

# Immunocytochemistry Protocol for ioOPC-like cells and ioOligodendrocyte-like cells

ioCells™

Immunocytochemistry Protocol for  
ioOPC-like cells and  
Oligodendrocyte-like cells

1.0

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# 1. Introduction

This immunocytochemistry (ICC) / immunofluorescence (IF) protocol, developed and optimised at bit.bio, has been designed for ioOPC-like cells (cat no io1100) and ioOligodendrocyte-like cells (cat no io1028), and associated derivative products.

Immunocytochemistry (ICC) staining is a powerful technique that uses antibodies for the specific detection and visualisation of proteins or other molecules in a cellular context. As such, ICC staining can provide insights into the distribution, localisation, and abundance of target molecules.

For characterising oligodendroglial cells, scientists rely on cellular markers such as O4 and myelin basic protein (MBP). The O4 antibody recognises a lipid sulfatide, a common surface marker for oligodendroglial lineage cells. O4 is expressed from a late progenitor stage (late oligodendrocyte progenitor cells) towards a pre-myelinating oligodendrocyte stage. MBP is a myelin-associated protein, and its expression is characteristic of pre-myelinating and myelinating oligodendrocytes.

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 our range of oligodendroglial cells. 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 ioOPC-like cells User Manual and [ioOligodendrocyte-like cells User Manual](#).

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## 2. Materials and equipment

### 2.1. Cells

- ioOligodendrocyte-like cells (io1028) and derivatives
- ioOPC-like cells (io1100)

### 2.2. Reagents and equipment

- Biological safety cabinet with a carbon filter (MSC-CF)
- Normoxic cell culture incubator (37°C, 5% CO<sub>2</sub>)
- -80°C freezer
- 1000 µL, 200 µL, 20 µL and 10 µL micropipettes
- 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)
- 96-well plate TC-treated, sterile (Corning Costar®, 3596)
- DPBS, no calcium, no magnesium (ThermoFisher, 14190144)
- 16% paraformaldehyde (ThermoFisher, 11586711)
- Saponin (Sigma-Aldrich, 47036-50G-F)
- Goat serum (Sigma-Aldrich, G9023-10ML)
- Parafilm (Fisher Scientific, 10018130)



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

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



Throughout this protocol, use a micropipette to remove liquids from each well, 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.

## Before starting

### Select the protocol for your culture vessel format

24-well Plates	Follow Section 3. Protocol for 24-well plates
96-well Plates	Follow Section 4. Protocol for 96-well plates



If using another plate format, refer to the supplier's information for the recommended media volumes.

Refer to the latest version of the [ioOPC-like cells User Manual](#) and [ioOligodendrocyte-like cells User Manual](#) for complete details on cell coating, cell thawing, and cell culture.

If you need assistance, visit [www.bit.bio/support-hub](http://www.bit.bio/support-hub).

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## 3. Protocol for 24-well plates

### 3.1. Cell fixation

- 3.1.1. Prepare fixation solution (4% paraformaldehyde/DPBS) as described in section 5.
- 3.1.2. Carefully remove spent culture medium, without disturbing the cells.



ioOligodendrocyte-like cells and ioOPC-like cells 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.



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

- 3.1.3. Carefully add 200  $\mu$ L of DPBS by pipetting down the side of the wells.
- 3.1.4. Gently remove the DPBS without disturbing the cells.
- 3.1.5. Carefully add 200  $\mu$ L of room temperature (RT) fixation solution.
- 3.1.6. Incubate for 10 min at RT.
- 3.1.7. Gently remove the fixation solution without disturbing the cells.
- 3.1.8. Carefully add 200  $\mu$ L of DPBS to each well.
- 3.1.9. Gently remove the DPBS without disturbing the cells.
- 3.1.10. Add 400  $\mu$ L of DPBS per well, wrap the plate with parafilm and store at 4°C overnight or until staining. Staining should be done within 14 days of fixation.

### 3.2. Permeabilisation and blocking

- 3.2.1. Prepare the following reagents as described in section 5:
  - Permeabilisation solution (0.02% saponin in DPBS).
  - Blocking solution (10% normal goat serum in DBPS).
- 3.2.2. Gently remove the DPBS without disturbing the cells.
- 3.2.3. Carefully add 200  $\mu$ L of permeabilisation solution to each well.
- 3.2.4. Incubate for 15 min at RT.
- 3.2.5. Gently remove the permeabilisation solution without disturbing the cells.
- 3.2.6. Carefully add 200  $\mu$ L of blocking solution to each well.
- 3.2.7. Incubate for 30 min at RT.

### 3.3. Primary antibody labelling

- 3.3.1. Select one test well and one control well from each of your conditions and timepoints.
- 3.3.2. Prepare the primary buffer and primary antibody mixture described in section 5.
- 3.3.3. Following incubation step 3.2.7, gently remove the blocking solution using a micropipette.
- 3.3.4. Carefully add 200  $\mu$ L of primary antibody mixture by pipetting down the side of the test wells.
- 3.3.5. Carefully add 200  $\mu$ L primary buffer to the control wells.
- 3.3.6. Seal plates with parafilm and incubate overnight at 4°C.
- 3.3.7. Following the incubation, gently remove the solution without disturbing the cells.
- 3.3.8. Carefully add 200  $\mu$ L of DPBS to each well.

3.3.9. Incubate for 5 min at RT.

3.3.10. Repeat steps 3.3.7 to 3.3.9 a further two times, for a total of three wash steps, leaving the cells in 200  $\mu$ L of DPBS before moving on to secondary antibody labelling.

### 3.4. Secondary antibody labelling

3.4.1. Prepare the secondary antibody mixture (1% normal goat serum in DPBS + DAPI + secondary antibodies) described in section 5.

3.4.2. Gently remove the DPBS without disturbing the cells.

3.4.3. Carefully add 200  $\mu$ L of secondary antibody mixture by pipetting down the side of the wells.

3.4.4. Incubate for 1 h at RT.



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

3.4.5. Following the incubation, gently remove the solution from each well without disturbing the cells.

3.4.6. Carefully add 200  $\mu$ L 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 3.4.7 a further three times, for a total of four wash steps, leaving the cells in 400  $\mu$ L 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. Protocol for 96-well plates



Throughout this protocol, use a micropipette to remove liquids from each well, making sure not to disturb the cell layer.



Do not allow the cell layer to dry out; leave behind approximately 50  $\mu\text{L}$  in the well after removing media.

### 4.1. Cell fixation

4.1.1. Add 67  $\mu\text{L}$  of 16% paraformaldehyde (PFA) directly into the wells containing 200  $\mu\text{L}$  of medium.



ioOligodendrocyte-like cells and ioOPC-like cells are sensitive to mechanical shear stress and may detach from the culture surface if liquids are removed or added too quickly or if the cells dry out.



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

4.1.2. Incubate for 10 min.

4.1.3. Remove 75% of the liquid in the well (217  $\mu\text{L}$ ).

4.1.4. Add 150  $\mu\text{L}$  of DPBS to the well.

4.1.5. Remove 75% of the liquid in the well (150  $\mu\text{L}$ ).

4.1.6. Repeat steps 4.1.4 to 4.1.5 a further three times, for a total of four wash steps, leaving cells in a total of 200  $\mu\text{L}$ .

4.1.7. If not continuing with permeabilisation and blocking, wrap the plate with parafilm and store at 4°C for up to 1 week.

### 4.2. Permeabilisation and blocking

4.2.1. Prepare the following reagents as described in section 5:

- Permeabilisation solution (0.02% saponin in DPBS).
- Blocking solution (10% normal goat serum in DPBS).

4.2.2. Remove 75% of the liquid in the well (150  $\mu\text{L}$ ).

4.2.3. Add 150  $\mu\text{L}$  of permeabilisation solution to the well.

4.2.4. Repeat steps 4.2.2 and 4.2.3 once.

4.2.5. Incubate for 15 min at RT.

4.2.6. Remove 75% of the liquid in the well (150  $\mu\text{L}$ ).

4.2.7. Add 150  $\mu\text{L}$  of blocking solution to the well.

4.2.8. Repeat steps 4.2.6 and 4.2.7 a further two times.

4.2.9. Incubate for 30 min at RT.

### 4.3. Primary antibody labelling

- 4.3.1. Select one test well and one control well from each of your conditions and timepoints.
- 4.3.2. Prepare the primary buffer and primary antibody mixture described in section 5.
- 4.3.3. Following the incubation, gently remove 90% of the blocking solution using a micropipette (180  $\mu$ L).
- 4.3.4. Carefully add 100  $\mu$ L of primary antibody mixture by pipetting down the side of the test wells.
- 4.3.5. Carefully add 100  $\mu$ L primary buffer to the control wells.
- 4.3.6. Seal plates with parafilm and incubate overnight at 4°C.
- 4.3.7. Following the incubation, gently add 100  $\mu$ L of DPBS to the wells.
- 4.3.8. Remove 75% of the liquid in the well (150  $\mu$ L).
- 4.3.9. Add 150  $\mu$ L of DPBS to the well.
- 4.3.10. Repeat steps 4.3.8 and 4.3.9 a further three times for a total of four washes, leaving the cells in 200  $\mu$ L.

### 4.4. Secondary antibody labelling

- 4.4.1. Prepare the secondary antibody mixture (1% normal goat serum in DPBS + DAPI + secondary antibodies) described in section 5.
- 4.4.2. Gently remove 90% of the DPBS without disturbing the cells.
- 4.4.3. Carefully add 100  $\mu$ L of secondary antibody mixture by pipetting down the side of the wells.
- 4.4.4. Incubate for 1 h at RT.



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

- 4.4.5. Following the incubation, gently add 100  $\mu$ L of DPBS to the wells.
- 4.4.6. Remove 75% of the liquid in the well (150  $\mu$ L).
- 4.4.7. Add 150  $\mu$ L of DPBS to the well.
- 4.4.8. Repeat steps 4.4.6 to 4.4.7 a further four times, for a total of five wash steps, leaving the cells in 200  $\mu$ L of DPBS.
- 4.4.9. Image each well using a fluorescent microscope with the fluorescent channel most appropriate for each antibody.

## 5. Reagents and solutions preparation



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

### 5.1. 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.

- 5.1.1. Add 30 mL of DPBS to a 50 mL centrifuge tube.
- 5.1.2. Add 10 mL of 16% paraformaldehyde.
- 5.1.3. Allow the fixation solution to warm to RT before use.

### 5.2. Preparation of permeabilisation solution (0.02% saponin in DPBS)



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

- 5.2.1. To prepare a 5% saponin solution in DPBS:
  - Weigh 1 g of saponin powder into a 50 mL falcon tube.
  - Add 20 mL DPBS.
  - Vortex vigorously to fully dissolve the saponin.
  - Store solution at 4°C and use within 7 days of preparation.
- 5.2.2. To prepare permeabilisation solution (0.02% saponin in DPBS):
  - Add 80 µL of 5% saponin solution to 20 mL of fresh DPBS and vortex vigorously.

### 5.3. Preparation of blocking solution (10% normal goat serum in DPBS)

- 5.3.1. Mix 500 µL goat serum with 4.5 mL DPBS.

### 5.4. Preparation of primary buffer (0.02% saponin / 2% normal goat serum in DPBS)

- 5.4.1. Mix 10 µL of goat serum with 490 µL of permeabilisation solution (0.02% saponin in DPBS).

### 5.5. Preparation of primary antibody mixture (0.02% saponin / 2% normal goat serum in DPBS + primary antibodies)

- 5.5.1. Centrifuge the stock primary antibody tubes for 5 s using a mini centrifuge.
- 5.5.2. Dilute antibodies in the primary buffer according to the recommended dilution in Table 1.

### 5.6. Preparation of secondary antibody mixture (1% normal goat serum in DPBS + DAPI + secondary antibodies)

- 5.6.1. Add 10 µL goat serum to 1 mL DPBS.
- 5.6.2. Add 2 µL of DAPI (1 mg/mL stock concentration, for a final 2 µg/mL concentration) to the final 1 mL solution.

5.6.3. Centrifuge the stock secondary antibody tubes for 5 s using a mini centrifuge.

5.6.4. Dilute secondary antibodies according to the recommended dilution in Table 1.

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**Table 1** Validated antibody information for the general characterisation of ioOligodendrocyte-like cells.

Antibody	Supplier	Cat no	Storage	Species	Dilution
<u>Human/Mouse/Rat/Chicken Oligodendrocyte Marker O4 Antibody</u>	R&D Systems	MAB1326	-20°C	Mouse	1/100
O4 is supplied as a powder, bit.bio recommends reconstitution in 100 µL DPBS prior to use.					
<u>Anti-Myelin Basic Protein Antibody, a.a. 82-87</u>	Merck Millipore	MAB386	-20°C	Rat	1/100
<u>Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488</u>	Thermo Fisher	A-21202	2°C to 8°C	Donkey	1/500
<u>Donkey Anti-Rat IgG H&amp;L (Alexa Fluor 647)</u>	Abcam	ab150155	-20°C	Donkey	1/500
<u>DAPI (1mg/mL)</u> We recommend 1mg/mL as a stock solution dissolved in water.	Bio-Techne	5748/10	-20°C	-	1/500

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## 6. 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

**Fix**

- Always leave ~50  $\mu$ L when aspirating and use low-retention micropipette tips held at the side wall
- Confirm plate coating per the user manual; consider re-coating if detachment persists

**Problem:** Very weak or no specific fluorescence signal

**Likely causes**

- Insufficient incubation time or poor antibody penetration
- Antibody concentration too low or antibody degraded (freeze–thaw damage)
- Fluorophore bleached by light exposure
- Saponin permeabilisation is transient and can be reversed if removed from the system too quickly

**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 saponin is freshly prepared, included within the primary antibody solution and that incubation times are followed correctly.

**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'
- Ensure when leaving the plates for longer periods of time such as completion of the staining protocol and imaging, that a higher volume of DPBS is added to the wells

**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 other questions you may have about either the protocol or the cells, contact us at [technical@bit.bio](mailto:technical@bit.bio).

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