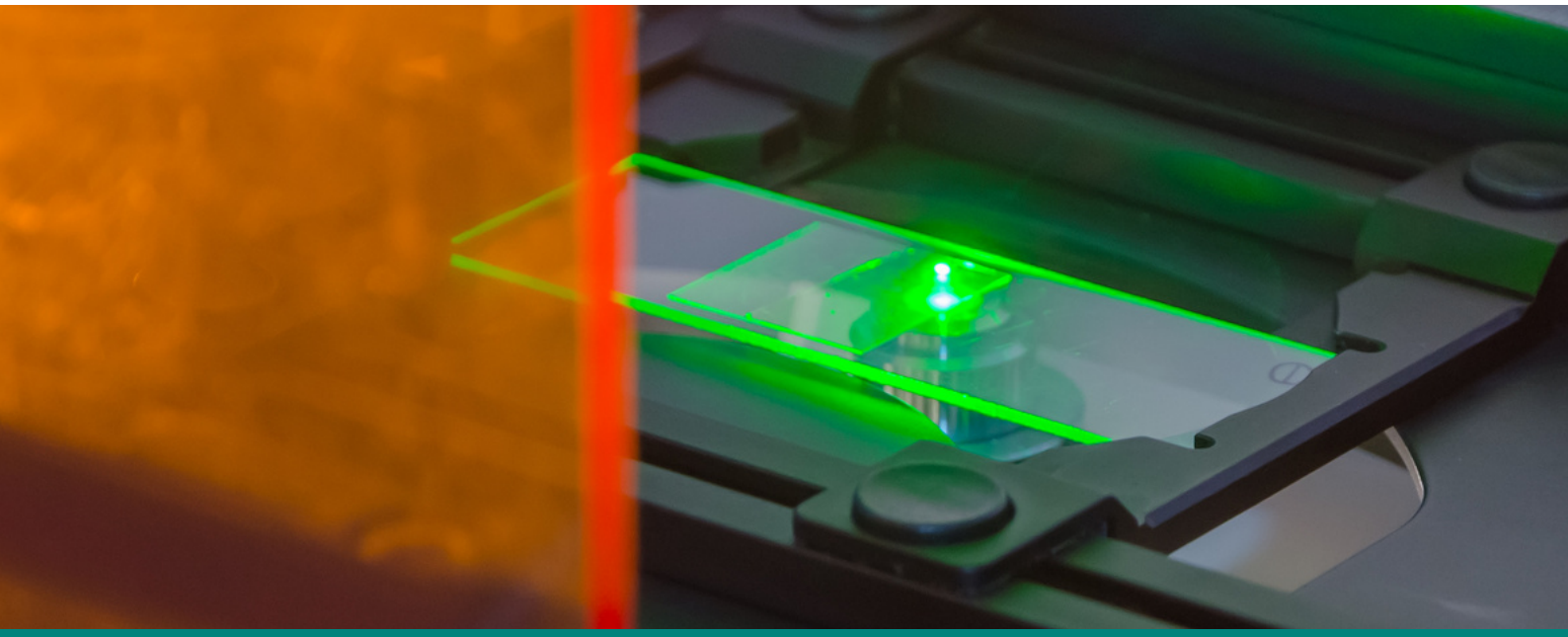

Immunohistochemistry Protocol

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CONTENTS

1. Introduction

Immunohistochemistry is an extremely popular and powerful technique that allows the visualisation of protein markers within thin sections of tissue. This can be used to analyse the distribution of receptors, look at the cellular makeup, expression of biomarkers and gross-morphology of a tissue amongst a myriad of other applications. Figure 1 shows some representative images that have been achieved using these protocols. The protocols within this guide are intended for frozen and fixed sections only and are not aimed to cover paraffin embedded sections although many of the stages will be similar.

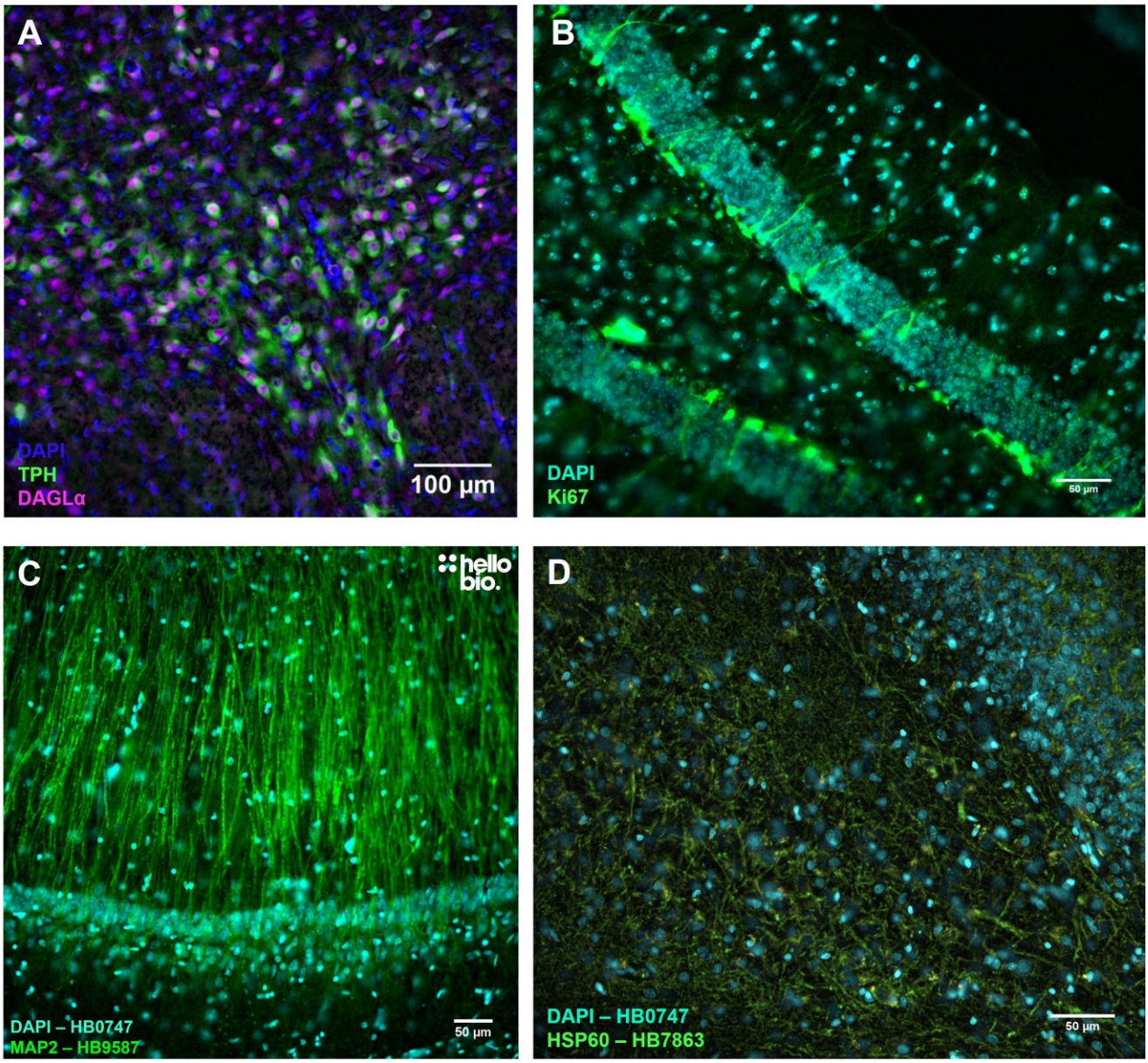


Figure 1. Representative immunohistochemistry images. A. Expression of tryptophan hydroxylase in 5-HT neurones of the dorsal raphe nucleus in rat alongside diacylglycerol lipase alpha expression. **B**. Expression of the neurogenesis marker Ki67 in granule cells of the mouse dentate gyrus. **C**. Hippocampal neurones from the CA1 of a rat visualised using MAP2 as a neuronal marker. **D**. Expression of HSP60 in the dense network of neurones and glia in the rat hippocampus.

2. Key Decisions

There are a few key decisions that need to be considered before starting any immunohistochemistry experiment. Some of these key decisions include:

- Whether to use a free-floating or slide mounted protocol (see section 2.1)

- Whether to include an isotype control (see section 2.2)

- Whether to use fluorescent or chromogenic mediated detection (see our stand-alone guide on choosing the correct secondary antibody)
    - If using fluorescent mediated detection then the choice of correct fluorophores is crucial. A brief overview is available in section 2.3 and more detail is available in the previously mentioned guide.

- What the best way is to prepare the tissue with regards to fixation, section thickness and cutting technique (see section 4)

- Whether it is necessary to use an antigen retrieval technique (see section 5.2)

- Whether additional steps are necessary to account for using tissue from the same species as the primary antibody (see section 5.3)

2.1 Free-floating and slide mounted immunohistochemistry

There are two primary methodologies in immunohistochemistry:-

Slide mounted:	Sections are cut straight from the cryostat onto microscope slides and all staining occurs directly on the slide.
Free-floating	Sections are kept in buffer after slicing then all staining occurs with the sections floating in an excess of buffer. Sections then need mounting at the end of the protocol.

Both techniques have their own distinct set of advantages and disadvantages which are detailed in table 1.

Method	Advantages	Disadvantages
Free floating	- Even penetration of antibody into tissue - Can use thicker sections - Lower risk of sections drying out - Can use lower antibody concentrations	- Risk of damaging sections with washes and other manipulations - Too difficult with very thin sections - Uses a higher volume of reagents
Slide mounted	- Can use much lower solution volumes - Less manipulation of sections means less damage.	- Often needs a higher antibody concentration - Needs to use thin sections otherwise diffusion distance too long into section

Table 1. Some advantages and disadvantages of slide mounted and free-floating immunohistochemistry techniques.

2.2 Choosing an isotype control

2.2.1 What are isotype controls?

Isotype controls are antibodies with no antigen specificity which are used in immunochemical experiments to control for non-specific interactions between antibodies and the sample. They are most commonly used in flow cytometry, immunohistochemistry and immunocytochemistry experiments where they are particularly useful due to the often high background staining levels. Background staining can be caused by many factors including:

- Binding of antibodies to Fc receptors on target cells. For example some antibodies (e.g. mouse IgG2a) bind to Fc receptors on human leukocytes independently of the primary antibody – antigen interaction.

- Cellular autofluorescence. Some cells and cellular structures contain either proteins or chemicals which naturally fluoresce and this can lead to background staining.

- Non-specific antibody interactions. The primary antibody may bind to non-intended targets meaning that the isotype control can be key in determining if this has occurred.

2.2.2 Choosing an isotype control

When choosing an isotype control it is important to bear in mind:

- The host species should be the same as that of the primary antibody

- The isotype (e.g. IgG2a, IgG1) should be the same as the primary antibody

- The isotype control should have the same modifications as the primary antibody (e.g. if the primary is biotinylated so should the isotype control).

2.1.3 Using Isotype controls

When isotype controls are included in an experiment they should always be used at the same concentration as the primary and following an identical protocol. Ideally the isotype control should generate minimal staining, indicating that there is minimal background staining. However this does not prove that the primary antibody is binding specifically!

2.3 Choosing secondary antibody fluorophores

It is important to choose secondary antibodies that have conjugated fluorophores that are sufficiently separated from each other in excitation/emission wavelengths. If there is too much overlap between spectra then each channel can bleed into each other meaning that it isn't possible to determine which signal comes from which antibody. In order to determine the best fluorophores to use:

1. Determine the compatibility of the microscope system you are planning on using with available fluorophores. Widefield microscopes will commonly be fitted with filter sets which are only compatible with limited numbers of fluorophores. Confocal microscopes are able to use a much wider range of fluorophores but will still be limited by the wavelengths of the installed lasers.

2. Use a spectral viewer tool (e.g. Spectraviewer) to check compatibility between various fluorophores.
    a. A preference should be towards using standard combinations e.g. DAPI and a fluorophore emitting in the green spectrum (e.g. Alexa Fluor 488, Dylight 488, Janelia Fluor 488, GFP).
    b. Make sure there is sufficient spacing between fluorophores such that there is no bleeding between channels.

3. Choose fluorophores that are as bright as possible with high resistance to photobleaching.

3. Equipment and consumables

Assuming a base level of standard laboratory equipment (e.g. pipettes, de-ionised water system, measuring cylinders etc) the specific equipment and consumables you will require for immunohistochemistry are detailed by stage within this section.

3.1 Tissue preparation

Tissue preparation will generally require specialised spaces with a range of expensive equipment. Many institutions have a histology service that will rent out the equipment or even perform the histology for a fee.

Equipment	Consumables
Cryostat / freezing microtome / other cutting equipment	Isopentane (for freezing fresh tissue)
Dissection tools	4% paraformaldehyde (PFA)
	Phosphate buffer (PB)
	30% sucrose in PBS
	Optimal cutting temperature (OCT) compound

3.2 Antigen retrieval

Equipment	Consumables
Water bath	Citrate buffer (for heat mediated retrieval)
	Trypsin retrieval buffer (for enzymatic retrieval)

3.3 Free floating (fluorescence detection)

Although not necessary, using nets to hold sections that fit into multi-well plates can hugely speed up the protocol although these do require higher solution volumes.

Equipment	Consumables
Cold room	Antibodies
Paintbrushes	Blocking solution
Rocker	Glycine
Subbed microscope slides	$NaBH_4$
Fluorescence microscope	PBS-Triton
	Mounting media
	DAPI
	Antibody solution
	Nail varnish

3.4 Free floating (chromogenic detection)

Although not necessary, using nets to hold sections that fit into multi-well plates can hugely speed up the protocol although these do require higher solution volumes.

Equipment	Consumables
Cold room	Antibodies
Paintbrushes	Blocking solution
Rocker	H_2O_2 (for HRP conjugated secondaries)
Subbed microscope slides	Levamisole (for AP conjugated secondaries)
Microscope	PBS-Triton
	Mounting media
	Counterstain (optional)
	Antibody solution
	Nail varnish
	Developer solutions

3.5 Slide Mounted (fluorescence detection)

Equipment	Consumables
Humidifying chamber	Hydrophobic pen
Cold room	PBS-Triton
Subbed microscope slides	Serum (same species as secondary antibody)
Fluorescence microscope	Antibodies
	DAPI
	Mounting media
	Nail varnish

3.6 Slide Mounted (Chromogenic detection)

Equipment	Consumables
Humidifying chamber	Hydrophobic pen
Cold room	PBS-Triton
Subbed microscope slides	Serum (same species as secondary antibody)
Microscope	Antibodies
	Mounting media
	Nail varnish
	Developer solutions
	H_2O_2 (for HRP conjugated secondaries)
	Levamisole (for AP conjugated secondaries)
	Counterstain (optional)

4. Tissue Preparation

High quality tissue preparation is absolutely essential for high quality immunohistochemistry results therefore it is always worth taking the time to make sure this is done to the highest possible standard to avoid disappointing results. Dependent upon the experimental requirements tissue may need to be used fresh or fixed.

4.1 Freezing tissue for later processing

It is sometimes necessary to freeze tissue before further processing or subsequent fixation. If not frozen properly this can create ice-crystals which damage cellular structures. By freezing properly this will maintain the quality of tissue to maximise the chance of successful immunostaining.

1. Pre-chill a beaker of isopentane (at least 500ml in a long beaker) to between -42°C and -45°C in a -80°C or using dry ice. Monitor the temperature regularly to ensure the temperature stays within this range.

2. Dissect the tissue using clean tools on ice.

3. Place tissue onto aluminium foil and remove excess liquid using filter paper.

4. Gently immerge the tissue (without aluminium foil) into the isopentane. The time taken to freeze depends upon tissue sample size but it takes around 5 minutes to freezer an adult rat brain.

5. Pre-chill the tips of forceps before removing the tissue from isopentane. Place the frozen tissue in pre-chilled tubes containing soft paper towels.

6. Store at -80°C until subsequent use.

4.2 Perfusion fixation

Generally the best quality tissue sections are achieved when the animal has been perfusion-fixed. This process involved replacing the blood and it's high autofluorescence with first buffer then fixative. Before attempting make sure this is covered under your regulatory regime and obtain training from an experienced practitioner. A general protocol for rodents is:

1. Overdose with anaesthetic by an appropriate route until the animal has no toe pinch and blink reflexes but the heart is still beating.

2. Open the ribcage to reveal the heart, snip the right atrium. Place a needle into the left ventricle and perfuse through ice-cold PBS (≈200ml for a rat, ≈15ml for a mouse) followed by a similar volume of 4% paraformaldehyde in PBS.

3. Remove the brain / other organs and place into a vial of 4% paraformaldehyde for 24hrs at 4°C.

4. Move the tissue into 30% sucrose in PBS at 4°C until the tissue has sunken to the bottom of the vial

Please see Gage et al., 2012 J. Vis. Exp. (65), e3564 for a detailed protocol with accompanying video

4.3 Immersion fixation

For small volumes of tissue it is often sufficient to incubate the tissue in fixative as opposed to using perfusion fixation. This will however lead to higher background as blood is not removed from small capillaries.

1. Dissect the tissue with clean tools on ice.

2. Wash briefly with ice-cold PBS

3. Place tissue into 4% paraformaldehyde in PBS for 24hrs at 4°C

4. Move the tissue into 30% sucrose in PBS at 4°C until the tissue has sunken to the bottom of the vial

4.4 Sectioning

Sections can be cut on an array of machines with the most common being cryostats and freezing microtomes. The choice of machine will be partially dictated by availability and partially by how thick sections need to be cut. Check that the specifications of the machines available match with the section thickness required.

Sections can either be cut directly onto the microscope slide in the case of slide-mounted immunohistochemistry or cut into PBS for free-floating immunohistochemistry.

Following cutting, sections can be frozen for processing at a later date:

Slide mounted:	After allowing the sections to dry then sections can be stored at -20°C to -80°C for up to a year
Free-floating	Transfer sections to cryoprotectant and then store at -20°C for up to a year.

5. Immunohistochemistry Protocols

5.1 Safety

Many of the chemicals used in immunocytochemistry have dangerous properties and can cause serious harm if not handled correctly. Always follow local rules and read the full COSHH document for any chemical that you have not used previously. Always wear appropriate PPE such as a lab coat and gloves.

Specifically highlighted hazards:

- Formaldehyde is a highly toxic poison, skin sensitizer and carcinogen and should only ever be used in a fume hood with full PPE.

- Sodium borohydride is toxic, causes severe skin burns and eye damage in addition to reacting rigorously with H_2O to create flammable gas which can ignite spontaneously.

5.2 Antigen retrieval

Antigen retrieval can be essential for the successful recognition of antigens by the primary antibody. During fixation by aldehydes, cross-links form between proteins which may hinder access to or directly modify the epitope bound to by the primary antibody. Two key methods are used for antigen retrieval: heat based method and enzyme based methods. Antigen retrieval can be challenging to get right and often requires attempting multiple methods before optimising based upon what is working best.

5.2.1 Heat mediated antigen retrieval

There are multiple protocols available using different buffers and steps. This is one that is well adapted to both free-floating and slide mounted sections and should be a first step.

1. Wash sections three times for 5 minutes in 0.1M PB

2. Transfer sections to citrate antigen retrieval buffer (10mM, pH8.5) that has been preheated to 80°C in a water bath and incubate for 30 minutes

3. Allow sections to cool to room temperature, remaining within the antigen retrieval buffer

4. Wash sections three times for 5 minutes in 0.1M PB

5. Continue into blocking.

5.2.2 Enzymatic antigen retrieval

There are many different enzymatic antigen retrieval protocols available using a range of proteases such as trypsin, pepsin and proteinase k amongst others. Enzymatic retrieval needs to be carried out carefully as it is easy to cause tissue damage by over-digestion of the section. Careful optimisation of incubation conditions and protease concentration is required for successful retrieval. This protocol is a good base to build upon:

1. Make a fresh trypsin retrieval buffer then heat to 37°C in a water bath.

2. Incubate sections for 10-20 minutes in the warmed trypsin solution in a water bath with gentle agitation.

3. Wash sections three times for 5 minutes in PBS-Triton

5.3 Using tissue from the same species the primary antibodies were raised in

Using primary antibodies from the same species as the tissue section can be extremely challenging. The primary antibody will readily bind to Fc receptors present in cells within the tissue while the secondary antibody will bind to endogenous antibodies that are present within the tissue section. This can lead to high background alongside non-specific staining. Resolving these issues can be challenging and two suggestions are described below.

1. Introduce an additional blocking step utilising a Fab fragment anti-mouse IgG antibody to bind to endogenous IgG antibodies.

2. Create an antibody complex by incubating the primary antibody and secondary antibody together separately before then adding mouse serum. After further incubation this can then be added to the tissue. This ensures that any secondary antibody not bound by primary is then sequestered by the mouse serum. See Goodpaster and Randolph-Habecker., 2014. J Histochem&Cytochem 62(3):197-204 for more details.

5.4 Free Floating Protocol (Fluorescent detection)

This protocol has been designed for use with sectioned brain tissue that has been fixed with 4% paraformaldehyde. We use 40µm sections cut on a freezing microtome although use of thinner and thicker sections is possible. Tissue sections should be placed immediately into PBS following sectioning for free-floating immunohistochemistry. For membrane bounds targets with extracellularly targeted antibodies the wash buffer should be swapped from PBS-Triton to PBS.

1. Wash sections three times with PBS-Triton for 5 minutes per wash

2. Incubate sections in 1% $NaBH_4$ for 30 minutes in PBS-Triton.
    a. Make up fresh as degrades rapidly

3. Wash sections three times with PBS-Triton for 5 minutes per wash

4. Incubate sections for 30 minutes in 0.05M glycine in PBS-Triton

5. Wash sections three times with PBS-Triton for 5 minutes per wash

6. Incubate sections in blocking solution for 2 hours at room temperature.

7. Incubate sections in primary antibody diluted into antibody solution overnight at 4ºC.
    a. If multiplexing is being carried out add all antibodies at the same time but ensure they are raised in different species.

8. Wash sections three times with PBS-Triton for 5 minutes per wash

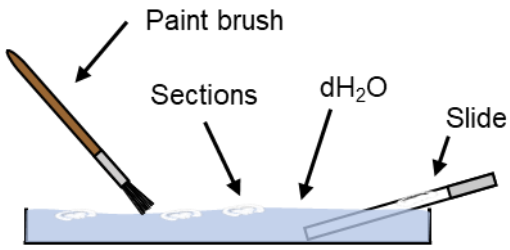
    From now onwards all steps should be done in the dark to avoid bleaching of fluorophores conjugated to the secondary antibody.

9. Incubate sections with secondary antibody diluted into antibody solution for 2 hours at room temperature.
    a. For multiplexing add all secondary antibodies at the same time but make sure they use different fluorophores.

10. Incubate slices with 1µg/ml DAPI for 5 minutes in PBS-Triton.

11. Wash sections three times with PBS-Triton for 5 minutes per wash

12. Fill a wide petri dish with dH_2O. One well at a time transfer sections to the petri dish with a paintbrush then use the paint brush to float each section in turn onto a microscope slide lowered into the petri dish. Mount closest to the frosted section first then one in place slowly pull the slide out of the dH_2O to add the next layer of sections.
    a. A slide should fit around 6 coronal rat brain sections or 8 coronal mouse brain sections.



13. Once a full slide has been mounted allow the sections to air dry until slightly moist
    a. It can often be helpful to use a paper towel to remove excess moisture from the slide to speed drying.

14. Add a drop or two of mounting medium to each slide (when dry enough) then slowly lower the coverslip over the slide taking care to avoid bubbles.
    a. If using a hard-set mounting medium then no sealing is necessary but if not then use clear nail varnish to seal the edges of the coverslip to stop the sample drying out.

15. Allow the mounting medium to dry then store the slides at 4ºC in the dark until ready to image.

5.5 Free Floating Protocol (Chromogenic detection)

This protocol has been designed for use with sectioned brain tissue that has been fixed with 4% paraformaldehyde. We use 40µm sections cut on a freezing microtome although use of thinner and thicker sections is possible. Tissue sections should be placed immediately into PBS following sectioning for free-floating immunohistochemistry. For membrane bounds targets with extracellularly targeted antibodies the wash buffer should be swapped from PBS-Triton to PBS.

1. Wash sections three times with PBS-Triton for 5 minutes per wash

2. Incubate sections in blocking solution for 2 hours at room temperature.

3. Incubate sections in primary antibody diluted into antibody solution overnight at 4ºC.

4. Wash sections three times with PBS-Triton for 5 minutes per wash

5. If using a HRP conjugated antibody, incubate sections in 0.3% hydrogen peroxide for 15 minutes.
    a. Note: If endogenous peroxidase activity is still high then the H_2O_2 concentration can be increased up to 3%

6. Incubate sections with secondary antibody diluted into antibody solution for 2 hours at room temperature.

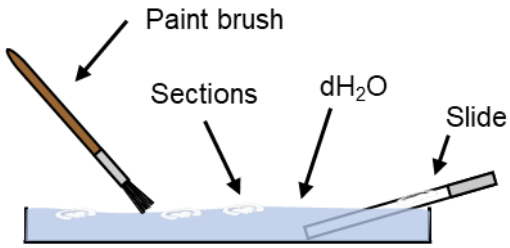
7. Wash sections three times with PBS-Triton for 5 minutes per wash

8. Incubate with detection solution following the manufacturer instructions. For Alkaline phosphatase conjugated secondary antibodies consider adding 1mM levamisole to the detection solution to inhibit endogenous phosphatases.

9. Wash sections three times with PBS-Triton for 5 minutes per wash to quench chromogen reaction.

10. Optional: Use a counterstain such as haematoxylin to visualise nuclei following manufacturer instructions.

11. Fill a wide petri dish with dH_2O. One well at a time transfer sections to the petri dish with a paintbrush then use the paint brush to float each section in turn onto a microscope slide lowered into the petri dish. Mount closest to the frosted section first then one in place slowly pull the slide out of the dH_2O to add the next layer of sections.
    a. A slide should fit around 6 coronal rat brain sections or 8 coronal mouse brain sections.



12. Once a full slide has been mounted allow the sections to air dry until slightly moist
    a. It can often be helpful to use a paper towel to remove excess moisture from the slide to speed drying.

13. Add a drop or two of mounting medium to each slide (when dry enough) then slowly lower the coverslip over the slide taking care to avoid bubbles.
    a. If using a hard-set mounting medium then no sealing is necessary but if not then use clear nail varnish to seal the edges of the coverslip to stop the sample drying out.

14. Allow the mounting medium to dry then store the slides at 4ºC until ready to image.

5.6 Slide Mounted Protocol (Fluorescent detection)

This protocol has been designed for use with sectioned brain tissue that has been fixed with 4% paraformaldehyde however is compatible with frozen sections too. Sections should be placed immediately onto microscope slides following sectioning for slide mounted immunohistochemistry.

1. Use a hydrophobic pen to draw round each section on the slide to prevent cross-contamination of liquids between sections.

2. Optional: use an antigen retrieval method (see section 4.2)

3. Wash sections 4 times for 10 minutes with PBS-Triton

4. Incubate sections with blocking solution in PBS-Triton for 30 minutes.
    a. The serum should be of the same species that the secondary antibody was raised in

5. Incubate sections in primary antibody diluted in antibody solution in PBS-Triton overnight at 4°C in a humidifying chamber.
    a. The antibody concentration will often need adjusting compared to when using a free-floating methodology. Generally a higher concentration will be needed.

6. Wash sections 3 times for 10 minutes with PBS-Triton

From now onwards all steps should be done in the dark to avoid bleaching of fluorophores conjugated to the secondary antibody.

7. Incubate sections with secondary antibody diluted in antibody solution in PBS-Triton for 2 hours

8. Wash sections 3 times for 10 minutes with PBS

9. Incubate sections in 1µg/ml DAPI in PBS for 5 minutes.

10. Wash sections once in PBS then once with dH_2O.

11. Air dry sections for 10 minutes until excess fluid has been removed but the sections remain moist.

12. Add a drop or two of mounting medium to each slide (when dry enough) then slowly lower the coverslip over the slide taking care to avoid bubbles.
    a. If using a hard-set mounting medium then no sealing is necessary but if not then use clear nail varnish to seal the edges of the coverslip to stop the sample drying out.

13. Allow the mounting medium to dry then store the slides at 4ºC in the dark until ready to image.

5.7 Slide Mounted Protocol (Chromogenic detection)

This protocol has been designed for use with sectioned brain tissue that has been fixed with 4% paraformaldehyde however is compatible with frozen sections too. Sections should be placed immediately onto microscope slides following sectioning for slide mounted immunohistochemistry.

1. Wash sections three times with PBS-Triton for 5 minutes per wash

2. Incubate sections in blocking solution for 2 hours at room temperature.

3. Incubate sections in primary antibody diluted into antibody solution overnight at 4ºC.

4. Wash sections three times with PBS-Triton for 5 minutes per wash

5. If using a HRP conjugated antibody, incubate sections in 0.3% hydrogen peroxide for 15 minutes.
    a. Note: If endogenous peroxidase activity is still high then the H_2O_2 concentration can be increased up to 3%

6. Incubate sections with secondary antibody diluted into antibody solution for 2 hours at room temperature.

7. Wash sections three times with PBS-Triton for 5 minutes per wash

8. Incubate with detection solution following the manufacturer instructions. For Alkaline phosphatase conjugated secondary antibodies consider adding 1mM levamisole to the detection solution to inhibit endogenous phosphatases.

9. Wash sections three times with PBS-Triton for 5 minutes per wash to quench chromogen reaction.

10. Optional: Use a counterstain such as haematoxylin to visualise nuclei following manufacturer instructions.

11. Allow the sections to air dry until slightly moist
    a. It can often be helpful to use a paper towel to remove excess moisture from the slide to speed drying.

12. Add a drop or two of mounting medium to each slide (when dry enough) then slowly lower the coverslip over the slide taking care to avoid bubbles.
    a. If using a hard-set mounting medium then no sealing is necessary but if not then use clear nail varnish to seal the edges of the coverslip to stop the sample drying out.

13. Allow the mounting medium to dry then store the slides at 4ºC until ready to image.

6. Image preparation

While images are often captured in proprietary software designed by microscope manufacturers we recommend using the free Fiji edition of ImageJ for further analysis (available at www.imagej.net). While excellent support is available (see www.imagej.net/learn), particular features to highlight for immunohistochemistry analysis includes the ability to:

- Remove background signal from images. Go to Process -> Subtract background

- Count cells either by hand or automatically (see this helpful guide)

- Calculate the area of part of the image. Use the drawing tools to make a shape then go to Analyse -> Measure.

- Where a Z-stack has been captured ImageJ is able to both Z project (Image -> Stacks -> Z-project) and 3D project (Image -> Stacks -> 3D project) the stack to maximise the information gained from the Z-stack.

When exporting images make sure to always save at as higher quality as possible using a .tif format. Avoid using formats such as .gif, .png or .jpeg as these can compress the image therefore losing potentially important details.

7. Solutions

10x PBS

Note: store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
NaCl	40g	80g	160g	1.37M
KCl	1g	2g	4g	27mM
Na_2HPO_4	7.2g	14.4g	28.8g	100mM
KH_2PO_4	1.2g	2.4g	4.8g	20mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

10x TBS

Note: store at room temperature

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
NaCl	40g	80g	160g	1.37M
Tris-base	12.1g	24.2g	48.5g	200mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

1x PBS-Triton / TBS-Triton

Notes:

- Triton X-100 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to made pipetting easier.

- The solution will need a good mixing with a stirring bar before being ready to use

- Generally make up fresh and don’t keep for longer than a few days.

- If staining for membrane bound proteins with an extracellular epitope consider omitting the Triton X-100. If over-permeabilisation is suspected then consider reducing the Triton X-100 concentration to 0.1% or substitute for 0.1% Tween-20

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
10x TBS / PBS	50ml	100ml	200ml	1x
dH_2O	450ml	900ml	1800ml	-
Triton X-100	1.5ml	3ml	6ml	0.3%

Cryoprotectant

Note: Ensure all sucrose is dissolved before adjusting to final volume.

Reagent	Amount to add			Final concentration
	250ml	500ml	1000ml	
Sucrose	75g	150g	300g	30%
0.1M Phosphate buffer	100ml	200ml	400ml	40mM
Ethylene glycol	75ml	150ml	300ml	30%
0.1M Phosphate buffer	Make up to final volume required			-

0.1M PB

Note: store at room temperature and make up fresh

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
Na_2HPO_4	5.45g	10.9g	21.8g	76.8mM
NaH_2PO_4	1.6g	3.2g	6.4g	26.7mM
dH_2O	≈400ml	≈800ml	≈1600ml	-
Conc HCl	Adjust to pH 7.4			-
dH_2O	Make up to final volume required			-

4% PFA in PBS

Notes:

- It is possible to buy either paraformaldehyde (PFA) in a powdered for or in a 40% solution. It is generally much easier to use the solution than powder.

- Over time 4% PFA can degrade therefore it is better to use a fresh solution than one that has been stored for a long period of time.

Solution

Notes:

- PFA is extremely toxic and a powerful skin irritant, only ever use in a fume hood.

Reagent	Amount to add			Final concentration
	50ml	100ml	500ml	
40% PFA	5ml	10ml	50ml	4%
PBS	45ml	90ml	450ml	-
Check pH and adjust to pH 7.4 if necessary				

Powder

Notes:

- Powdered PFA is extremely dangerous, only ever weigh out and prepare in a fume hood.

- Powdered PFA is not very soluble in water therefore the solution will require heating with a stirring bar until fully dissolved. This may take up to 3 hours.

- Take extreme caution not to overheat the solution while dissolving to avoid boiling of the solution and do not leave.

Reagent	Amount to add			Final concentration
	50ml	100ml	500ml	
PFA	2g	4g	20g	4%
PBS	50ml	100ml	500ml	-
Check pH and adjust to pH 7.4 if necessary				

DAPI staining solution

Notes:

- DAPI is normally stored as a 1mg/ml stock solution.
- Protect both the stock and working solution from light to avoid bleaching.
- DAPI is a potential mutagen therefore care should be taken to always handle with gloves while wearing correct PPE.

Reagent	Amount to add					Final concentration
	10ml	20ml	50ml	100ml	500ml	
1mg/ml DAPI	10µl	20µl	50µl	100µl	500µl	1µg/ml
PBS	10ml	20ml	50ml	100ml	500ml	-

Citrate antigen retrieval buffer

Note: Can be stored at room temperature for 2-3 months or longer at 4°C.

Reagent	Amount to add			Final concentration
	100ml	500ml	1000ml	
Tri-sodium citrate	0.294g	1.47g	2.94g	10mM
dH_2O	≈80ml	≈400ml	≈800ml	-
Conc HCl or conc NaOH	Adjust to pH 8.5			-
dH_2O	Make up to final volume required			-

Trypsin antigen retrieval buffer

Notes:

- Make up from freshly thawed trypsin stock to avoid degradation of the trypsin enzyme

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
0.5% Trypsin	0.5ml	1ml	5ml	0.05%
1% $CaCl_2$	0.5ml	1ml	5ml	0.1%
dH_2O	3ml	6ml	30ml	-
Conc NaOH	Adjust to pH 7.8			-
dH_2O	Make up to final volume required			-

1% Sodium borohydride

Notes:

- Make up fresh immediately before use

- Use in a well ventilated area away from any sources of ignition as $NaBH_4$ releases hydrogen gas when dissolved in water.

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
Tri-sodium citrate	0.05g	0.1g	0.5g	264mM
dH_2O	Make up to final volume required			-

0.05M Glycine

Note: Make up fresh as will rapidly suffer from microbial growth

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
Glycine	18.7mg	37.5mg	187.7mg	0.05M
dH_2O	Make up to final volume required			-

Blocking solution

Notes:

- Make up fresh immediately before use

- Blocking solutions can sometimes require a bit of optimisation to get perfect results therefore this recipe may need adjusting or replacing for an alternative. Increasing the concentration of serum would be the suggested first thing to change if there are struggles with high background.

- Use serum from the same species as that the secondary antibody was raised in.

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
BSA	0.1g	0.2g	1g	2%
Serum (from secondary host species)	0.15ml	0.3ml	1.5ml	3%
PBS-Triton	4.85ml	9.7ml	48.5ml	-

Antibody solution

Notes:

- Make up fresh immediately before use

- Use serum from the same species as that the secondary antibody was raised in.

Reagent	Amount to add			Final concentration
	5ml	10ml	50ml	
Serum (from secondary host species)	0.15ml	0.3ml	1.5ml	3%
PBS-Triton	4.85ml	9.7ml	48.5ml	-

8. Troubleshooting

Immunohistochemistry is a long multi-step process with many things that can go wrong and numerous factors that should be considered for a successful experiment. At some point it is inevitable that something will go wrong or not be optimal. Below are compiled some of the most common pitfalls that can cause sub-optimal immunohistochemistry results.

Problem	Potential cause	Suggested solutions
Weak or no staining	Too low antibody concentration	- Try increasing the concentration of primary antibody - Consider using an amplification system using biotinylated secondary antibodies
	Antibody incompatibility	- Ensure that the secondary antibody is compatible with the species and antibody subclass of the primary antibody
	High background is obscuring signal	- Try following suggestions detailed under the “High background or non-specific staining” section of this guide.
	Membrane damaged by permeabilisation reagents	- Try using a lower concentration of detergent. - Try using a weaker detergent (e.g. substitute Triton X-100 for Tween-20)
	Antibody is not suitable for IHC	- Some antibodies are not suitable for the way the antigen is presented in IHC. Check the datasheet to see if it has been previously tested in IHC or ICC and if not consider using a different antibody. - Try using a different fixation method which will present the antigen in a different way therefore potentially allow the antibody to bind.
	Low abundance of target protein	- Try increasing the primary antibody concentration - Try using an amplification method such as biotinylated secondary antibodies
	Fixation may be obscuring the target epitope	- Try one of the antigen retrieval methods described in section 5.2

Problem	Potential cause	Suggested solutions
Weak or no staining	Poor antibody penetration into tissue section	- Try incubating the antibody for longer or at higher concentration - Try using thinner tissue sections - Try using smaller primary antibodies (e.g. use IgG not IgM)
	Insufficient permeabilisation	- Try increasing the concentration of Triton-X100 in buffers. - If Tween-20 is being used consider switching to Triton-X100 which is a stronger detergent.
	Incompatible secondary fluorophores used with detection system	- Double check that the microscope system being used has the correct excitation and emission filters for the fluorophores being used. - Consider using different fluorophore conjugated secondary antibodies. At a minimum nearly all fluorescent microscopes have filter sets enabling imaging of DAPI and FITC / Alexa Fluor 488 / Dylight 488.
	Degradation of fluorophore following mounting	- Image sections within 2 days of mounting in immunofluorescent sections.
	Degraded buffers	- Check the pH of buffers before use - Be vigilant for signs of bacterial growth and throw out if observed. - Make fresh where necessary.
	Degraded primary antibody	- Ensure that the antibody is within the best before date supplied by the manufacturer. - Consider aliquoting antibodies, snap freezing then storing at -80°C in the future to avoid the risk of antibodies degrading.

Problem	Potential cause	Suggested solutions
Weak or no staining	Damaged fluorophore conjugated secondaries due to light bleaching	- Ensure that all steps carried out when using fluorophore conjugated secondaries are carried out either in dim light or the dark - When imaging try to use the minimum illumination possible to capture the best image. This is particularly important on confocal microscopes where high laser power settings can bleach some fluorophores extremely quickly. - Consider using fluorophores that are more resilient to bleaching compared to older compounds such as FITC or TRITC.
	Incompatible buffers	- Some antibodies perform better in TBS based buffers while others prefer PBS. Trying changing buffer to see if this increases staining.
	Over-fixation	- Try reducing the amount of time that the tissue is incubated with fixative. - Consider trying an alternative fixative solution
Bleed through between channels when multiplexing	Overlapping fluorophore excitation/emission spectra	- Try using well known pairs of fluorophores with limited overlap (e.g. DAPI, Alexa Fluor 488 and Alexa Fluor 594) - Use a spectral analysis tool (e.g. Spectraviewer) to make sure there is limited overlap between fluorophore pairs. - Try single antibody controls to see if signal in one channel is real or just due to bleed through. - Some advanced image analysis software has functions that can correct (to a degree) for bleed through.
	Unsuitable excitation and emission filters on imaging system	- Check compatibility between fluorophores and filters to ensure that each filter only captures on fluorophore. - For confocal microscopes consider tightening the window of light wavelengths allowed into the detector and use sequencing so only one laser is active for each fluorophore.

Problem	Potential cause	Suggested solutions
Altered tissue morphology	Ice damage	- Ensure in future experiments that tissue is frozen following established protocols such as those listed in this document. Where tissue sections are placed into 30% sucrose ensure that the tissue has fully sunk otherwise there may not have been sufficient time for the sucrose to diffuse into the tissue. - Consider introducing a scoring system for damage into analysis to assess if ice damage is influencing the experimental results.
	Rough handling of free-floating sections	- Using devices such as nets can help reduce the amount of handling that tissue sections require throughout the protocol. - Use high quality fine paintbrush for picking up and moving sections. Ensure that the tip has not been degraded by cryostat / microtome blades. - When mounting free-floating sections try to avoid touching sections as much as possible and instead use the brush to waft onto the slide.
	Degradation due to poor storage	- Sections can become contaminated with microbial growth in not properly stored. Consider using 0.05% sodium azide to prevent growth in storage solutions or freezing sections as detailed in section 4.4
	Too harsh antigen retrieval	- Consider switching antigen retrieval methods to a less harsh method
	Frozen sections detach from the slide	- Ensure that slides are always treated to ensure sections stick more effectively. While commercial varieties are available slides can be subbed in the lab following established protocols such as CSH Protocols; 2008; doi:10.1101/pdb.prot4804
	Incomplete fixation	- Incomplete fixation can cause tissue to degrade rapidly. Try increasing the amount of time tissue is incubated with fixative or consider increasing the concentration of fixative.

Problem	Potential cause	Suggested solutions
High background or non-specific staining	Auto fluorescent molecules in tissue section	- If tissue hasn't been perfused, consider this for the next experiment due to the strong autofluorescence caused by remaining red blood cells. - Autofluorescence can be caused by the fixative. Trying using a different fixative. - Ensure that $NaBH_4$ is freshly prepared - Try incubating sections with dyes that quench fluorescence (e.g. Pontamine sky blue, Sudan black or Trypan blue). - Try using fluorophores that are in a different wavelength from the autofluorescence (e.g. red shifted dyes such as Alexa Fluor 680)
	Non-specific secondary binding	- Run a no primary control to assess if the secondary is binding to the tissue section. - If the primary antibody is from the same species as the tissue this can cause major problems. See section 4.3 of this protocol or consider using a different species for the primary antibody.
	Too high primary antibody concentration	- Try reducing the concentration of primary antibody
	Too high secondary antibody concentration	- Try reducing the concentration of secondary antibody. We find a 1:300 to 1:500 dilution a good place to start.
	Insufficient purification of antibodies	- Un-purified polyclonal antibodies can contain antibodies that are cross-reactive with a range of non-target proteins. Check the datasheet; polyclonal antibodies should ideally be purified by affinity chromatography using the immunogen as bait. - While issues with monoclonal antibodies are rarer they should still be purified at least using Protein A or G affinity chromatography. - Where other alternatives are available consider switching to a better purified antibody or where alternatives are not possible consider purifying the antibody in-house.

Problem	Potential cause	Suggested solutions
High background or non-specific staining	Insufficient blocking	- Ensure that the blocking serum is from the same species as the secondaries were raised in. - Try using a different blocking solution. - Try increasing the length of the blocking step or increasing the concentration of serum
	Insufficient washing	- Try increasing the length and/or number of washes. - Ensure that there is sufficient agitation during washes to aide unbound antibody diffusion out of tissue
	Primary antibody is from the same species as the tissue sections.	- Run a no primary control to assess if the secondary is binding to the tissue section. - If the primary antibody is from the same species as the tissue this can cause major problems. See section 4.3 of this protocol or consider using a different species for the primary antibody.

	Endogenous enzyme activity (for chromogenic detection)	- Attempt to block endogenous enzyme activity with specific inhibitors.
		  ○ For HRP conjugated secondaries use 0.3% H_2O_2 for 10-15 minutes. If blocking is not successful at this concentration consider increasing to 3%.
		  ○ For AP conjugated secondaries use 2mM levamisole.

	Sections have dried out	- Ensure that sections always are kept moist and in a humidifying chamber for long incubations with slide mounted protocols.
	Too much substrate (for chromogenic detection)	- Try reducing the concentration of substrate or the incubation time.
	Too high signal amplification (if using biotinylated secondary antibodies)	- Try reducing the concentration of amplification reagents or secondary antibody. - Consider if signal amplification is necessary or whether a standard approach would work.

Problem	Potential cause	Suggested solutions
High background or non-specific staining	Thickness of tissue section	- The thicker the tissue section the more scattered light in a widefield microscope. Try either using thinner sections of switch to a confocal microscope.

9. Further Reading

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