

Immunocytochemistry Protocol

Say **hello** to ValidAbs™
Highly validated, data-rich antibodies

hellobio.com

CONTENTS

1. Introduction to ICC.....	3
2. Key Decisions.....	4
2.1 Isotype Controls	4
2.1.1 What are isotype controls?	4
2.1.2 How to choose isotype controls	5
2.2 How to choose a fixative	5
2.3 When to permeabilise	6
2.4 Choosing secondary antibody fluorophores	6
3. Equipment and consumables	7
4. ICC Protocols	8
4.1 Safety.....	8
4.2 Fixation	8
4.3 Antigen Retrieval (optional).....	8
4.4 Permeabilisation	9
4.5 Immunofluorescent staining	9
5. Image preparation	10
6. ICC solution recipes and preparation	12
10x PBS	12
1x PBST	12
1x PBS – Triton X-100.....	13
4% PFA in PBS	13
BSA + Glycine blocking solution.....	14
Serum blocking solution	14
Antibody incubation solution (1% BSA PBS-T).....	15
DAPI staining solution	15
Antigen retrieval buffer	15
7. Troubleshooting ICC problems	16
8. Further Reading.....	18

1. Introduction to ICC

Immunocytochemistry is a type of immunofluorescence technique in which antibodies are used to label cells directly upon either a microscope slide or coverslip. Adherent cells can either be grown directly on coverslips or cells in suspension can be pipetted onto a coverslip to proceed with the staining process. Figure 1 shows some example images that have been achieved following the protocols detailed within this guide.

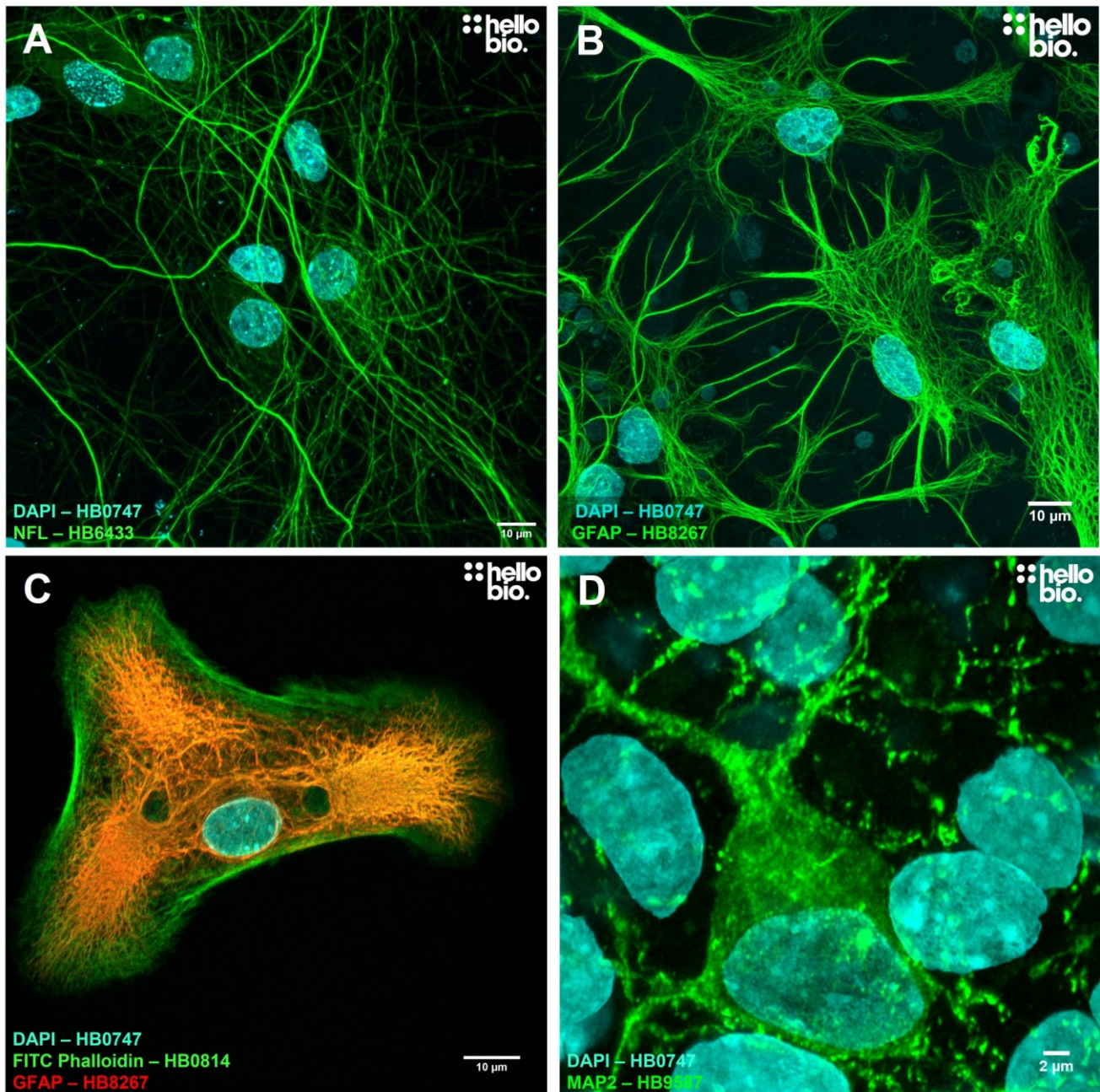


Figure 1. Example ICC images from cultured rat neurones using Hello Bio antibodies. A Neurofilament L staining reveals the dense cytoskeletal network of cultured neurones. **B and C** GFAP staining (HB8267) of astrocytes with FITC Phalloidin (HB0814) in C additionally being used to label actin filaments. **D** MAP2 staining (HB9587) reveals the cell body and processes of a cultured neurone. In all images DAPI (HB0747) is used to reveal cell nuclei.

2. Key Decisions

Before starting any immunocytochemistry it is important to consider the following points:

- Is it necessary to use an isotype control or will a no-primary replicate provide sufficient confidence that any staining seen is genuine (see section 2.1).
- What fixative will provide the best staining while also preserving structural morphology (see section 2.2).
- Whether to permeabilise the cells or not (see section 2.3)
- Whether antigen retrieval is necessary (see section 4.3)
- Which fluorophore combinations should be used for optimal signal (see section 2.4)

2.1 Isotype Controls

2.1.1 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.

2.1.2 How to choose isotype controls

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 How to use 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!

2.2 How to choose a fixative

Fixation prevents degradation of the sample alongside helping to maintain cellular structures to enable crisp images. Two methods are most commonly employed: methanol and paraformaldehyde. Fixation can however cause artefacts and interfere with detection of certain epitopes therefore it is important to consider which is the best fixative to use for each experiment.

Blocking solution	Recipe	Mechanism	Advantages	Disadvantages
Methanol	100% methanol (-20°C)	Causes precipitation of cellular proteins	<ul style="list-style-type: none">- Good when epitope is masked by PFA fixation.	<ul style="list-style-type: none">- Some antibody epitopes may be made inaccessible by precipitation.- May change membrane protein structure as dissolves lipids.- Causes cell shrinkage and structural changes- Not recommended for overexpressed fluorescent proteins

Blocking solution	Recipe	Mechanism	Advantages	Disadvantages
Para-formaldehyde (PFA)	4% PFA in PBS	Causes protein cross-linking via free amine groups	<ul style="list-style-type: none"> - Excellent preservation of morphology - Recommended standard fixative 	<ul style="list-style-type: none"> - Some antibody epitopes may be modified by cross-linking - Over-fixation can reduce epitope availability and antibody binding.

2.3 When to permeabilise

Antibodies are large protein structures therefore are unable to transit the plasma membrane. Dependent upon whether the target protein is intra-cellular or extracellular it may be necessary to permeabilise the cells to allow antibodies to cross the cell membrane and access the epitope.

Target protein	
Permeabilise	<ul style="list-style-type: none"> - Intracellular proteins - Membrane proteins where the antibody epitope is on the intra-cellular side of the membrane
Don't permeabilise	<ul style="list-style-type: none"> - Membrane proteins where the antibody epitope is on the extracellular side of the membrane

2.4 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 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)

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 required for immunocytochemistry are detailed within this section. A suitable imaging system is also needed for detection of fluorescently labelled cells with both widefield and confocal systems producing excellent images suitable for publication.

Equipment	Consumables
Rocker	PBS containing 0.1% Triton X-100
Cold room / fridge	PBS-T
Cell culture plates	Blocking solution
Microscope slides	Primary antibody
Small forceps	Secondary antibody
Optional: Water bath (able to heat to 95°C)	DAPI (available as HB0747 and HB8199)
Optional: Cover glass staining jar	BSA
	Mounting medium
	Clear nail varnish (if not using a hardset mounting medium)
	Optional: Antigen retrieval buffer

4. ICC Protocols

4.1 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:

- Methanol is highly flammable and toxic leading to blindness and kidney failure. Avoid breathing methanol vapors and avoid skin contact.
- Formaldehyde is a highly toxic poison, skin sensitizer and carcinogen and should only ever be used in a fume hood with full PPE.

4.2 Fixation

Fixation is the first stage in any immunocytochemistry protocol. Remove the cells from the incubator and optionally wash 3 times with ice-cold PBS. This step removes debris however if cells are poorly adhered can cause them to detach. If cells are poorly adherent omit this step.

PFA	Methanol
1. Incubate cells in 4% PFA in PBS (pH7.4) for 10 minutes at room temperature in a fume hood.	1. Incubate cells in chilled 100% methanol (-20°C) at room temperature for 5 minutes.
2. Wash cells three times (5 minutes per wash) with ice-cold PBS	2. Wash once with PBS

4.3 Antigen Retrieval (optional)

Fixation with formaldehyde based fixatives causes cross-linking of proteins which may obscure the epitope in the target protein therefore inhibiting antibody binding. Antigen retrieval aims to help re-expose the epitope to allow antibody binding however can cause damage to the morphology of cells.

Antigen retrieval in an optimal step and should only be done if previous attempts have not been successful or if the antibody vendor recommends use of antigen retrieval.

1. Preheat antigen retrieval buffer (100 mM Tris, 5% [w/v] urea, pH 9.5) to 95°C in a cover glass staining jar.
2. Place the coverslips in the antigen retrieval buffer for 10 minutes, maintaining the temperature at 95°C, contained within staining jar making sure that the side cells are on is always facing a consistent direction.
3. Remove the coverslips from the cover glass staining jar and return then to a 6-well culture plate making sure that the side with cells is always facing up.
4. Wash coverslips in PBS three times for 5 minutes per wash.

4.4 Permeabilisation

If permeabilisation is desired (see section 2.3) then proceed by:

1. Incubate the coverslips in PBS containing 0.1% Triton X-100 for 10 minutes.
 - a. This is a good starting point but may need optimizing if permeabilisation is too extensive or too poor.
 - i. Triton can be used in a range of 0.1 to 0.25% while other detergents such as Tween20 are less aggressive and can be used if permeabilisation is too high.
2. Wash cells in PBS three times for 5 minutes per wash.

4.5 Immunofluorescent staining

1. Incubate coverslips in blocking solution for 30 minutes at room temperature
 - a. There are multiple blocking solutions however the best starting point is 1% BSA, 300mM glycine in PBS containing 0.1% Tween20 (PBS-T).
 - b. If blocking is insufficient or the antibody datasheet recommends then another good solution is 2% BSA, 3% serum (ideally from the same species as the secondary antibody) and 300mM glycine in PBS containing 0.1% Tween20.
2. Incubate cells in diluted primary antibody in 1% BSA in PBS-T in a humidified chamber for 1 hour at room temperature or overnight at 4°C.
 - a. Follow the antibody datasheet for an initial suggested dilution then optimise for each application

- b. Tip: a plastic box containing a paper towel soaked in dH₂O can be a good alternative where a more expensive humidifying chamber is not available.
3. Wash coverslips three times in PBS for 5 minutes per wash with agitation.

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

4. Incubate cells with the secondary antibody in 1% BSA in PBS-T for 1 hour at room temperature.
 - a. Tip: Counterstains such as FITC Phalloidin ([HB0814](#)) or Rhodamine Phalloidin-TRITC ([HB8621](#)) can be added during the secondary incubation.
5. Wash three times with PBS for 5 minutes per wash.
6. Incubate cells with 1µg/ml DAPI for 1 minute to visualise cell nuclei
7. Wash with PBS once then briefly with dH₂O
8. Add a drop of mounting medium to each microscope slide then slowly lower the coverslip onto the slide taking care to avoid air 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.
9. Allow the mounting medium to dry then store the slides at 4°C in the dark until ready to image.

5. 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 immunocytochemistry 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.

6. ICC solution recipes and preparation

10x PBS

Notes:

- Store at room temperature
- If using PBS tablets (such as [HB5330](#)) then add 1/10 of the dH₂O specified by the manufacturer to make a 10x solution

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

1x PBST

Notes:

- Tween20 is extremely viscous therefore it is often helpful to cut the end off the pipette tip using scissors to make 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.
- Alternatively if using PBS tablets (such as [HB5330](#)) then follow manufacturer instructions to make 1xPBS then add Tween20 to a final concentration of 0.1% (1ml Tween20 / 1L of PBS)

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
10x TBS / PBS	50ml	100ml	200ml	1x
dH ₂ O	450ml	900ml	1800ml	-
Tween20	0.5ml	1ml	2ml	0.1%

1x PBS – Triton X-100

Notes:

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

Reagent	Amount to add			Final concentration
	500ml	1000ml	2000ml	
10x TBS / PBS	50ml	100ml	200ml	1x
dH ₂ O	450ml	900ml	1800ml	-
Triton X-100	0.5ml	1ml	2ml	0.1%

4% PFA in PBS

Notes:

- It is possible to buy either paraformaldehyde (PFA) in a powdered form 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				

BSA + Glycine blocking solution

Notes:

- Make up fresh and ensure BSA is fully dissolved before use

Reagent	Amount to add					Final concentration
	10ml	20ml	50ml	100ml	500ml	
BSA	0.1g	0.2g	0.5g	1g	5g	1%
Glycine	0.23g	0.45g	1.12g	2.25g	11.26g	300mM
PBS-T	10ml	20ml	50ml	100ml	500ml	-

Serum blocking solution

Notes:

- Make up fresh and ensure BSA is fully dissolved before use
- Use the same species serum as the secondary antibody for best results

Reagent	Amount to add					Final concentration
	10ml	20ml	50ml	100ml	500ml	
BSA	0.2g	0.4g	1g	2g	10g	2%
Serum	0.3ml	0.6ml	1.5ml	3ml	15ml	3%
PBS-T	9.7ml	19.4ml	48.5ml	97ml	485ml	-

Antibody incubation solution (1% BSA PBS-T)

Notes:

- Make up fresh and ensure BSA is fully dissolved before use

Reagent	Amount to add					Final concentration
	10ml	20ml	50ml	100ml	500ml	
BSA	0.1g	0.2g	0.5g	1g	5g	1%
PBS-T	10ml	20ml	50ml	100ml	500ml	-
Antibody	As incubated on datasheet					

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 (available as HB8199)	10µl	20µl	50µl	100µl	500µl	1µg/ml
PBS	10ml	20ml	50ml	100ml	500ml	-

Antigen retrieval buffer

Notes:

- If stored for a period of time, re-check pH before use

Reagent	Amount to add					Final concentration
	10ml	20ml	50ml	100ml	500ml	
Tris	0.12g	0.24g	0.61g	1.21	6.06g	100mM
Urea	0.5g	1g	2.5g	5g	25g	5%
PBS	10ml	20ml	50ml	100ml	500ml	-
Conc NaOH	Adjust pH to 9.5					

7. Troubleshooting ICC problems

Immunocytochemistry is a long multi-step process with many different factors that need to be considered. 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 immunocytochemistry to not work.

Problem	Potential cause	Suggested solutions
Weak or absent staining	Cells have detached from the coverslip	<ul style="list-style-type: none"> - Be more gentle during washes or reduce the number of washes
	Cells were poorly permeabilised	<ul style="list-style-type: none"> - Try increasing the concentration of Triton X-100
	Cells dried out	<ul style="list-style-type: none"> - Ensure that cells are always covered in an excess of liquid throughout the protocol
	Cells are over fixed	<ul style="list-style-type: none"> - Reduce the amount of time that cells are incubated with the fixative
	Too little primary antibody	<ul style="list-style-type: none"> - Increase the primary antibody concentration - Increase the incubation period - Consider moving to a three step detection system (e.g. biotin-streptavidin based)
	Light exposure	<ul style="list-style-type: none"> - Ensure that samples are never exposed to light once the secondary antibody has been applied - Use the minimum exposure possible while imaging and be cautious of bleaching (especially using confocal microscopes)
	Wrong secondary antibody	<ul style="list-style-type: none"> - Ensure the secondary antibody is specific for the species the primary was raised in
	Protein not present in target cells	<ul style="list-style-type: none"> - Check literature to see if protein is expected to be present in cell population - Consider using a positive control
	Epitope obscured by fixation	<ul style="list-style-type: none"> - Try a different fixative - Try adding an antigen retrieval step
Too short exposure	<ul style="list-style-type: none"> - Increase exposure time and/or gain when imaging 	

Problem	Potential cause	Suggested solutions
High background	Non-specific binding	<ul style="list-style-type: none"> - Try testing the secondary antibody without a primary to ensure the secondary is not binding to targets within the cells
	Poor blocking	<ul style="list-style-type: none"> - Try changing the blocking solution - Try increasing the blocking incubation time
	Too high antibody concentration	<ul style="list-style-type: none"> - Try reducing primary antibody concentration - Try reducing secondary antibody concentration
	Reactive aldehyde groups	<ul style="list-style-type: none"> - Consider introducing 1% NaBH₄ in PBS incubation step
	Spectral overlap	<ul style="list-style-type: none"> - Ensure that the absorption/emission spectra of secondary antibodies do not overlap
	Insufficient washing	<ul style="list-style-type: none"> - Increase washing steps after secondary incubation
Non-specific straining	Antibody is binding to epitopes present in other proteins	<ul style="list-style-type: none"> - Try a different antibody and see if the staining patterns correlate.
	Antibody concentrations too high	<ul style="list-style-type: none"> - Try reducing the primary and secondary antibody concentration
	Reactivity with endogenous IgGs	<ul style="list-style-type: none"> - Try using antibodies raised in species other than the source of the cells (especially in primary cell culture)

8. Further Reading

Glynn, M., McAllister, A. 2006. Immunocytochemistry and quantification of protein colocalization in cultured neurons. *Nat Protoc* 1, 1287–1296.

Burrry RW. 2011. Controls for immunocytochemistry: an update. *J Histochem Cytochem.* 59(1):6-12.

Matos LL, Trufelli DC, de Matos MG, da Silva Pinhal MA. 2010. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights.* 5:9-20.