Immunocytochemistry Protocol for ioMicroglia

ioCells™

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1.0

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1. Introduction

This immunocytochemistry (ICC) / immunofluorescence (IF) protocol, developed and optimised at bit.bio, has been designed for ioMicroglia (cat no io1021 and io1029) 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 microglia. 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 ioMicroglia User Manual.

1.1. Applicable ioCell products

This protocol was developed using wild-type ioMicroglia Male (io1021) and is suitable for the whole ioMicroglia product family of ioDisease Models, CRISPR-Ready ioCells, and ioTracker Cells.

For the full list of applicable products, please visit the ioMicroglia 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
- Rocking platform shaker, set to low speed
- 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)
- Bovine Serum Albumin (BSA) (Sigma-Aldrich, A7906)
- DAPI (Hoechst) (Bio-Techne, 5748/10)
- Parafilm (Fisher Scientific, 10018130)

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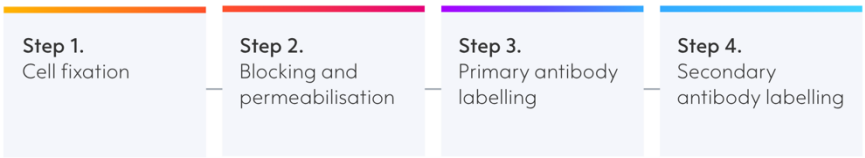
3. Protocol

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 ioMicroglia 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:
16% paraformaldehyde (PFA)
Permeabilisation buffer
Blocking buffer

3.1.2. Per well, slowly add 167 µL of 16% PFA to the 500 µL of media. With the existing medium in the well, the final concentration will be 4% PFA.

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

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3.1.3. Incubate for 10m at room temperature (RT).
3.1.4. Slowly remove the solution without disturbing the cells.
3.1.5. Gently add 500 µL of DPBS to each well.
3.1.6. Incubate plate for 5m at RT.
3.1.7. Repeat steps 3.1.4 to 3.1.6 a further two times, for a total of 3 DPBS washes, leaving the cells in 500 µL of DPBS.
3.1.8. Inspect cells down the microscope and record any changes to morphology, such as an active morphology characterised by rounded cells.
3.1.9. 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 from the wells.
3.2.2. Gently add 500 µL of permeabilisation buffer down the side of each well.
3.2.3. Incubate for 5m at RT while rocking the plate on the rocking platform shaker.
3.2.4. Carefully remove the permeabilisation buffer and replace it with 500 µL of blocking buffer.
3.2.5. Incubate for 1h at RT while rocking the plate on the rocking platform shaker.

3.3. Primary antibody labelling

3.3.1. Prepare the primary antibody solutions described in section 4.
3.3.2. After incubation step 3.2.5, aspirate blocking buffer using a micropipette.
3.3.3. Carefully add 500 µL of primary antibody dropwise down the side of the appropriate wells.
3.3.4. Add 500 µL blocking buffer to the two 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 500 µL of DPBS to each well.
3.3.8. Incubate cells for 5m at RT.
3.3.9. Repeat steps 3.3.6 to 3.3.8 a further two times, leaving the cells in 500 µL of DPBS before moving on to secondary antibody labelling.

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 using a micropipette.
3.4.3. Carefully add 500 µL of secondary antibody solution (with DAPI) to the appropriate wells.
3.4.4. Incubate cells with the secondary antibody mixture for 1h at RT while rocking the plate on the rocking platform shaker.
3.4.5. Cover plates with foil to prevent fluorophore bleaching.
3.4.6. After incubation, remove the liquid from each well without disturbing the cells.
3.4.7. Carefully add 500 µL of DPBS to each well.
3.4.8. Incubate cells for 5m at RT; wrap the plates in foil or place them in a cupboard to prevent fluorophore bleaching.
3.4.9. Repeat steps 3.4.6 to 3.4.8 a further two times, leaving the cells in 500 µL of DPBS.
3.4.10. 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 16% paraformaldehyde (PFA)

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

4.1.1. Remove the lid from a fresh 50 mL falcon tube.
4.1.2. Break the glass vial of the 16% PFA across the seal using an ampule breaker.
4.1.3. Add 10 mL of 16% PFA to the falcon tube.
4.1.4. Store at RT in a chemicals cabinet until used.

4.2. Preparation of permeabilisation buffer

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

4.3.1. Weigh 5 g of BSA powder.
4.3.2. Add 5 g of BSA powder to 100 mL of DPBS containing 0.1% Triton-X-100 to make a 5% BSA blocking buffer.
4.3.3. Mix thoroughly and store at 4°C until use.

4.4. Preparation of primary and secondary antibody solutions

4.4.1. Centrifuge the stock primary antibody tubes for 5s using a mini centrifuge.
4.4.2. Dilute antibodies in blocking buffer according to the recommended dilution described in Table 1.

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

Antibody	Supplier	Cat no	Storage	Species	Dilution
IBA1 Polyclonal	ThermoFisher	PA5-27436	-20°C	Rabbit	1/1000
TREM2 Monoclonal (clone 9H4L26)	ThermoFisher	702886	-20°C	Rabbit	1/100

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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 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)

Suggested solution

- Ensure fixation is exactly 10m at RT with 4% PFA; do not exceed
- Confirm that the installed filter sets match the specific requirements for the fluorophores selected 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

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6. Related resources

ioMicroglia User Manual
https://www.bit.bio/resources/user-manuals/microglia

Video tutorial on how to culture ioMicroglia
https://www.bit.bio/resources/video-tutorials/how-to-culture-microglia

ioMicroglia protocols page
https://www.bit.bio/products/glial-cells/microglia-wild-type-io1021?tab=protocols

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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