Immunocytochemistry Protocol for ioGlutamatergic Neurons

ioCells™

Immunocytochemistry Protocol for ioGlutamatergic Neurons

0.10

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bit.bio
The Dorothy Hodgkin Building
Babraham Research Campus
Cambridge CB22 3FH
United Kingdom
www.bit.bio

Customer support:
technical@bit.bio
orders@bit.bio

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THE CELL CODING COMPANY

bit.bio
The Dorothy Hodgkin Building
Babraham Research Campus
Cambridge CB22 3FH
United Kingdom
www.bit.bio

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technical@bit.bio
orders@bit.bio

1. Introduction

This immunocytochemistry (ICC) / immunofluorescence (IF) protocol, developed and optimised at bit.bio, has been designed for ioGlutamatergic Neurons (cat no io1001) and derivative products.

Immunocytochemistry (ICC) staining is a powerful tool that enables specific proteins or other molecules to be quantitatively visualised in a cellular context. As such, ICC can provide insights into the distribution, localisation, and abundance of specific proteins or other molecules. While ICC staining of iPSC-derived cells is a relatively straightforward process, it does require careful consideration of antibody pairings, delicate treatment of cells, and optimisation to reduce non-specific signals.

The protocol outlines recommended reagents and concentrations for the fixation, permeabilisation, and antibody staining of key markers relevant to glutamatergic neurons. While these specific antibodies and dilutions are recommended for reliable outcomes, the core protocol can be adapted by researchers for use with other primary antibodies of interest.

This document focuses on the ICC procedure itself. It assumes that the thawing and culturing of the cells has been performed according to the instructions in the latest version of the ioGlutamatergic Neurons User Manual.

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2. Materials and equipment

2.1. Cells

- ioGlutamatergic Neurons (io1001) and derivatives

2.2. Reagents and equipment

- Biological safety cabinet with a carbon filter (MSC-CF)
- Normoxic cell culture incubator (37°C, 5% CO_2)
- -80°C freezer
- 1000 µL, 200 µL, 20 µL and 10 µL pipettes
- Standard light microscope
- Epifluorescent microscope
- 1.5 mL microcentrifuge tubes (Starlab, S1615 5510)
- 15 mL centrifuge tube, conical (Greiner Bio-one, 188271)
- 50 mL centrifuge tube, conical (Greiner Bio-one, 227261)
- 24-well plate TC-treated, sterile (Corning Costar®, 3526)
- DPBS, no calcium, no magnesium (ThermoFisher, 14190144)
- 16% paraformaldehyde (ThermoFisher, 11586711)
- Triton-X-100 (Sigma-Aldrich, T8787-50ML)
- Donkey serum (Sigma-Aldrich, D9663)
- Goat Serum (Sigma-Aldrich, G9023-5ML)
- DAPI (Hoechst) (Bio-Techne, 5748/10)

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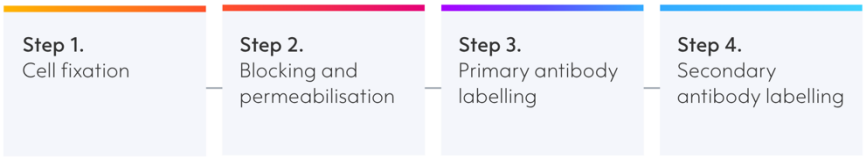
3. Protocol

The following protocol recommends general guidelines. We encourage users to optimise the critical steps according to their experimental conditions.

This protocol is specifically designed for 24-well plates. If using another plate format, refer to the supplier's information for the recommended media volumes.

This protocol is split into 4 main steps:

Step 1: Cell fixation
Step 2: Blocking and permeabilisation
Step 3: Primary antibody labelling
Step 4: Secondary antibody labelling



Do not allow the cell layer to dry out; leave behind approximately 50 µL in the well after removing media.

Before starting

Refer to the latest version of the ioGlutamatergic Neurons User Manual for complete details on cell coating, cell thawing, and cell culture.

If you need assistance, visit www.bit.bio/support-hub.

3.1. Cell fixation

3.1.1. Prepare 4% paraformaldehyde/DPBS, according to the instructions in section 4.1.
3.1.2. Carefully remove spent culture medium, without disturbing the cells.

Neuronal cells are sensitive to mechanical stress. Perform all media additions slowly and on the side of the well.

Always use micropipettes, not serological pipettes, to prevent cell detachment.

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3.1.3. Add 500 µL of DPBS to each well.
3.1.4. Incubate for 5 min at RT.
3.1.5. Remove the DPBS without disturbing the cells.
3.1.6. Carefully add 500 µL of cold (2-8°C) 4% paraformaldehyde/DPBS to each well.
3.1.7. Incubate for 10 min at RT.
3.1.8. Remove the solution from each well without disturbing the cells.
3.1.9. Carefully add 500 µL of DPBS to each well.
3.1.10. Incubate the cells for 5 min at RT.
3.1.11. Remove 450 µL DPBS without disturbing the cell layer.
3.1.12. Repeat 3.1.9 to 3.1.11 steps once more.
3.1.13. If staining immediately, proceed to section 3.2. Otherwise leave the cells in 500 µL DPBS and wrap the plate with parafilm. Store the plate 4°C and use within two weeks.

3.2. Blocking and permeabilisation

3.2.1. Prepare blocking solution as described in section 4.3
3.2.2. Carefully remove the DPBS.
3.2.3. Gently add 500 µL of blocking solution down the side of each well.
3.2.4. Incubate for 1 h at RT.

3.3. Primary antibody labelling

3.3.1. Prepare the primary antibody solutions described in section 4.4.
3.3.2. Following incubation step 3.2.4, aspirate blocking solution.
3.3.3. Carefully add 500 µL of the primary antibody mixture to the appropriate wells.
3.3.4. Add 500 µL of blocking solution to the negative control wells.
3.3.5. Seal plates with parafilm and incubate overnight at 4°C.

3.4. Primary antibody washes

3.4.1. Remove the liquid from each well without disturbing the cells.
3.4.2. Carefully add 500 µL of DPBS to each well.
3.4.3. Incubate cells for 5 min at RT.
3.4.4. Repeat steps 3.4.1 to 3.4.3 a further two times, leaving the cells in 500 µL of DPBS before moving on to the preparation of the secondary antibody solution.

3.5. Secondary antibody labelling

3.5.1. Prepare the secondary antibody solutions described in section 4.4.
3.5.2. Aspirate the DPBS from the wells.
3.5.3. Carefully add 500 µL of secondary antibody (with DAPI) to the appropriate wells.
3.5.4. Incubate cells with the secondary antibody mixture for 1 h at RT.

Protect the plate from light to prevent fluorophore bleaching; cover plates with foil.

3.5.5. After incubation, remove the liquid from each well without disturbing the cells.
3.5.6. Carefully add 500 µL of DPBS to each well.
3.5.7. Incubate the cells for 5 min at RT. Wrap the plates in foil to prevent the fluorophore from bleaching.
3.5.8. Repeat steps 3.5.5 to 3.5.7 a further two times, leaving the cells in 500 µL DPBS.
3.5.9. Image each well using fluorescent microscope with appropriate channel for each antibody

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4. Reagents and solutions preparation

4.1. Preparation of 4% paraformaldehyde/DPBS

Any handling of paraformaldehyde should be performed in an appropriate safety cabinet. Refer to the paraformaldehyde SDS for specific handling instructions.

4.1.1. Add 30 mL of DPBS to a 50 mL centrifuge tube.
4.1.2. Add 10 mL of 16% paraformaldehyde.
4.1.3. Mix gently and store at 4°C until use.

4.2. Preparation of 0.1% Triton-X-100/DPBS

4.2.1. Add 500 µL of Triton-X-100 stock solution to 500 mL of DPBS.

Triton-X-100 is viscous, consider using wide bore tips and/or a reverse-pipetting technique.

4.2.2. Mix thoroughly and store at RT until use.

4.3. Preparation of blocking solution

4.3.1. Add 28.2 mL of 0.1% Triton-X/DPBS to 50 mL centrifuge tube
4.3.2. Add 900 µL of Donkey serum
4.3.3. Add 900 µL of Goat serum
4.3.4. Mix gently and store at 4°C until use.

Goat Serum and Donkey Serum should be sterile filtered using a 0.22 µm filter prior to using.

4.4. Preparation of primary and secondary antibody solutions

4.4.1. Prepare the base antibody solution by diluting 1 mL of blocking solution in 9 mL of DPBS.
4.4.2. Refer to Table 1 (below) for antibody dilution recommendations. Dilute each antibody in a relevant volume of the base antibody solution.

Table 1 Validated antibody information for the general characterisation of ioGlutamatergic Neurons.

Antibody	Supplier	Cat no	Storage	Species	Dilution
Anti-Vesicular Glutamate Transporter 2 Antibody (VGLUT2)	Millipore	MAB5504	2°C to 8°C	Mouse	1/500
Anti-MAP2 antibody	Abcam	ab5392	2°C to 8°C	Chicken	1/2000
Purified anti-Tubulin β 3 (TUBB3) Antibody	Biolegend	801202H	2°C to 8°C	Mouse	1/1000
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher	A-21202	2°C to 8°C	Donkey	1/1000
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 647	Thermo Fisher	A-21449	2°C to 8°C	Goat	1/1000
DAPI (1mg/mL) We recommend 1mg/mL as a stock solution dissolved in water.	Bio-Techne	5748/10	2°C to 8°C	-	1/500

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5. Troubleshooting

Problem: Cells lifting or detaching during washes or antibody incubations

Likely causes
- Too vigorous aspiration/dispensing (serological pipettes, high suction)
- Over-permeabilisation (Triton-X-100 concentration too high or incubation too long)
- Inadequate coating of the plate surface

Fix
- Always leave ~50 µL when aspirating and use low-retention micropipette tips held at the side wall
- Always adhere to recommended Triton-X-100 concentration and incubation time
- Confirm plate coating per the user manual; consider re-coating if detachment persists

Problem: Very weak or no specific fluorescence signal

Likely causes
- Antibody concentration too low or antibody degraded (freeze–thaw damage)
- Insufficient incubation time or poor antibody penetration
- Fluorophore bleached by light exposure

Fix
- Prepare fresh primary antibody or dilute at the upper end of recommended range (e.g. 1:200 instead of 1:500)
- Always adhere to recommended incubation time
- Keep samples in the dark—wrap plates in foil during all secondary antibody incubation steps

Problem: Uneven staining (edge-brightening or centre-dullness)

Likely causes
- Poor reagent distribution (static plate)
- Evaporation at plate edges

Fix
- Make sure antibodies distribute evenly
- Avoid using only the outer wells for critical samples; include perimeter wells as ‘buffer’

Problem: Autofluorescence or bleed-through between channels

Likely causes
- Over-fixation (excessive PFA concentration or time)
- Imaging settings (filters or gain too broad/high)

Fix
- Ensure fixation is exactly 10 min at RT with 4% PFA; do not exceed
- Confirm that the installed filter sets match the specific requirements for Alexa Fluor 488, Alexa Fluor 647, and DAPI
- Reduce the exposure and/or gain to a lower level to establish a baseline and avoid overexposure

Problem: DAPI signal uneven or weak

Likely causes

- DAPI concentration too low or degraded

Fix

- Prepare fresh DAPI at 0.5 to 1 µg/mL in DPBS.

Our technical support team at bit.bio is available to answer any questions you may have about either the protocol or the cells, contact us at technical@bit.bio.

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