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Immunohistochemistry (IHC) Protocols for Frozen Sections: Indirect Methods

Detailed protocols to take you through tissue processing and on to indirect IHC methods for floating sections.

Immunohistochemistry (IHC) allows you to detect a specific protein in tissue sections. In our experience, the optimal type of section is from frozen tissue fixed by transcardial perfusion with a buffered paraformaldehyde solution. This tissue is cut frozen in a cryostat and sections collected either by thaw mounting on slides (10–12 µm thick sections) or floating (30–36 µm thick sections).

These protocols describe IHC with floating sections. Typically, floating sections are from tissues like adult brain and so require a thin brush to transfer them from one well to the next in a multi-well plate.

With indirect IHC methods, the primary antibody binds your protein of interest, and a secondary antibody conjugated to a reporter binds the primary antibody. There are more steps compared to the direct IHC method, but it is benefits from signal amplification since multiple secondary antibodies can bind the primary antibody. On the other hand, the indirect IHC methods can also produce more background than direct IHC methods in some situations.

This is the IHC protocol we use with rabbit-raised polyclonal primary antibodies on rat floating tissue sections.

Sacrifice and Tissue Processing

1. Anesthetize the rats with pentobarbital sodium (Pental).
2. Perform the transcardial perfusion, first with 50 ml of IHC phosphate-buffered saline (IHC-PBS), then with 220 ml of the fixative (ice-cold IHC-PBS with 4% paraformaldehyde (PFA) and 4% sucrose).

IHC-PBS (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	80 ml
KH_2PO_4	0.2 M	16 ml
NaCl		8 g
Double distilled water		860 ml

Fixative Buffer (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	120 ml
KH_2PO_4	0.2 M	24 ml
NaCl		1.31 g
Sucrose		11.5 g
Paraformaldehyde		144 ml

3. Divide the tissue into coronal blocks and further fix by immersion in the fixative described above.
4. Incubate at 4–8°C overnight.

5. Transfer the tissue blocks to Perfusion Buffer.

Perfusion Buffer (pH 7.4)

Reagent	Concentration	Volume/Weight
Na_2HPO_4	0.2 M	220 ml
KH_2PO_4	0.2 M	50 ml
NaCl		2.45 g
Sucrose		81 g
Double distilled water		270 ml

6. Cut the tissue in a cryostat within 21 days.

7. Float the tissue sections, 30 µm thick, in Cryopreservation Buffer and preserve at -20°C.

Cryopreservation Buffer (pH 6.5)

Reagent	Concentration
Ethylene glycol	40%
Polyvinylpyrrolidone	1%
Potassium acetate buffer	0.1 M

Protocols for Indirect IHC

To detect indirect IHC signals, use a bright-field or fluorescent microscope. It's important to know which type of microscope you plan to use before beginning the experiment.

Option A: **Detection by Bright-Field Microscopy**
Option B: **Detection by Fluorescent Microscopy**

Option A:
Detection by Bright-Field Microscopy

1. Rinse the floating sections wit IHC-PBS for 2 x 5 minutes.
2. Quench endogenous peroxidase activity by incubating the sections with 0.2% hydrogen peroxide in IHC-PBS with 0.2% Triton X-100* and 20% methanol for 25 minutes at room temperature.
3. Rinse the sections with IHC-PBS for 2 x 5 minutes.
4. If antigen retrieval is necessary, proceed to the **Antigen Retreival** protocol at the end of this one. Please note that treatment with hydrogen peroxide (step 2) induces moderate antigen retrieval.
5. Rinse the sections with IHC-PBS for 2 x 5 minutes.
6. Incubate the sections with the rabbit primary antibody in Antibody Solution for 1 hour at room temperature.

Antibody Solution

Reagent	% of final volume
IHC-PBS	97.65
Triton X-100*	0.3
Tween-20	0.05
Normal serum**	2

**If your primary antibody targets an extracellular protein, reduce the Triton X-100 to 0.05% in both the primary and secondary antibody solutions.*

***Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use normal donkey serum (NDS). Likewise, if your secondary antibody was raised in goats, use normal goat serum (NGS).*

7. Transfer to 4°C overnight.
8. Rinse the sections with IHC-PBS containing 2% NS** for 2 x 5 minutes.

Two options for indirect bright-field IHC with secondary antibodies are available here:

A1: **Secondary Antibody Conjugated to Biotin**
A2: **Secondary Antibody Conjugated to HRP**
Option A1

Secondary Antibody Conjugated to Biotin

1. Incubate the sections with biotinylated donkey anti-rabbit antibody (Merck, catalog no. AP182B) diluted 1:400 in Antibody Solution, for 1 hour at room temperature.
2. Transfer to 4°C overnight.
3. Rinse the sections with IHC-PBS containing 2% NDS** for 2 x 5 minutes.
4. Incubate the sections with extravidin-peroxidase (Merck, catalog no. E2886) diluted 1:200 in IHC-PBS, for 1 hour at room temperature.
5. Proceed to **Detection with a Bright-Field Microscope**.

Option A2

Secondary Antibody Conjugated to HRP

1. Incubate the sections with horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody (Merck, catalog no. AP182P) diluted 1:400 in Antibody Solution for 1 hour at room temperature.
2. Transfer to 4°C overnight.
3. Rinse the sections with IHC-PBS containing 2% NDS** for 2 x 5 minutes.

   ***Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use NDS. Likewise, if your secondary antibody was raised in goats, use NGS.*
4. Proceed to **Detection with a Bright-Field Microscope.**

Detection with a Bright-Field Microscope

1. Incubate the sections with a solution containing 0.0125% diaminobenzidine (DAB; Merck, catalog no. D5637) and 0.05% nickel ammonium sulfate for 10 minutes at room temperature.
2. Transfer the sections to the same DAB solution described above, which has been supplemented with hydrogen peroxide at a final concentration of 0.0015%. This is necessary to monitor the color reaction.
3. Rinse the sections with IHC-PBS for 4 x 10 minutes.
4. Mount the sections on glass slides (gelatinized or coated with any other type of adhesive material) and allow them to dry.
5. Dehydrate the sections by incubation with increasing ethvanol concentrations (70%, 90%, and 100%; 5 minutes at each concertation). Delipidate in xylene for 10 minutes and apply a coverslip with Permount (or any other xylene diluted adhesive).
6. Detect with a bright-field microscope.

Option B

Detection by Fluorescent Microscopy

1. Rinse the floating sections with IHC-PBS for 2 x 5 minutes.
2. If antigen retrieval is necessary, proceed to the protocol on enzymatic retrieval in the troubleshooting section at the end of this protocol.
3. Rinse the sections with IHC-PBS for 2 x 5 minutes.
4. Incubate the sections with the rabbit primary antibody in Antibody Solution for 1 hour at room temperature.
5. Transfer to 4°C overnight.
6. Rinse the sections with IHC-PBS containing 2% NDS for 2 x 5 minutes.

Two options for indirect fluorescent IHC with secondary antibodies are available here:

B1: **Secondary Antibody Conjugated to Biotin**
B2: **Secondary Antibody Conjugated to a Fluorophore**

Option B1

Secondary Antibody Conjugated to Biotin

1. Incubate the sections with biotinylated donkey anti-rabbit antibody (Merck, catalog no. AP182B) diluted 1:400 in Antibody Solution for 1 hour at room temperature.
2. Transfer to 4°C overnight.
3. Rinse the sections with IHC-PBS containing 2% NDS** for 2 x 5 minutes.

   ***Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use NDS. Likewise, if your secondary antibody was raised in goats, use NGS.*
4. Incubate the sections with streptavidin-Cy3 (Sigma, catalog no. S6402) diluted 1:200 in IHC-PBS, for 1 hour at room temperature, protected from light.
5. Proceed to **Detection with a Fluorescent Microscope**.

Option B2

Secondary Antibody Conjugated to a Fluorophore

1. Incubate the sections with a fluorophore-conjugated goat anti-rabbit antibody diluted 1:200 in Antibody Solution for 1 hour at room temperature.
2. Transfer to 4°C overnight.
3. Rinse the sections with IHC-PBS containing 2% NGS** for 2 x 5 minutes.

   ***Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use NDS. Likewise, if your secondary antibody was raised in goats, use NGS.*
4. Proceed to **Detection with a Fluorescent Microscope**.

Detection with a Fluorescent Microscope

1. Mount the sections on glass slides in IHC-PBS (pH 7.4).
2. Dry the glass slides in a fume hood for 1 hour.
3. Stain the sections on the slides with DAPI by placing DAPI solution (5 mg/ml stock solution in deionized water or dimethylformamide (DMF); dilute to 500 nM in IHC-PBS for final use) on each slide. Next, cover each slide with a piece of parafilm to spread the solution evenly on each slide.
4. After 2 minutes, remove the parafilm and rinse the slide with 0.5 ml IHC-PBS using a pipette (repeat twice).
5. Dry the slides in a fume hood for 30 minutes.
6. Apply coverslips using the adhesive Immu-Mount™ (Shandon™).
7. Dry the slides overnight, protected from light.
8. Store at -18°C until they ready to view under the microscope.

Antigen Retrieval Protocol

If there is no observed staining after your IHC experiment, then we recommend that one of the following antigen retrieval protocols. You can use both methods, but you will need to determine the optimal conditions with careful testing.

Hydrogen peroxide treatment (moderate treatment)

1. Incubate the sections with 0.2% hydrogen peroxide in IHC-PBS (pH 7.4), 0.2% Triton X-100*, and 20% methanol, for 25 minutes at room temperature.

   **Use a serum based on the species that your secondary antibody was raised in. For example, if your secondary antibody was raised in donkeys, use NDS. Likewise, if your secondary antibody was raised in goats, use NGS.*

Enzymatic retrieval (aggressive treatment)

Stock solutions required for trypsin treatment:

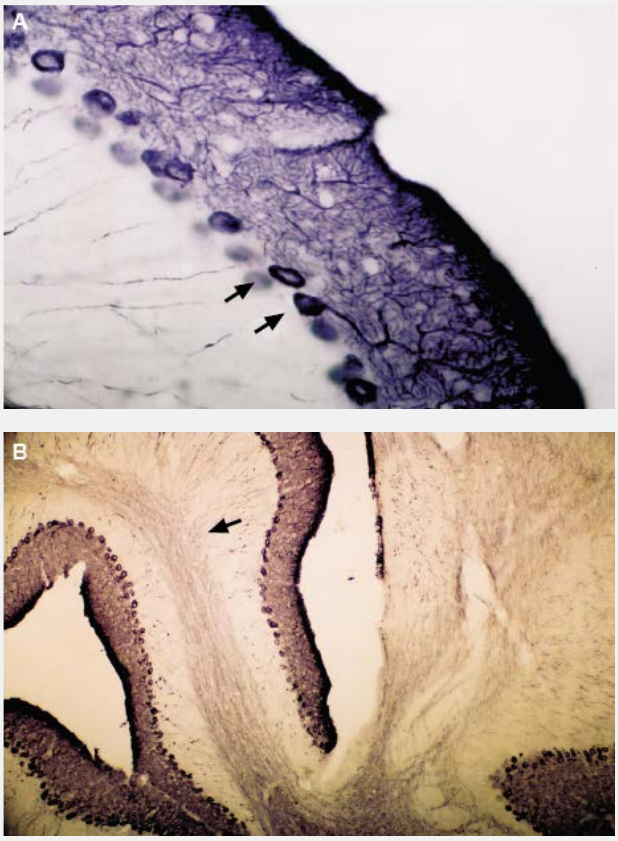
- Trypsin type II-S (Sigma, catalog no. T-8128): 0.1% trypsin dissolved in IHC-PBS. Store as frozen aliquots.
- Trypsin type II-S inhibitor (Sigma, catalog no. T-9128): 0.1% trypsin inhibitor diluted in IHC-PBS. Store as frozen aliquots.

1. Dilute the trypsin stock solution 1:100 to obtain a final trypsin concentration of 0.001%
2. Add CaCl2 to the trypsin stock solution for a final concentration of 0.001%.
3. Incubate the sections in this solution for 5–7 minutes at 37°C.
4. Rinse the sections with IHC-PBS for 2 x 5 minutes.
5. Dilute the trypsin inhibitor stock solution 1:100 into the primary antibody solution*.

   It's incredibly important to remember that if you use trypsin in this step, then **you must also add 0.001% trypsin inhibitor to the primary antibody solution.*

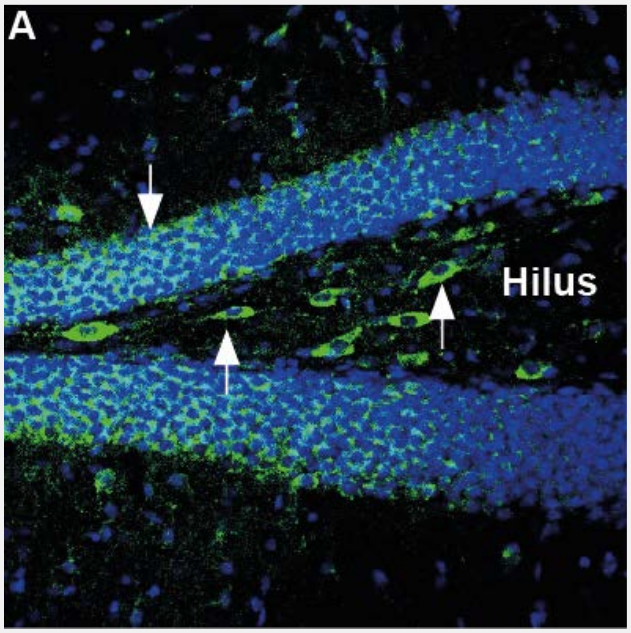
Example data

Figure 1
Expression of IP3 receptor 1 in mouse cerebellum.
Immunohistochemical staining of mouse cerebelluvm with Anti-IP3 Receptor-1 (ITPR1) Antibody (ACC-019). A) Immunoreactivity appeared in Purkinje cells (indicated with arrows) and their dendritic trees. B) Axonal processes coursing through cerebellar white matter were visualized (shown by the arrow).



Example data

Figure 2
Glucose transporter 3 (GLUT3) expression in the murine hippocampus and cerebellum.
Immunohistochemical staining of perfusion-fixed frozen mouse brain sections with Anti-GLUT3 (extracellular) Antibody (AGT-023) (1:200) and goat anti-rabbit Alexa Fluor 488. A) GLUT3 staining (green) in the mouse hippocampal dentate gyrus is detected in interneurons (arrows pointing up) in the hilus and granule layer arrow pointing down). The cell nuclei are stained with DAPI (blue). B) GLUT3 staining (green) in the mouse cerebellum is observed in Purkinje cells (vertical arrows) and dendrites (horizontal arrows) in the molecular layer (Mol). The cell nuclei are stained with DAPI (blue).



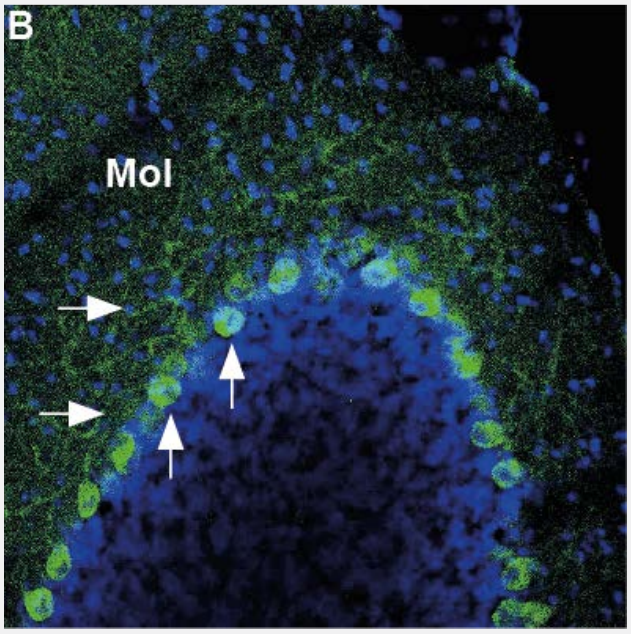


Figure 3
P2RY6 expression in the rat parietal cortex.
Immunohistochemical staining of perfusion-fixed frozen rat brain sections with Anti-P2Y6 Receptor (extracellular) Antibody (APR-106) (1:1,000), donkey anti-rabbit biotin-conjugated antibody, and streptavidin-Cy3. P2RY6 immunoreactivity (red) appeared in the pyramidal neurons (arrows). The cell nuclei are stained with DAPI (blue).