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Immunohistochemical Staining for Neuroscience

The following protocol is a guideline for staining various neurological antigens. This protocol will require optimization for a particular antigen or antibody. Please read the entire procedure before beginning.

I. Rat Brain Fixation by Perfusion

1. Prepare the following perfusate solutions:
    - saline (0.9% NaCl), kept at RT
    - 4% paraformaldehyde (PFA)* chilled to 4°C

2. Anesthetize rat into a deep surgical plane using approved animal care protocols.

3. Expose the heart and insert a large bore needle (18 gauge) into the left ventricle. This will be the entry site for the perfusate.

4. Snip the right atrium to allow the blood, normal saline and fixative to leave the body during perfusion.

5. Using a peristaltic pump, perfuse the rat with 25–50 ml of the RT saline for 10–15 min. Gravity method may also be employed if a pump is not available.

6. Perfuse with saline until the fluid is clear. Any blood remaining in the brain will cause staining of RBC's in subsequent immunohistochemical staining procedures.

7. Perfuse the rat with cold 4% PFA in the same manner as with the saline solution.

8. Remove the brain, place in fresh 4% PFA and allow to post-fix overnight at 4°C.

    NOTE: *This is suggested as a basic protocol. Not all antibodies will stain optimally with 4% PFA perfusion and overnight post-fixation. Some antibodies may require lower concentrations of PFA or shorter post-fixation times. Lower concentrations and shorter fixation times will result in softer tissue, which will be more fragile.*

9. Transfer the brain to 30% Sucrose at 4°C for 24–48 hr (up to 4 days is not harmful) to cryoprotect prior to sectioning. If a vibratome is to be used for sectioning, cryoprotection is not necessary.

II. Sectioning of Brain for Immunohistochemical Staining

A. Sliding Microtome

This is the most common and appropriate method for sectioning brain and flat retina. A sliding microtome with a freezing stage cuts 25-50 µm (thicker if desired) sections of fixed, cryoprotected tissues, which retain outstanding morphology when completed. Remove cut sections from the knife blade with a soft paintbrush (e.g., round #6) and place into PBS. A container with multiple compartments is convenient if the brain is to be "reconstructed" after staining. For example in a 24 well tray, sections #1, 25, 49, 73, 97, etc., will all be in the first well, sections #2, 26, 50, 74, 98, etc. will be in the second well, etc.

B. Vibratome

A vibratome can be used if the antigen is altered or destroyed by freezing. Fixed tissues sectioned using a vibratome do not require cryoprotection because this method of sectioning does not involve freezing the tissue. Sections less than 50 mm can be difficult to prepare using this method.

C. Cryostat

Fixed, cryoprotected specimens can be cut on a cryostat and the sections can be picked up directly onto Superfrost Plus® or coated slides. The slides are stained as frozen sections (PharMingen's Standard Immunohisto-chemical Staining Procedure) with the exception that the incubation with primary antibody will be overnight at 4°C and the incubations with the secondary antibody and the tertiary reagent will be 1 hr each at RT. This method is most frequently used for thin sections (less than 20 µm) or very fragile tissue (e.g., transverse retina), or if a sliding microtome or vibratome is not available. It is possible to cut thick sections on a cryostat and stain the sections free floating , but intact tissue sections are difficult to remove from the cryostat. Fresh frozen cryostat sections, in general, are less useful because morphology is often adversely affected. If the antigen must have a special fixative (e.g., acetone) that cannot be perfused, the animal may be perfused with normal saline (to remove RBC's), and then the tissue is removed and snap frozen. The frozen tissue is sectioned, then fixed after it is placed on the slide.

D. Paraffin

Paraffin sections can be used for some neuroscience applications; however, many antigens are altered or destroyed by the processing. Microwave pretreatment may assist in reviving some antigens, but paraffin sections are not recommended for most antibodies.

NOTE: *Store cut, free floating sections covered at 4°C. Best results are obtained when sections are stained as soon as possible. Sodium azide (0.1%) may be added to the PBS in which the tissues are stored to limit bacterial contamination. However, if the sections are stored in PBS with azide, wash thoroughly with PBS to remove any trace of the azide in the sections prior to staining.*

III. Free Floating Immunohistochemical Staining

The preferred method of immunostaining for most neuroscience applications is to stain the sections free floating, which provides access to both surfaces of the tissue sections by the staining reagents. Free-floating sections are normally stained with a three-step permanent procedure (purified primary antibody, biotinylated secondary antibody, and ABC with DAB). These sections may also be used for single or multi-color indirect immunofluorescent staining. After staining is complete, the sections are mounted on chrome alum -coated slides. *

1. Place sliding microtome or vibratome cut brain sections into a staining net and rinse 3 times for 10 min each in PBS†. The use of a staining net and crystallizing dish will allow the buffer to be changed without handling the actual sections.

2. Transfer brain sections to a microcentrifuge tube which contains 1 ml of primary antibody, appropriately diluted in PBS containing 0.3% Triton® and 5% normal serum*. Incubate sections overnight at 4°C with gentle rotation. One eighth of an average size rat brain, depending on the plane of section, can be stained at one time in each tube.

3. The following day, empty each microcentrifuge tube into the staining net and rinse 3 times for 10 min each in PBS, with gentle rotation. Check each tube to be sure no sections remain.

4. Transfer sections to another microcentrifuge tube which contains 1 ml biotinylated secondary antibody, appropriately diluted in PBS containing 0.3% Triton® and 5% normal serum*. Incubate for 1 hr at RT with gentle rotation.

5. Prepare tertiary reagent from ABC Elite Kit, by adding 980 µl PBS/Triton® High Salt* and 10 µl each reagent A and B to a microcentrifuge tube. The added salt decreases background staining. ABC reagent should be prepared and allowed to sit for 30 min prior to use.

6. Rinse sections 3 times for 10 min each with gentle rotation.

7. Transfer brain sections to the microcentrifuge tube containing the 1ml of tertiary reagent. Incubate for 1 hr at RT with gentle agitation.

8. Rinse sections as above in Step 6. Meanwhile, remove a 25 mg DAB* aliquot from freezer to allow to thaw. DAB is a suspect carcinogen and should be used in an area dedicated for that purpose. Always wear gloves when using DAB.

   DAB chromogen:
   a. Add 100 ml PBS to crystallizing dish in DAB area.
   b. Add thawed 25 mg DAB aliquot and mix.
   c. Add 2-3 ml 0.3% H202 .
   d. Immediately immerse the brain sections, using the staining net, into the DAB solution and allow to react with gentle agitation until desired brown intensity is obtained, usually 3-5 min, although 30 sec to 10 min is not uncommon.

9. Rinse the brain sections twice in PBS. Collect the discarded DAB staining solution and the first rinse solution for neutralization. A one gallon plastic bottle can be used to store DAB solution for treatment in 1–2 L batches.

10. Rinse sections again, 2 times for 10 min each in PBS.

11. Mount sections on chrome alum slides* and allow to dry upright overnight.

12. Osmicate sections in 0.05% osmium tetroxide* for 10-30 sec to intensify the DAB stain.

13. Rinse well in water.

14. Dehydrate in 4 changes of 100% alcohol, clear in 3-4 changes of xylene (or xylene substitute) and cover slip. Always allow slides to drain 15-20 sec between containers to prevent carry over from dish to dish.

__NOTE:__ Some xylene substitutes may cause artifacts with Nomarski optics.

***NEUROSCIENCE SOLUTIONS**

2X Phosphate Buffer Stock Solution (2X PO_4)

NaOH	7.7 g
NaH_2PO_4 (monobasic)	33.6 g
deionized water	1 litre

10X Saline (9% NaCl)

NaCl	90 g
deionized water	1 litre

PBS, low molarity (used in all staining procedures)

10X saline	100 ml
2X PO_4	50 ml
deionized water	850 ml

PBS, high molarity (used only in paraformaldehyde and sucrose solutions)

2X PO_4	500 ml
deionized water	500 ml

PBS/ 0.3% Triton®

Triton X-100	300µl
PBS, low molarity	100 ml

PBS/ 0.3% Triton® and 5% normal serum

Triton X-100	300 µl
normal serum	5.0 ml
PBS, low molarity	95 ml

PBS/ 0.3% Triton® and High Salt

Triton X-100	300 µl
NaCL	2.0 g
PBS, low molarity	100 ml

30% Sucrose

Sucrose	300 g
PBS, high molarity	to 1 litre

Osmium tetroxide, 0.05%

4% Osmium tetroxide	3 ml
PBS, low molarity	240 ml