Immunocytochemistry Protocol for ioAstrocytes 2.0

ioCells™

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1.0

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1. Introduction

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This immunocytochemistry (ICC) / immunofluorescence (IF) protocol, developed and optimised at bit.bio, has been designed for ioAstrocytes 2.0 (cat no. io1110S) 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 astrocyte lineage markers, nuclear transcription factor SOX9, the intermediate filament protein Vimentin and the cytoplasmic marker S100b, generating high-quality data suitable for advanced analysis, including high-content imaging (HCI).

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 protocol has been optimised and validated at bit.bio to preserve the delicate morphology and ensure optimal epitope preservation necessary for ioAstrocytes 2.0. The use of a standard 4% Paraformaldehyde (PFA) fixation is balanced to prevent cellular damage while maximising antibody accessibility to all targets. Adherence to the exact 10-minute fixation time is essential, and users must always include a 'No Primary Antibody' control to validate the secondary antibody specificity.

This protocol has been optimised for use from 7 days post-thaw, which is the key characterisation window.

This document focuses on the ICC procedure itself. It assumes that the thawing and culturing of the cells have been performed according to the instructions in the latest version of the ioAstrocytes 2.0 User Manual.

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2. Materials and equipment

2.1. Cells

- ioAstrocytes 2.0 (io1110S)

2.2. Application-specific reagents and equipment

- Biological safety cabinet with a carbon filter (MSC-CF)
- Epifluorescent microscope
- 24-well plate TC-treated (Corning Costar, 3526; IBIDI, 82426)
- DPBS, no calcium, no magnesium (Thermo Fisher, 14190144)
- 16% paraformaldehyde (Thermo Fisher, 11586711)
- Triton-X-100 (Sigma-Aldrich, T8787-50ML)
- Heat-inactivated Fetal Bovine Serum (Thermo Fisher, 10100-147)
- Anti-SOX9 Antibody (Merck Millipore, AB5535)
- Anti-S100b Antibody (Sigma-Aldrich - S2532)
- Anti-Vimentin Antibody (abcam, ab8978)
- Donkey anti-Mouse IgG (H+L) Alexa Fluor™ 488 (Invitrogen, A-21202)
- Donkey anti-Rabbit IgG (H+L) Alexa Fluor™ 568 (Invitrogen, A10042)
- DAPI (Bio-Techne, 5748/10)

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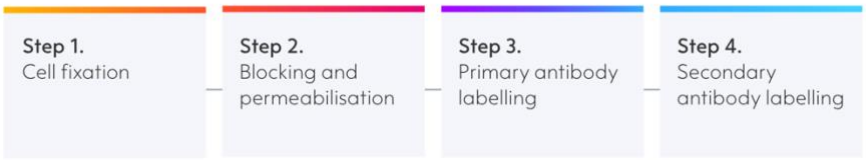
3. Protocol

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

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

This protocol has 4 steps:

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



Throughout this protocol, use a micropipette to remove and add liquid to wells, making sure not to disturb the cell layer.

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

ioAstrocytes 2.0 are sensitive to mechanical shear stress and may detach from the culture surface if liquids are removed or added to quickly or if the cells dry out.

Before starting:

Refer to the latest version of the ioAstrocytes 2.0 User Manual for complete details on cell coating, cell thawing and cell culture. At bit.bio, ICC is routinely performed at day 7 and day 14 of the culture.

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

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3.1. Cell Fixation

3.1.1. Prepare fixation solution (4% paraformaldehyde/DPBS) as described in section 4.1.
3.1.2. Carefully remove the medium from each well.
3.1.3. Carefully add 250 µL of fixation solution dropwise to the centre of each well.
3.1.4. Incubate at room temperature (RT) for 10 min.
3.1.5. Remove fixation solution without disturbing the cells.
3.1.6. Carefully add 250 µL of DPBS dropwise to the centre of the well and incubate at RT for 5 min.
3.1.7. Remove DPBS from the wells.
3.1.8. Repeat 3.1.6 to 3.1.7 two additional times.
3.1.9. Add 500 µL of DPBS dropwise to the centre of the well.
3.1.10. If not staining immediately, wrap the plate with parafilm and store at 4°C until use (up to 2 weeks). Otherwise, continue to the next section.

3.2. Blocking and permeabilisation

3.2.1. Prepare blocking solution as described in section 4.3.
3.2.2. Carefully remove the DPBS from the wells intended for staining and controls.
3.2.3. Add 250 µL of blocking solution dropwise to the centre of the well.
3.2.4. Incubate at RT for 1 hr.

3.3. Primary antibody labelling

3.3.1. Select test wells for each panel, pairing every test well with a corresponding secondary control well for each timepoint.
3.3.2. Prepare the primary antibody solutions as described in section 4.4.
3.3.3. Following incubation step 3.2.4, gently remove the blocking solution using a micropipette.
3.3.4. Carefully add 250 µL of primary antibody dropwise to the centre of the appropriate wells.
3.3.5. Carefully add 250 µL of blocking solution dropwise to the centre of the appropriate wells designated for secondary controls.
3.3.6. Seal plates with parafilm and incubate overnight at 4°C.
3.3.7. After the incubation, remove the liquid from each well using the micropipette without disturbing the cells.
3.3.8. Carefully add 250 µL DPBS dropwise to the centre to each well.
3.3.9. Incubate cells for 5 min at RT.
3.3.10. Repeat steps 3.3.7 to 3.3.9 two additional times, for a total of three washes, leaving the cells in 250 µL of DPBS before proceeding to secondary antibody labelling.

3.4. Secondary antibody labelling

3.4.1. Prepare the secondary antibody solutions (with DAPI) described in section 4.4.
3.4.2. Carefully remove DPBS from the wells.
3.4.3. Carefully add 250 µL of secondary antibody solutions (with DAPI) to all wells, including those designated for secondary controls.
3.4.4. Incubate cells with secondary antibody solutions for 1 hr at RT.

Protect the plate from light by covering in foil to prevent fluorophore bleaching.

3.4.5. After incubation, carefully 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 in the dark.
3.4.8. Repeat steps 3.4.5 to 3.4.6 two additional times.
3.4.9. Remove DPBS from each well and add 500 µL of fresh DPBS.
3.4.10. Image each well using a fluorescent microscope with the appropriate fluorescent channel for each antibody.

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4. Reagents and solutions preparation

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Volumes are indicative, please calculate exact volumes for your experimental setup, considering the number of wells and antibodies being tested.

4.1. Preparation of fixation solution (4% paraformaldehyde/DPBS):

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 0.25% Triton-X-100/DPBS

4.2.1. Add 1250 µL of Triton-X-100 stock solution to 500 mL of DPBS.
4.2.2. Mix thoroughly and store at RT until use.

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

4.3. Preparation of blocking solution

4.3.1. Add 47.5 mL of 0.25% Triton-X-100/DPBS to a 50 mL centrifuge tube.
4.3.2. Add 2.5 mL of heat-inactivated FBS to the 50 mL centrifuge tube to obtain a final concentration of 5% FBS in 0.25% Triton-X-100.
4.3.3. Mix gently and store at 4°C until use. Blocking solution is best used fresh, however it can be stored at 4°C for up to 7 days. Inspect the solution for visible contaminants before use; if any are present, prepare a fresh solution.

4.4. Preparation of primary and secondary antibody solutions

4.4.1. Prepare primary antibody solutions by diluting stock antibodies in blocking solution prepared according to sections 4.2 and 4.3. See Table 1 for dilutions of each antibody.

As the recommended antibodies are raised in the same species, two separate test wells and primary antibody solutions should be prepared.

4.4.2. Mix primary solution before use.
4.4.3. Prepare secondary antibody solutions by diluting stock antibodies in DPBS including DAPI.
4.4.4. Mix secondary solutions before use.

Table 1. Validated antibody information for the general characterisation of ioAstrocytes.

Antibody	Supplier	Cat no	Storage	Species	Dilution
Primary Antibody					
Panel 1					
Anti-Vimentin	abcam	ab8978	-20°C	Mouse	1/500
Panel 2					
Anti-SOX9	Merck	AB5535	2-8°C	Rabbit	1/500
Anti-S100b	Merck	S2532	-20°C	Mouse	1/1000
Secondary Antibody					
Panel 1					
Anti-mouse IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific	A-21202	2-8°C	Donkey	1/500
DAPI (1 mg/mL) We recommend 1 mg/mL as a stock solution dissolved in water.	Bio-Techne	5748/10	-20°C	-	1/1000
Panel 2					
Anti-rabbit IgG (H+L) Alexa Fluor 568	Thermo Fisher Scientific	A10042	2-8°C	Donkey	1/500
Anti-mouse IgG (H+L) Alexa Fluor 488	Thermo Fisher Scientific	A-21202	2-8°C	Donkey	1/500
DAPI (1 mg/mL) We recommend 1 mg/mL as a stock solution dissolved in water.	Bio-Techne	5748/10	-20°C	-	1/1000

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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).
- Inadequate coating of the plate surface.
- Pipette tip made contact with the cell layer.

Fix

- Always leave ~50 µL when aspirating and use low-retention micropipette tips held at the side wall without touching the bottom of the well.
- 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.
- Under- or over-fixation.

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.
- Ensure fixation with 4% PFA is incubated for 10 min at RT; do not exceed.

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 and ensure they have been well mixed in the solutions prior to adding to the plate.
- Avoid using only the outer wells for critical samples; include perimeter wells as ‘buffer’.
- If leaving the plates for extended periods, such as between the staining protocol and imaging, add a larger volume of DPBS to the wells.

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Problem: Autofluorescence or bleed-through between channels

Likely causes

- Over-fixation (excessive PFA concentration or incubation 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 568 and DAPI.
- Reduce the exposure time 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 reagent has degraded.

Fix

- Prepare fresh DAPI at 0.5 to 1 µg/mL in DPBS and perform a 5 min incubation with DAPI in PBS solution. Remove and add DPBS to the wells and continue imaging.

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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