Laboratory for Integrative Neuroscience Immunofluorescence Protocol Version 2

Margaret I. Davis

Abstract

This is the general immunofluorescence protocol used in the Laboratory for Integrative Neuroscience at NIAAA. This protocol can be adapted for most primary antibodies but specific antibodies and concentrations need to be determined by the user and the protocol adapted accordingly. Secondary antibodies listed are optimal for the Axiozoom and LSM880 filters and excitation sources and may need to be changed to match the microscope available. I also provide tips for optimizing staining, reducing background, and characterizing antibodies.

Citation: Margaret I. Davis Laboratory for Integrative Neuroscience Immunofluorescence Protocol. **protocols.io** dx.doi.org/10.17504/protocols.io.kz3cx8n
Published: 07 Dec 2017

Before start

- If unfamiliar with fluorescence microscopy, see tutorial: https://micro.magnet.fsu.edu/primer/techniques/fluorescence/fluorhome.html
- Before starting, validate and titrate all antibodies. Select concentrations that result in a strong signal that does not saturate and does not result in non-specific staining. The titration should result in a signal that is dependent on the concentration of the primary antibody such that a change in concentration result in a change in exposure time when imaging (i.e. 1:1000 is a 1 second exposure, while 1:2000 requires 2 seconds of exposure. Details on titration are provided in the protocol.
- If possible, test in a knock out.
- Our preferred sources for primary antibodies include Millipore, Frontier Biosciences (Japan), Immunostar, Abcam, Encor, Alamone, and Cayman.
- Order the cross-adsorbed, purified versions of the secondary antibodies and carefully titrate using tissue with no primary antibody. We generally use them between 1:1000 and 1:3000. Test secondaries in combination for cross reactivity in multi-labels. Mouse and rat will cross react.

Materials

Triton X-100 T8787-50ML by Sigma Aldrich

cell culture plate 24 well View by Sigma-aldrich

10x Phosphate Buffered Saline 70011044 by Gibco, ThermoFisher

Sudan black B 199664 by Sigma Aldrich

sodium borohydride 452882 by Sigma Aldrich

Bovine Serum Albumin A2153 by Sigma

Fluoromount F4680 by Sigma

4,6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) D1306 by Thermo Fisher Scientific

TO-PRO™-3 Iodide T3605 by Thermo Fisher Scientific

Goat anti-Chicken IgY (H L) Secondary Antibody, Alexa Fluor 488 A11039 by Thermo Fisher Scientific

Goat anti-Rabbit IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 A11011 by Thermo Fisher Scientific

Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 A11057 by Thermo Fisher Scientific

Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 A21447 by Thermo Fisher Scientific

Donkey anti-Goat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 A11055 by Thermo Fisher Scientific

Donkey anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 350 A10035 by Thermo Fisher Scientific

Donkey anti-Rabbit IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 A31573 by Thermo Fisher Scientific

Goat anti-Mouse IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 A32723 by Thermo Fisher Scientific

Donkey anti-Rat IgG (H L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 A21208 by Thermo Fisher Scientific

Goat anti-Rat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 A11006 by Thermo Fisher Scientific

Goat anti-Rat IgG (H L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 A11077 by Thermo Fisher Scientific

Alexa Fluor® 488 AffiniPure Donkey Anti-Chicken IgY (IgG) (H L) 703-545-155 by Jackson Immunoresearch

Rhodamine Red™-X (RRX) AffiniPure Donkey Anti-Chicken IgY (IgG) (H L) 703-295-155 by Jackson Immunoresearch

Protocol

Step 1.

Fix brain via perfusion with 4% freshly prepared and filtered formaldehyde in Phosphate-Buffered Saline (PBS). Rinse vasculature first with 37C PBS, then perfuse formaldehyde. Post fix overnight-BUT NOT LONGER than 12 hrs- in 10 volumes of 4% formaldehyde at 4C. Wash at least 1hr in PBS before sectioning. See video from the Shain lab linked below.

LINK:
https://www.jove.com/video/3564/whole-animal-perfusion-fixation-for-rodents

Step 2.

Vibratome section at 40 µm and collect in a 24-well culture plate in PBS, treadmilling after 6 wells back to the first well. It will take 180-200 coronal sections to section from olfactory bulbs to the nigra. If you need to cut past nigra/VTA, like locus coeruleus, you will have to cut the brain in half or use the cryostat. Agarose (2%) helps with this but isn't usually necessary. It also tends to bind antibodies if not removed.

Step 3.

Save sections in PBS at 4C until ready to use. Ideally, use within a few days, otherwise, you will need to freeze in cryoprotectant or at 4C with 0.01% sodium azide in PBS. Prolonged storage frequently increases autofluorescence. Wrap in foil if the brains contain fluorescent protein or tracers. Dispose of sodium azide according to institution regulations if used.

Step 4.

Select columns to stain and place into PBS in the staining well. The number of columns that you choose depends on the region that you want to sample. If you just want to check expression, one well from 2-3 mice is sufficient (1/6 series). For smaller regions, every other or every third section may be required for adequate sampling. Around 25-30 sections/well (in 500 µL) is typical in a 24-well plate so use the smaller plates to conserve antibody. Paint brushes are great for moving sections to new wells for staining and you can combine columns in 12-well trays (800 µL).

Step 5.

Optional. Incubate 2 x 5 min with 5 mg/mL $NaBH_4$ in water to quench autofluorescence. You will need to do this if tissue has been stored for longer than 1 month. Wash twice with PBS. This step is easier in larger wells or with net cup inserts (EM Sciences) because the sections float more in the borohydride bubbles. Optional Step 13 to block lipid fluorescence can also be performed here.

Step 6.

Carefully remove and replace PBS with PBST (0.2% Triton X-100) and incubate for at least 1 hr. A pipet tip attached to a plastic transfer pipet works well for removing solutions. Avoid touching the tissue with suction because this will rip the sections.

Step 7.

Block with 5% Bovine Serum Albumin (BSA) prepared in PBST for 4 hr to overnight. 500 µL/well is sufficient in a 24-well tray. Overnight incubations should be on an orbital shaker in the cold. Sometimes it may be necessary to block with the serum (0.5% + 5% BSA) from the species in which the secondary antibody was created (usually goat or donkey). If the antibodies are good, BSA is fine and generally gives less background autofluorescence than serum.

Step 8.

Remove BSA solution and replace with primary antibody prepared in PBST. For example, chicken anti-GFP, 1:2000 (0.5 µL/mL). Manuscripts citing this protocol should list the antibodies and concentrations used for the specific experiments.

Step 9.

Incubate overnight-72 hrs at 4C on the orbital shaker. Shorter incubations are possible but antibody penetration may suffer. If you have to choose, dilute the primary more and incubate longer as opposed to a higher concentration for a shorter time.

Step 10.

Wash at least 3 x 30 minutes with PBST on the oribital shaker at room temperature. Longer washes are always better.

Step 11.

Add the species appropriate Alexa-conjugated secondary antibody diluted in PBST and incubate overnight at 4C on an orbital shaker. For example, use AF488 goat anti-chicken for the GFP antibody. It is possible to incubate all day but, again, penetration and signal suffer. We can image AF350/DAPI, AF405, AF488, AF555, AF568, and AF633/647 on the Axiozoom. The LSM880 will image AFs 405, 488, 543, 555, 568, 633, 647, and has 2P tunable 720-1100 nm excitation. Use 760 nm for 2P excitation of DAPI and AF350. DAPI will also excite with the 405 nm laser. Wrap in foil to protect from light after adding the secondary antibodies.

Step 12.

Wash 3 x 30 mins. DAPI (1:20,000 of the 5 mg/mL stock; 0.5 µL into 10 mL of PBST) can be added in the first wash. Collect DAPI waste in the appropriate chemical waste container. Alternatively, you may also use ToPro-3 (dilute 1:20,000) for staining nuclei in far red (647 nm) channel. If using suspected mutagens like DAPI, wash a few extra times. Wear gloves and clean well. Some mounting media contain the DNA stain. These tend to increase background and expose the sections and user to more dye than necessary.

Step 13.

Optional here or after $NaBH_4$ Step #5. Block myelin fluorescence by incubating 2 x 5 mins with filtered 0.1% Sudan Black in 70% EtOH. Wash once with water, then three times for 15 minutes with PBST before continuing to the next step. Increase Triton concentration 2-fold if destaining is not sufficient or destain overnight. This step is best performed in a 6 or 12 well tray since the sections tend to stick together in the presence of ethanol.

Step 14.

Mount sections in order from PBS onto Superfrost plus slides. Adding a few drops of PBS, ordering the sections in the PBS, and then spreading them with paintbrushes is the easiest method for some. Others prefer to slide sections onto the slides from a petri dish or PBS. Carefully blot excess PBS and allow sections to dry upright long enough to stick (3-5 mins) but do not over-dry. This will cause autofluorescence. Avoid the far right portion of the slide because the slide holders on the higher power scopes obscure this end of the slide. Use 3 drops of Fluoromount and seal with Sally Hansen Extreme Wear nail polish. Use #1.5 coverslips for the Zeiss microscopes.

Step 15.

Image every section on the Axiozoom at low power (11-16x or tiled from multiple 100x images) to document- generally 27 snaps and it will take 30 minutes. If you don't see a signal on the Axiozoom at 250x, you may still see one on the Axiovert so check! Finally, image on confocal for high power or tiling of high power images above 10x, if needed. You can select regions to image from the axiozoom images. Save everything in the original Zeiss format. This saves all of the metadata such as exposure times, objective/zoom, scaling, and filters. You can export later from Zen or Fiji on your desktop.

Step 16.

Helpful Hints

If you need to store slides for a long time, store slides at 4C in sealed, humidified slide boxes in a plastic bag or they will dry out over time.

It is possible to use pre-made formaldehyde but it will likely give you autofluorescence. Freshly prepared according to our lab protocol is best. NEVER use formalin.

It is possible to "drop fix" brains until PN21 for 24 hr, beginning at 37C and transferring to 4C. This is not ideal but is often the only option for very young mice where perfusion would be difficult and mice do not respond to anesthesia. Expect strong yellow-green autofluorescence in the vasculature to appear with storage.

Use a small natural hair or teklon paint brush to transfer and mount sections. Michaels has good ones in the craft section. Brain sections stick to cheap plastic so try a few until you find the best brand.

800 µL of PBS per well is sufficient to collect the sections for storage in a 24-well tray. If the wells are too full, it is difficult to remove without disturbing the slices. Surface tension holds the sections best at 500 µL.

Squirt bottles are great for PBS and PBST washes.

Titrate antibodies when they first arrive and every new lot that you receive. Take a few sections from the area of interest and put them into 4 different wells. Dilute the antibody at the suggested concentration, half, and twice. For example, 1:1000 or 1 µg/mL is "suggested". Do 1:500, 1:1000, and 1:2000 + a negative control. Use a well-characterized secondary at the standard concentration (i.e. 1:1000 for AF488 goat anti-rabbit).

The most common background and lack of specificity problems are caused by using too much antibody. Less is more, especially if you plan to quantify something.

If in doubt, 2 different antibodies against 2 different regions of the protein can help resolve things. Do them together in 2 different colors. If GFP is the issue, stain it red with an antibody.

Some antigens require heating in 10 mM sodium citrate, pH 6 (95C for 10 mins with slow cooling on the bench). In my experience, nuclear antigens and proteins in large complexes may require antigen retrieval.

There are ways to amplify the signal, such as TSA and avidin-biotin. Both have issues in the striatum and require extra controls and blocking steps.

If the experiment is a multi-label and everything colabels perfectly, then you probably did something wrong. Check your secondary antibody species. If using a goat primary, all secondaries must be in donkey. This is a common mistake.

Too much of any secondary antibody will stain somthing, particularly in hippocampus and cortex. Dilute the secondary antibody two fold and see if it goes away in the negative control while still detecting your real signal.

Anti-mouse secondaries sometimes pick up blood vessels because of the endogenous mouse immunoglobulins. The control will show what’s artifact. AF647 donkey anti-mouse recognizes blood vessels in B6 mice but not all secondary antibodies react with B6 immunoglobulins. Jackson Immunolabs has some isotype-specific secondaries if you really need them but the antibody companies rarely tell you which mouse IgG isotype or strain was used for the immunizing antigen. Sometimes they are a mixture. It is possible to purchase mouse blocking kits, such as "Mouse on Mouse" but these have not proven particularly useful in our lab.

Monoclonal antibodies (rabbit, mouse, rat) will increase specificity but sometimes produce a lower signal than a polyclonal because they only recognize one epitope.

Sudan Black (0.1% in 70% EtOH, 2 x 10 mins) can be used to quench mounted slides that have accumulated autofluorescence during storage. Destain in a Coplin jar overnight. A Pap pen will hold

Sudan Black on the slide during the incubation.

Older animals are terrible for fluorescence because of lipofuscin. Less than 90 days is best. If you have to use older animals, be sure to do controls. This is especially important for punctate and intracellular antigens, or high power imaging. If it's lipofuscin, it will fluoresce in more than one channel. It will also be present in the no primary control.

It's better to start with a nice specimen than to try to make a poorly fixed section with background look pretty. If you have to do a lot of post hoc image enhancement, start over.

Think about what you're seeing before you believe it. Is it a receptor inside of a cell or on the surface where it should be? A peptide that doesn't stain terminals? A transporter clearly associated with a nucleus? Something that looks like a Nissl stain in the hippocampus? Is it where it's supposed to be in the brain? In the correct proportions?

Look at the whole brain. This is particularly important when doing virus injections because many transport due to damage to fibers en passant, or via projections to the injected region and retrograde transport back to source. Injections into amygdala sometimes label projections to auditory thalamus because the needle passes through the auditory cortex. Sometimes it doesn't matter, but injections into mice are extremely variable and need to be verified. See Kupferschmidt at al., 2015 for an easy way to do this in tissue after ex-vivo experiments. Thin sections are best but you can stain thick sections after optogenetic experiments.

Multi-labels take a lot of thought and a lot of species. Mouse, rabbit, chicken, guinea pig, goat, and rat all work. DO CONTROLS and different combinations/species/antibodies before believing colocalization.

SOFTWARE PACKAGE
Fiji/Image J

Warnings

Use caution when working with DNA binding agents as counterstains (DAPI, ToPro). Dispose of waste accoringly and wear PPE.