50 mL Falcon tubes.
    11. Put into freezer for long term storage, store in fridge when actively using. Avoid repeated freeze-thaws.
        1. If it is somewhat opaque after thawing, heat or microwave briefly to resolve this. Avoid bubbling, and use a microwave-safe container!
    - Notes: The antifade agent n-propyl gallate, **NPG, seems to dim both background and specific staining**, so we probably won't use this unless necessary. NPG does not dissolve as 50% w/v in DMSO, but does dissolve at 25%.

- *PVP mountant:*
    - Add 25 g Polyvinylpyrrolidone (M.W. 10,000) to 25 mL 0.1 M NaPB (pH 7.4).
    - Dissolve the PVP by warming and stirring for several hours.
    - Add 1 mL glycerol
    - Add anti-fade agent if desired
    - Notes:
        - Developed by John Kiernan (Kiernan, 1990).
        - Keeps for 2 to 3 years at room temperature.
        - Add azide to 0.05% to ensure complete prevention of microbial growth.
        - Discard if it becomes too viscous.
        - Not prone to bubbles.

- The refractive index is 1.46 (Pearse, 1968), but increases as the water evaporates at the edges of the coverslip until unstained structures are barely visible.
- I've observed ***increased*** green fluorescent background compared to ProLong Gold (a proprietary mountant that is likely PVA + NPG), so making this may not be worth the trouble!

Cresyl violet solution

- Recipe (0.2%, working solution):
  - Use 50 mL conical tube. Avoid using beaker, as this stuff stains containers. (Stain can come out with ethanol, though).
  - Add 0.1 g (100 mg) cresyl violet acetate powder.
  - Add 47.5 mL dH2O
  - Add 2.5 mL distilled white vinegar (assuming it is 5% acetic acid).
  - Leave rocking to dissolve overnight.
  - Use within 1 week. Store in fridge to prevent mold growth.
- Recipe (2%, concentrated stock solution):
  - Use 50 mL conical tube. Avoid using beaker, as this stuff stains containers. (Stain can come out with ethanol, though).
  - Add 1 g cresyl violet acetate powder.
  - Add 47.5 mL dH2O
  - Add 2.5 mL distilled white vinegar (assuming it is 5% acetic acid).
  - Leave rocking to dissolve overnight.
  - Store in freezer. Thaw partially to withdraw desired amount for making working Nissl solution.
- Notes:
  - Used for Nissl staining.
  - Suspected carcinogen, but unclear.
  - Binds to RNA, but can be unbound by alcohols, detergents, and acidic solutions.
  - Use alcohol to get rid of stain residues.

CUBIC Technique Reagents:

- Reagent 1 – Clearing solution (makes roughly 100 mL):

1. Grab 100 mL beaker, add small stir bar, zero out scale.
2. Add the following: 25g urea, 35g double distilled water
3. Cover beaker top with parafilm to prevent evaporation.
4. Stir to dissolve.
5. Next, add 25g N,N,N',N'-tetrakis(2-hydroxypropyl) ethylenediamine (a.k.a. Quadrol), 15g polyethylene glycol mono-p-isooctylphenyl ether (a.k.a. Triton X-100).
6. Cover beaker top with parafilm to prevent evaporation.
7. Stir without heat for a few hours until dissolved.
8. Store in fridge. Shelf life uncertain. May precipitate, but can be heated to 37°C and stirred to re-dissolve. Use incu-shaker for this rather than a hotplate.

- Reagent 2 – Imaging / Refractive Index Matching Solution (makes roughly 20 mL):

1. Grab 40-50 mL beaker, add small stir bar, zero out scale.
2. Add the following: 10 g sucrose, 5 g urea, 2 g of 2,2′,2″-nitrilotriethanol (a.k.a. Triethanolamine; liquid, not HCl version), 3g double distilled water.
3. Cover beaker top with parafilm to prevent evaporation.
4. Stir with low heat for a few hours until dissolved.
5. Let solution cool to RT.

6. Add 20 uL polyethylene glycol mono-p-isooctylphenyl ether (a.k.a. Triton X-100).
7. Stir more without heat.
8. Store at room temp; seems to separate out when stored in fridge. Shelf life uncertain. Consider adding azide if possible.

DAB (3,3'-diaminobenzidine)

- General notes:
    - Carcinogen. Dispose of as hazardous waste.
    - Powder should be off-white.
    - Can store powder at -20 degrees C.
    - Reaction product is brown and insoluble, except when bleach is applied.
    - Considering the brown precipitate can deposit on staining tools and basins, plan to have a dedicated staining receptacle for DAB reactions exclusively.
    - If a heavy metal is added to intensify the stain, that too is hazardous (toxic). Dispose of as hazardous waste when finished using.
    - Similar to how tyramide signal amplification can be done in multiple rounds and multiple colors, DAB staining can be done this way, too.
        - After DAB staining is complete, expose sections to PBS + 0.05% sodium azide for 10 minutes, rinse in PBS for 5 minutes, expose sections to 1% H2O2 for 10 minutes, rinse 3x (5 min each) in PBS, then apply your next antibody that has HRP attached. After the incubation with this second HRP antibody is done, you can do the DAB reaction as stated above - but be sure to use either a different metal to color it, or omit the metals to obtain the normal brown color.
    - Background staining in DAB reactions is kind of inevitable, but a lot of the staining will actually appear fainter under higher magnification on a microscope than it looks when just observing the sections by eye. That said, try to avoid having the sections sit in reaction solution for too long.
- Stock aliquots (10%):
    - Add 100 mg DAB to 1 mL distilled water. Separate into 100 uL aliquots and store in freezer.
    - Notes:
        - Discard if solution turns fully turbid brown (should be mostly clear).
        - IHC World page notes that 15 - 25 uL of 10 N HCl should be added to the DAB stock solution to turn it light brown. They're still working with DAB * 4HCl, so I don't know why this is done if the product is fine dissolving in regular distilled water. https://www.ihcworld.com/_protocols/chromogen_substrates/POD_DAB_brown.htm
        - There is a freebase variant (lacking tetrahydrochloride) that is dark brown. I'm not sure that the freebase form can be reacted (buyer beware!), but again according to IHC World, 15 - 25 uL of 10N HCl can be added to every 1 mL of stock solution to allow it to dissolve and turn it "a light brown color".
- Dilution for working solution (0.05%):
    - Note: Make right before you will perform the DAB reaction.
    - Add one stock 100 uL, 10% aliquot of DAB to 20 mL of working solution 1x PBS.

        - The pH value should be between 7.2 to 7.4. According to IHC World, pH < 7.0 will reduce staining intensity. pH > 7.6 will cause background staining.
    - Add 100 uL of 3% H_2O_2 (hydrogen peroxide; from store-bought brown bottle) to your working solution; makes a 0.015% concentration. This is to catalyze the color-changing reaction.
    - Mix well.
- Nickel intensification: For a **gray** reaction product, use **nickel chloride** with DAB.
    - Stock vial (1%) Add 10 mg NiCl to 1 mL distilled water. Add this whole stock vial to your 20 mL working DAB solution.
- Other metal intensification options are possible, but not all are great.
    - Copper sulfate isn't recommended because of potential increased background despite still intensifying the stain (Hsu & Soban, 1982).
    - Cobalt chloride is a good alternative for a dark blue product. IHC World page recommends final conc of 0.05%; https://www.ihcworld.com/_protocols/chromogen_substrates/POD_DAB_blue.htm .
    - Nickel ammonium suflate might be really good, as it's a black reaction product as opposed to nickel chloride's gray-ish blue. Working solution conc recommended at 0.05%; https://www.ihcworld.com/_protocols/chromogen_substrates/POD_DAB_black.htm
    - Most other metals don't work. Significant testing done by others, though the authors (Hsu & Soban, 1982) had a final concentration of 0.02% rather than 0.05% as I and IHC World do above.

DAPI (4′,6-diamidino-2-phenylindole)

- Notes:
    - DAPI stains all cell nuclei blue. It is excited by UV/violet light. Thus, it shouldn't be used with any blue fluorescent stains.
    - DAPI dissolves fine in distilled water but will precipitate in phosphate buffer. Only dilute it in distilled water, and rinse all sections briefly with distilled water beforehand.
    - DAPI is carcinogenic since it binds to DNA. Avoid skin exposure.
    - Discard as hazardous chemical waste.
    - Working solution does not keep long (>1 week) even if kept in fridge.
- Stock aliquots:
    - Sometimes DAPI is sold in small amounts, like 10 mg. This is hard to weigh out, and thus you should dissolve it in its original vial if possible
    - When using a 10 mg vial, dilute with 1 mL distilled water to make a 10 mg/mL solution (a 1% solution). Shake until dissolved.
    - Separate this 1 mL amount into the desired number of stock aliquots. You can make 10x 100 uL aliquots.
    - These should be stored in the freezer. Stick with one aliquot and use until depleted. Chemical appears resistant to freeze-thawing, so you can re-use one aliquot a moderate amount.
- Performing the stain:
    - This stain can be done before OR after IHC. I prefer afterward to avoid contaminating later solutions with trace amounts of DAPI.

- Make a working solution of DAPI - a 1:10,000 dilution from aliquot to distilled water should make a sufficiently bright stain, but solution could be diluted to 1:20,000 or even more dilute if the stain comes out too bright.
    - Using the solution from stock may involve you trying to pipette out sub-microliter amounts. Instead, you might want to dilute one aliquot 1:100, and then take out a small amount from that and dilute it again to the desired amount.
- If you have sections sitting in PBS, rinse them in distilled water 2x 5 min each prior to DAPI staining.
- Incubate in DAPI working solution for 20 min room temperature and shaking/rocking. You can cover the wellplate with an opaque lid to protect from light, but this may be unnecessary.
- After staining, rinse 5 min with distilled water, then 2x 5 min each in PBS. Discard the first rinse leftovers as hazardous chemical waste. The working solution can be retained in a vial, or discarded as hazardous chemical waste.
- Sections are now ready to mount or can be stored until ready to mount.

Paraformaldehyde (PFA)

(Technically it's formaldehyde once dissolved into solution.)

- Stock Solution (8% in distilled water, pH ~7.4)
    - Add 40 g Paraformaldehyde to 450 ml distilled water.
    - Add 1 ul of 10 N sodium hydroxide (NaOH) per ml of water; in other words, 500 ul for 500 ml.
    - Apply medium heat while stirring at medium speed to dissolve over 15-20 min in a fume hood. Solution should not go above 65º C.
    - Eventually, granules will fully dissolve and the solution will become translucent. Stop stirring at this point! If stirring continues, a cloudy precipitate will form that will be filtered out and reduces the final concentration of PFA in solution.
    - Once the granules have dissolved and the solution clears, turn off the heat and equilibrate to pH 7.4 with approx 1.5 ml of 20% HCl.
    - Bring volume to 500 ml with distilled water.
    - Filter while still warm with 0.45 um (or 0.2 um) filter. Coffee filter may suffice.
    - Store at 4º C. This 8% stock solution in water is good for 30-60 days at 4º C. Alternatively, stock may be aliquoted and frozen for very long term storage.

- Working solution (4% PFA):
    - Mix 8% PFA stock with 2x concentrated buffer of choice (borate buffer, phosphate buffer) at desired pH. For a less strong fixative solution of about 2% PFA, mix 250 mL 8% PFA stock, 250 mL distilled water, and 500 mL buffer. The buffer added to PFA should result in a final osmolarity range of 400-600 mOsm (PFA is excluded).
    - Warm to body temperature before use.
    - Note: PFA diluted in buffer is only good for 1 week at 4º C. Thus, PFA stock and buffer should be mixed immediately before use.

- My notes for devising a super concentrated 40% PFA stock solution recipe:

- May require additional NaOH compared to 8% stock.
- Prior attempts created an almost gel-like substance. Haven't attempted again since 2016.

Phosphate Buffer

(~0.2 M PB, pH 7.4)

- Notes:
  - Not to be confused with phosphate buffered SALINE
  - The following recipes are based on their anhydrous forms. You MUST adjust the weight for any forms with "hydrate" in their name.
  - Is there a difference between sodium and potassium versions? Not much for most applications. Just be mindful of the molecular weight differences.

- Recipe if using sodium phosphate powders (572 mOsm):
  - Add the following to 1 L of distilled water (doesn't need to be double distilled if using with PFA)
  - 5.52 g monobasic sodium phosphate (NaH_2PO_4; MW = 119.98, anhydrous, 46 mmol, 92 mOsm/L)
  - 22.72 g dibasic sodium phosphate (Na_2HPO_4; MW = 141.96, anhydrous, 160 mmol, 480 mOsm/L)
  - Once dissolved, pH to 7.4 (should be close)
  - Store at room temp.
  - Dilute 1:1 with distilled water for 0.1 M NaPB (286 mOsm)

- Recipe if using sodium phosphate stock solutions (555 mOsm):
  - Create a 0.5 M Na2HPO4 (anhydrous, dibasic, MW 141.96) stock by dissolving 3.55g in 50 mL distilled water.
    - (Calculation: 141.96 * .5 * .05)
    - 1500 mOsm
  - Create a 1 M NaH2PO4 (anhydrous, monobasic, MW 119.98) stock by dissolving 6g in a separate 50 mL distilled water.
    - (Calculation: 119.98 * .5 * .05)
    - 2000 mOsm
  - In a separate beaker, mix 15.5 mL dibasic PB, 2.3 mL monobasic PB, and fill to the 50 mL line with distilled water to get a 0.2 M buffer at around pH 7.4 (determined with various buffer tables online, but check with pH meter to be sure). 465 mOsm from dibasic, 90 mOsm from monobasic.
  - Store at room temp.
  - Dilute 1:1 with distilled water for 0.1 M NaPB.

Phosphate Buffered Saline

10x Stock using powders, 1 liter (0.1 M PBS, pH 7.4, 3390.5 mOsm/L):

- Add the following to ~900 mL distilled water:
  - 10.9 g Na2HPO4 (anhydrous, dibasic, MW 141.96, 76.8 mmol, 230.4 mOsm/L)
    - If not anhydrous, check its formulation.
  - 3.2 g NaH2PO4 (anhydrous, monobasic, MW 119.98, 26.7 mmol, 80.1 mOsm/L)
    - If not anhydrous, check its formulation. If dihydrate, MW = 156.01. 26.7 mol would instead be 4.17 g

- Mix to dissolve. Adjust pH to 7.4 with more of either Na2HPO4 or NaH2PO4 (don't use NaOH or HCl), fill to 1 L with distilled water.
- Add 90 g NaCl (MW 58.44, 1540 mmol, 3080 mOsm/L)
    - Added afterwards as some pH meters are thrown off by addition of this ingredient.
- 1x working solution is ~339 mOsm/L.

10x stock if using stock solutions, 500 mL:

- Create a 0.5 M Na2HPO4 (anhydrous, dibasic, MW 141.96) stock by dissolving 7.1g in 100 mL distilled water.
    - (Calculation: 141.96 * .5 * .1)
- Create a 1 M NaH2PO4 (anhydrous, monobasic, MW 119.98) stock by dissolving 6g in a separate 50 mL distilled water.
    - (Calculation: 119.98 * .5 * .05)
- Create a 4 M NaCl (MW 58.44) stock by dissolving 46.8g in a separate 200 mL distilled water.
    - (Calculation: 58.44 * 4 * .05)
- In a separate beaker, mix 77 mL dibasic PB, 11 mL monobasic PB, and fill to ~350 mL total volume with distilled water.
- The pH should be around 7.4 according to online calculators, but check to be sure.
    - Use a pH meter to check. If it's outside of the range ph 7.3-7.5, let me know and I can adjust it later.
    - FYI: Never adjust buffer solutions with straight up concentrated acids (hydrochloric) or bases (sodium hydroxide). Not because something bad will happen, but because buffers resist pH changes - *hence their name*. Instead, adding more of either the acidic component or the basic component of the buffer is warranted.
    - pH is checked before addition of NaCl, as concentrated NaCl seems to throw off pH readings on lower quality meters.
- Add 154 mL NaCl stock.
- (Calculation for NaCl: Final concentration is supposed to be 9%. A 4 M stock is a 23.4% solution. 9 / 23.4 = 0.384, so the NaCl stock must compose 30.8% of the mixture's final volume. 50 mL * .384 = 19.2 mL NaCl stock)
- 23 mM monobasic, 77 mM dibasic, and 1536 mM NaCl
- 46 mOsm from monobasic, 231 mOsm from dibasic, and 3072 mOsm from NaCl = 3349 mOsm
- The resulting 1x PBS ("working") solution will be 335 mOsm. This doesn't need to be measured in our lab; it's assumed based on the amount of solutes dissolved.

- Recipe if using Potassium phosphate powders (to make potassium phosphate buffered saline - KPBS - stock solution @ 0.12 M, 6x working solution, 2329.6 mOsm)
    - Add the following into 980 ml distilled water
    - 2.96 g KH_2PO_4 (21.7 mmol; 86.8 mOsm)
    - 17.2 g K_2HPO_4 (98.7 mmol; 394.8 mOsm)
    - 54 g NaCl (924 mmol; 1848 mOsm)
        - Shouldn't this be potassium chloride if we were doing a sodium free mixture? Doesn't really matter for IHC on fixed tissue, but it just seems inconsistent.
    - pH should be around 7.4. Balance if necessary.
    - Store at room temp.

Saline Flush Solution for perfusions

Volume depends on number of rats to be perfused. If clamping the descending vein, one may use ~150 mL per rat. Unused solution should be stored in fridge. Heparin is an anticoagulant; it should not be

frozen. Nitrite and procaine are vasodilators. Use 0.06 – 1% benzyl alcohol as a preservative if necessary.
In 500 mL distilled water, add:

- 4.5 g NaCl; makes 0.9%
- 500 units heparin; remember to divide by number of units per mg
- 5 g sodium nitrite OR 2.5 g procaine HCl
- pH balance to 7.4
- Warm to body temperature (37°C) during use.

Sodium Azide

(SEE COMMENTS IN PART 2 SECTION II OF THIS DOCUMENT BEFORE USE)

- Stock aliquots (10%):
  - Dissolve 1 g sodium azide into 10 mL. Can keep at room temp.
  - Use extreme caution when handling this powder. It is very toxic. Read the MSDS, then use full protective equipment and a fume hood. Use caution for cleaning up any powder spills. Avoid contact with copper and lead – can create explosive compounds.

- Working dilutions:
  - Dilute to 0.05% concentration in your antibody solutions to use as a preservative.
  - Effective in blocking endogenous peroxidase activity at this concentration. DO NOT MIX WITH HRP REAGENTS UNLESS YOU WANT TO STOP THEIR FUNCTION!

Sucrose solution

(30%):

- Recipe: Add 3 g sucrose to 10 mL 1x PBS.
  - (not distilled water; salt is needed to pull more water out of the tissue and keep ice crystals small)
- Notes:
  - Sucrose MW = 343.3. Concentration is 3 g / 10 mL = 300 g/L. Molarity is 300 / 343.3 = 0.874 M.
  - Osmolaties are 874 mOsm from sucrose and 335 mOsm from 1x PBS (see earlier recipe calculations), so total osmolarity is 1209 mOsm.

Triton stock solution

(10% in dH2O)

- Recipe:
  - Obtain a beaker that can hold 100 mL or greater.
  - Put the stirbar in.
  - Put the beaker on top of the magnetic stir plate.
  - Add 45 mL distilled water
  - Turn stirplate on to low spin. No heat!
  - Slowly add 5 mL of 100% Triton X-100 (black cabinet, orange rack, in 50 mL tube)
  - Turn up stir speed to a point where the solution is mixing but it is not going fast enough to foam or splash.
  - Wait for a while, as it will take many minutes to dissolve.

Appendix G: Miscellaneous, unsorted new info [AXG]

Confocal notes:
All modern (post 2008) confocal microscopes have a single pinhole for all detectors. However, when scanned on a line-basis (Zeiss "MultiTracking") this results in slightly different confocal slice thicknesses between excitation wavelengths. When the pinhole is set to approximately 1 Airy, confocal slice thickness is a function of pinhole diameter and Emission wavelength. The mismatch in confocal slice thickness, as a result of a "source" single pinhole is particularly apparent when imaging a sample at widely differing Em wavelengths. e.g., DAPI vs Cy3. Slice thickness mismatch can be avoided if the pinhole diameter is normalized for each Ex wavelength during Z-scanning. (Source: http://microscopy.berkeley.edu/courses/tlm/clsm/index.html)
Use this equation to correct for pinhole size between channels:

$$FWHM_{det,axial} = \sqrt{\left(\frac{0.88 \cdot \lambda_{em}}{n-\sqrt{n^2-NA^2}}\right)^2 + \left(\frac{\sqrt{2} \cdot n \cdot PH}{NA}\right)^2} \quad (4)$$

λ_{em} = *emission wavelength*
PH = *object-side pinhole diameter [μm]*
n = *refractive index of immersion liquid*
NA = *numerical aperture of the objective*

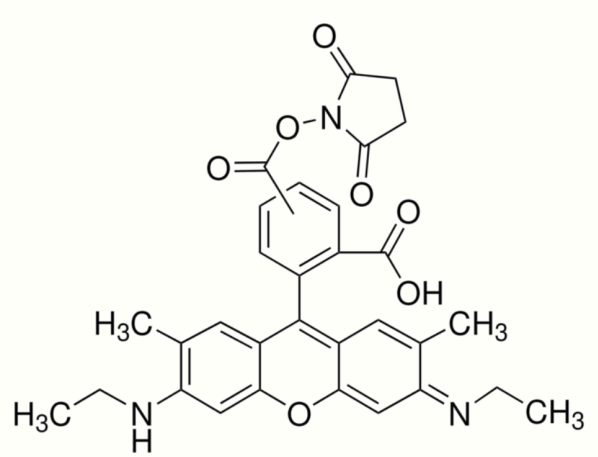
In case the above image is broken:
FWHM(det,axial) = sqrt[[(0.88*Emitmax)/(n-sqrt(n^2-NA^2))]^2 + [(sqrt(2)*n*PH)/NA]^2]*4

Appendix H: Product info

[Be advised that this section will contain info that can easily go out of date, or the prices change enough that a new search for a different vendor and lesser price is warranted.]

Dye esters for making tyramide conjugates:

- Rhodamine
  - Main name is 5(6)-Carboxytetramethylrhodamine N-succinimidyl ester.
  - Alternate names:
    - 2-(3,6-bis(dimethylamino)xanthylium-9-yl)benzoate--2,5-dioxopyrrolidin-1-yl acetate



- (Chem diagram from millipore-sigma site, specific product discontinued: https://www.sigmaaldrich.com/US/en/product/sial/36422)
- Variants may add spacers, but some may be functionally different.
    - Tetramethylrhodamine
    - 5-carboxytetramethylrhodamine (sometimes called 5-TAMRA)
    - Rhodamine 6G
    - Rhodamine X
    - Comparison of the fluorescent properties of these three: https://pdf.benchchem.com/15599/A_Head_to_Head_Comparison_5_TAMRA_Versus_Other_Rhodamine_Derivatives_for_Peptide_Labeling.pdf
    - See dye x filter matching notes document for more details of what will work with my microscope.
- Vendors
    - TAMRA
        - 5 mg for $169 (6 isomer was way more pricey but otherwise identical in specs): https://www.lumiprobe.com/p/tamra-nhs-ester-5
        - 25 mg for $198 (called NHS-rhodamine, but is actually TAMRA): https://www.thermofisher.com/order/catalog/product/46406
        - 25 mg for $123: https://broadpharm.com/product/bp-25604,
        - 25 mg for $125: https://vectorlabs.com/products/56-tamra-nhs-ester/
        - AA Blocks Inc is wildly inexpensive: https://www.aablocks.com/prod/246256-50-8
            - Same product distributed by via milliporesigma https://www.sigmaaldrich.com/US/en/product/aablocksinc/aabh9a953fc3
    - Rhodamine 6G
        - ThermoFisher product is wildly overpriced. https://www.thermofisher.com/order/catalog/product/C6127
        - Single isomer, somewhat pricey (mixed isomer version discontinued): https://www.aatbio.com/products/6-cr6g-se-6-carboxyrhodamine-6g-succinimidyl-ester-single-isomer
        - Other isomer from same company, also pricey: https://www.aatbio.com/products/5-cr6g-se-5-carboxyrhodamine-6g-succinimidyl-ester-single-isomer

    - Even Lumiprobe is kinda pricey: https://www.lumiprobe.com/p/r6g-nhs-ester-5
- ROX (a.k.a. 5(6)-ROX N-succinimidyl ester)
    - (be advised - not ideal for my current filter set as of 2026)
    - https://www.targetmol.com/compound/5%286%29-ROX%20N-succinimidyl%20ester 1 mg $68, 5 mg $190, 10 mg $320
    - MedChemExpress is overpriced.
    - SCBT pricing ain't bad. https://www.scbt.com/p/5-6-carboxy-x-rhodamine-n-succinimidyl-ester-114616-32-9