Immunofluorescent Labeling

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Introduction

Immunofluorescent labeling is a straight-forward technique to assessing the presence and subcellular localization of an antigen or a protein. Several labeling methods are available depending on the biological sample, cell preparation, and availability of antibodies against the target.

The method presented here has demonstrated utility in detecting the presence of triggering receptor expressed on myeloid cells 2 (TREM2), ionized calcium-binding adapter molecule 1 (IBA1), CX3-C motif chemokine receptor 1 (CX3CR1), spi-1 proto-oncogene (PU.1), purinergic receptor P2Y12 (P2RY12), and integrin subunit alpha M (CD11b) in iCell® Microglia.

This protocol describes plating the iCell Microglia on polyethyleneimine (PEI) coated surfaces as the cells do not adhere well to commonly used cell culture surfaces, such as Poly-D-Lysine and laminin, and the labeling of iCell Microglia for fluorescent immunocytochemistry assays.

Required Equipment, and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Microglia Quick Guide.

Item	Vendor	Catalog Number
Equipment		
High Content Imaging System or Fluorescent Microscope with Digital Camera	Multiple Vendors	
Consumables		
iCell Microglia Kit, 01279	FUJIFILM Cellular Dynamics	R1131
8% Paraformaldehyde (formaldehyde) Aqueous Solution	Electron Microscopy Sciences	157-8-100
Blocker™ BSA (10X) in PBS	ThermoFisher	37525
Borate Buffer, 20X	ThermoFisher	28341
Cell Culture Microplate, µClear, Black, CELLCOAT	Greiner Bio-One	655090 (96-well)
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (DPBS)	ThermoFisher	14190
Poly(ethyleneimine) Solution	Millipore Sigma	181978-100g
Sterile Tissue Culture Grade Distilled Water (Sterile Water)	Multiple Vendors	
Triton™ X-100 Detergent Solution	ThermoFisher	85111

Recommended Antibodies

The following table of primary and secondary antibodies provides the recommended dilution factor for labeling iCell Microglia. Select the appropriate combination of primary and secondary antibodies.

Item	Vendor	Catalog Number	Dilution Factor[1]
Primary Antibodies			
Goat Anti-TREM2	R&D Systems	AF1828	5 µg/ml[2]
Rabbit Anti-IBA1	FUJIFILM Wako Chemicals	0019-19741	1:250
Rabbit Anti-CX3CR1	Novus Biologicals	NBP1-76872	1:25
Rabbit Anti-Pu.1	Cell Signaling Technologies	2266S	1:50
Rabbit Anti-P2RY12	Millipore Sigma	HPA014518	1:25
Mouse Anti-CD11b/Integrin alpha M	R&D Systems	MAB1699	10 µg/ml[2]
Secondary Antibody			
Donkey anti-Rabbit IgG Alexa Fluor 488	ThermoFisher	A21206	1:250
Donkey anti-Mouse IgG Alexa Fluor 647	ThermoFisher	A31571	1:250
Donkey anti-Goat IgG Alexa Fluor 647	ThermoFisher	A21447	1:250
Nuclear Stain			
NucBlue™ Fixed Cell ReadyProbes™ Reagent	ThermoFisher	R37606	4 drops/ml

[1] 2X dilution

[2] The dilution factor varies based upon the lot-specific concentration.

Methods

Preparing the Cell Culture Surface

Plate iCell Microglia onto 0.7% poly(ethyleneimine) solution (PEI) coated vessels to improve adherence and minimize cell loss.

The following procedure details coating a 96-well cell culture plate with 0.7% PEI solution. Scale volumes appropriately for other vessel formats.

1. Prepare 100 ml of 1X borate buffer by diluting 5 ml of 20X borate buffer in 95 ml sterile water.
2. Pour approximately 1 ml of 50% PEI solution into a 15 ml centrifuge tube. Centrifuge the tube briefly to collect the PEI solution in the bottom of the tube.

   Note: *Due to the high viscosity of 50% PEI solution, pipetting is not recommended.*
3. Prepare an intermediate 7% PEI solution by adding 6 ml of 1X borate buffer per 1 ml of 50% PEI solution.
4. Mix the 7% PEI solution by vortexing.
5. Prepare a final 0.07% PEI solution by diluting 0.5 ml of 7% PEI solution in 49.5 ml of 1X borate buffer. Filter through 0.2 µm filter unit.
6. Add 100 µl/well of 0.07% PEI solution to the 96-well plate.
7. Incubate plate at 37 ºC for ≥ 1 hour.
8. Aspirate the PEI solution from the plate. Do not allow the wells to dry.
9. Rinse the plate 3 times with 200 µl/well of DPBS.
10. Allow the final DPBS rinse to remain in the plate until plating the cells.

Culturing iCell Microglia

1. Prepare the Maintenance Medium according to the iCell Microglia Quick Guide.
2. Thaw the iCell Microglia according to their Quick Guide.
3. Aspirate the DPBS from the PEI-coated plate immediately before dispensing the cells.
4. Culture the cells at 37°C, 5% CO_2.
5. Maintain the cells according to their Quick Guide until ready to perform immunofluorescent labeling.

Day 1

Labeling iCell Microglia: Fixation, Permeabilization, and Primary Antibody Incubation

The following procedure details labeling the iCell Microglia cultured in 96-well cell culture plates. Scale volumes appropriately for other vessel formats.

iCell Microglia are loosely adherent and require precautions to minimize cell loss during volume exchange steps including pipetting gently against the side of wells with a slow pipetting speed and allow some volume to remain in the wells during medium exchange steps.

1. Fix the cells with 4% paraformaldehyde solution by adding 100 µl of 8% Paraformaldehyde solution to each well containing 100 µl of maintenance medium.
2. Incubate the plate at room temperature for 15 minutes.
3. Prepare permeabilization buffer by diluting BSA solution to 1.5% (v/v), and Triton X-100 to 0.25% (v/v) in DPBS. Filter the permeabilization buffer through a 0.2 µM filter unit.
4. Slowly remove 175 µl of the paraformaldehyde solution from each well.
5. Slowly add 175 µl of permeabilization buffer to each well.
6. Incubate the plate at room temperature for 20 minutes.
7. Slowly remove 175 µl of permeabilization buffer from each well.
8. Slowly add an additional 175 µl of permeabilization buffer to the wells to further dilute the residual paraformaldehyde solution.

   Note: *The cells can be labeled immediately or stored at 4 ºC for up to 1 month when the plate is properly sealed to prevent evaporation.*
9. Prepare blocking buffer by diluting BSA Solution to 1.5% (v/v) in DPBS. Filter the blocking buffer through a 0.2 µM filter unit.
10. Dilute the primary antibody in blocking buffer. Use the dilution factor specified in the table above.
11. Slowly remove 150 µl/well of solution.
12. Add 50 µl/well of diluted primary antibody solution.
13. Incubate the plate at 4 °C overnight.

Day 2

Labeling iCell Microglia: Secondary Antibody Incubation and Nuclei Labeling

1. Wash the microglia by removing 75 µl/well of solution and slowly adding 175 µl/well of blocking buffer.

2. Wash the cells two additional times by removing 175 µl/well of solution and slowly adding 175 µl/well of blocking buffer.
3. Prepare the secondary antibody in blocking buffer. Use the dilution factor specified in the table above.

   Tip: *Include nuclear stain with the secondary antibody by adding it to the blocking buffer.*
4. Remove 150 µl/well of the blocking buffer wash.
5. Add 50 µl/well of diluted secondary antibody.
6. Incubate the plate at room temperature for 30 minutes, protected from light.
7. Wash the cells by removing 75 µl/well of solution and slowly adding 175 µl/well of DPBS.
8. Wash the cells two additional times by removing 175 µl/well of solution and slowly adding 175 µl/well of DPBS.
9. Acquire images of the plate using a high content imaging system or fluorescent microscope.

   Note: *If necessary, store plates with labeled cells at 4 ºC for up to 1 month, protecting from light and properly sealed to prevent evaporation.*

Example Data

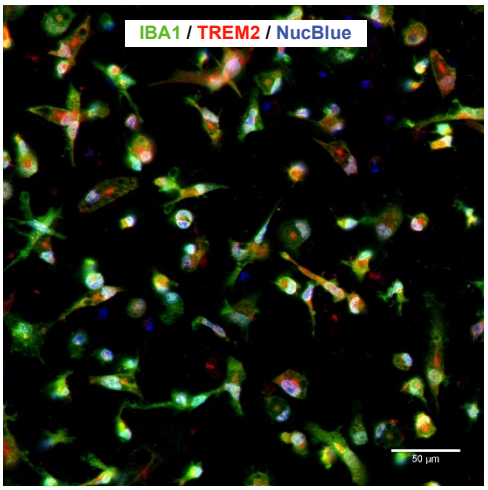

Figure 1: Immunofluorescent-labeled iCell Microglia

This image shows the majority of iCell Microglia, 01279 labeled with IBA1 and TREM2, characteristic microglia markers, one day post-plating. Nuclei were labeled with NucBlue Reagent.

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