

Immunocytochemistry Protocol for ioSkeletal Myocytes

ioCells™

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ioSkeletal Myocytes

1.0

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1. Introduction

This immunocytochemistry (ICC) / immunofluorescence (IF) protocol, developed and optimised at bit.bio, has been designed for ioSkeletal Myocytes (cat no io1002) 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 skeletal myocytes. 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 [ioSkeletal Myocytes User Manual](#).

2. Materials and equipment

2.1. Cells

- ioSkeletal Myocytes (io1002) and derivatives

2.2. Reagents and equipment

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- Biological safety cabinet with a carbon filter (MSC-CF)
- Normoxic cell culture incubator (37°C, 5% CO₂)
- -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)
- DAPI (Hoechst) (Bio-Techne, 5748/10)

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.

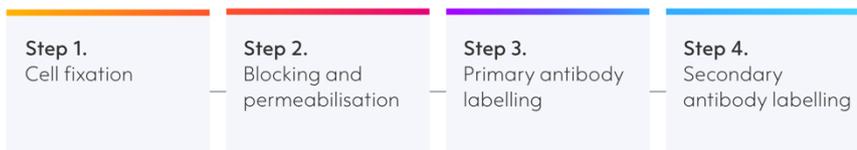
This protocol is split into 4 main steps:

Step 1: Cell fixation

Step 2: Blocking and permeabilisation

Step 3: Primary antibody labelling

Step 4: Secondary antibody labelling



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 μ L in the well after removing media.

Before starting

Refer to the latest version of the [ioSkeletal Myocytes 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 4% paraformaldehyde/PBD, 0.1% Triton-X-100/DPBS and Blocking solution according to the instructions in section 4.

3.1.2. Carefully remove spent culture medium, without disturbing the cells.



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

3.1.3. Add 500 μ L of DPBS to each well.

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- 3.1.4. Incubate for 5 min at room temperature (RT).
- 3.1.5. Remove the DPBS without disturbing the cells.
- 3.1.6. Carefully add 250 μ L of 4% paraformaldehyde/PBS to each well.
- 3.1.7. Incubate for 15 min at RT.
- 3.1.8. Remove the solution from each well without disturbing the cells.
- 3.1.9. Carefully add 500 μ L of DPBS to each well.
- 3.1.10. Incubate cells for 5 min at RT.
- 3.1.11. Repeat steps 3.1.8 to 3.1.10 once more, leaving the cells in 500 μ L of DPBS.
- 3.1.12. 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 500 μ L 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 500 μ L of the primary antibody mixture to the appropriate wells.
- 3.3.4. Add 500 μ L of 0.1% TritonX-100/DPBS 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 500 μ L 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 500 μ L 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.
- 3.4.3. Carefully add 500 μ L 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 500 μ L of DPBS to each well.
- 3.4.7. Incubate cells for 5 min at RT; wrap the plates in foil or place them in a cupboard 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 μ L of DPBS.
- 3.4.9. Image each well using a fluorescent microscope with the fluorescent channel most appropriate for each antibody.

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 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 29.1 mL of 0.1% Triton-X-100/DPBS to a 50 mL centrifuge tube.
- 4.3.2. Add 900 µL of donkey serum.



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

- 4.3.3. 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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Table 1 Validated antibody information for the general characterisation of
ioSkeletal Myocytes.

Antibody	Supplier	Cat no	Storage	Species	Dilution
Recombinant Anti-Desmin antibody [Y66]	Abcam	ab32362	2°C to 8°C	Rabbit	1/500
Anti-Dystrophin Antibody, clone 2C6	Merck	MABT827	2°C to 8°C	Mouse	1/100
Recombinant Anti-Myogenin antibody	Abcam	ab124800	2°C to 8°C	Rabbit	1/500
MF 20 antibody	UIOWA	MF-20	2°C to 8°C	Mouse	1/100
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	ThermoFisher	A21202	2°C to 8°C	Donkey	1/1000
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 647	ThermoFisher	A31573	2°C to 8°C	Donkey	1/2000
DAPI (1mg/mL) We recommend 1mg/mL as a stock solution dissolved in water.	Bio-Techne	5748/10	-20°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 μ L 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
- Consider using dPBS +/- for washes

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
- Incomplete incubation during washing steps

Fix

- Make sure antibodies distribute evenly
- Avoid using only the outer wells for critical samples; include perimeter wells as 'buffer'
- Ensure to incubate for full 5 minutes during every washing step

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

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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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