Immunocytochemistry Protocol for ioGABAergic Neurons

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1. Introduction

This immunocytochemistry (ICC) / immunofluorescence (IF) protocol, developed and optimised at bit.bio, has been designed for ioGABAergic Neurons (cat no io1003) 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 GABAergic 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 ioGABAergic Neurons User Manual.

1.1 Applicable ioCell products

This protocol was developed using wild-type ioGABAergic Neurons (io1003) and is suitable for the whole ioGABAergic Neurons product family of ioDisease Models, CRISPR-Ready ioCells, and ioTracker Cells.

For the full list of applicable products, please visit the ioGABAergic Neurons catalogue page linked here.

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2. Materials and equipment

2.1. 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)
- Goat Serum (Sigma-Aldrich, G9023-5ML)
- Donkey Serum (Sigma-Aldrich, D9663-10ML)
- DAPI (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 ioGABAergic 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 the following reagents, according to the instructions in section 4:

- 4% paraformaldehyde/PBS
- 1% Triton-X-100/DPBS
- Blocking Solution

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.

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

3.1.3. Add 500 µL of DPBS to each well.
3.1.4. Remove the DPBS without disturbing the cells.
3.1.5. Carefully add 250 µL of 4% paraformaldehyde/PBS to each well.
3.1.6. Incubate for 10 min at RT.
3.1.7. Remove the solution from each well without disturbing the cells.
3.1.8. Carefully add 250 µL of DPBS to each well.
3.1.9. Incubate cells for 5 min at RT.
3.1.10. Repeat steps 3.1.7 to 3.1.9 once more, leaving the cells in 250 µL of DPBS.
3.1.11. If the staining will not proceed immediately, wrap the plate with parafilm and store at 4°C overnight. Otherwise, continue to the next section.

3.2. Blocking and permeabilisation

3.2.1. Carefully remove the DPBS.
3.2.2. Gently add 250 µL of blocking solution down the side of each well.
3.2.3. Incubate for 1 h at RT.

3.3. Primary antibody labelling

3.3.1. Prepare the primary antibody solutions described in section 4.
3.3.2. After incubation step 3.2.3, aspirate blocking solution.
3.3.3. Carefully add 250 µL of the primary antibody mixture to the appropriate wells.
3.3.4. Add 250 µL of blocking solution to the negative control wells.
3.3.5. Seal plates with parafilm and incubate overnight at 4°C.
3.3.6. Remove the liquid from each well without disturbing the cells.
3.3.7. Carefully add 250 µL of DPBS to each well.
3.3.8. Incubate cells for 5 min at RT.
3.3.9. Repeat steps 3.3.6 to 3.3.8 a further two times, leaving the cells in 250 µL of DPBS before moving on to the preparation of the secondary antibody solution.

3.4. Secondary antibody labelling

3.4.1. Prepare the secondary antibody solutions described in section 4.
3.4.2. Aspirate the DPBS from the wells.
3.4.3. Carefully add 250 µL of secondary antibody (with DAPI) to the appropriate wells.
3.4.4. 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.

3.4.5. After incubation, remove the liquid from each well without disturbing the cells.
3.4.6. Carefully add 250 µL of DPBS to each well.
3.4.7. Incubate cells for 5 min at RT; wrap the plates in foil to prevent fluorophore bleaching.

3.4.8. Repeat steps 3.4.5 to 3.4.7 a further two times, leaving the cells in 500 µL of DPBS.
3.4.9. Image each well using a fluorescent microscope with the fluorescent channel most appropriate for each antibody.

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4. Reagents and solutions preparation

4.1. Preparation of 4% paraformaldehyde/PBS

Any handling of paraformaldehyde (PFA) 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 1% Triton-X-100/DPBS

4.2.1. Add 500 µL of Triton-X-100 stock solution to 50 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 12.5 mL of 1% Triton-X-100/DPBS to a 50 mL centrifuge tube.
4.3.2. Add 35 mL of PBS to obtain a final concentration of 0.25% Triton-X-100.
4.3.3. Add 1.25 mL of Goat Serum and 1.25 mL of Donkey Serum to the 50 mL centrifuge tube to obtain a final concentration of 5% of combined sera.

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

4.3.4. Mix gently and store at 4°C until use.

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 for antibody dilution recommendations. Dilute each antibody in a relevant volume of the base antibody solution.

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Antibody	Supplier	Cat no	Storage	Species	Dilution
VGAT Antibody (F-2)	Santa Cruz	sc-393373	2°C to 8°C	Mouse	1/200
Anti-GABA antibody	Sigma	A2052-100UL	-20°C to -80°C	Rabbit	1/500
Anti-MAP2 antibody	Abcam	ab5392	2°C to 8°C	Chicken	1/2000
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A-21202	2°C to 8°C	Donkey	1/1000
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 555	ThermoFisher	A-31572	2°C to 8°C	Donkey	1/1000
Goat anti-Chicken IgY (H+L) Secondary Antibody, Alexa Fluor 647	ThermoFisher	A-21449	2°C to 8°C	Goat	1/500
DAPI	Bio-Techne	5748/10	-20°C	-	1/500

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

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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

Suggested solution
- 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

Suggested solution
- 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

Suggested solution
- Make sure antibodies distribute evenly
- Avoid using 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)

Suggested solution
- 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 555, 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

Suggested solution

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

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

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