Immunofluorescent Labeling

Introduction

Immunofluorescent labeling is a straight-forward technique for assessing the presence and the subcellular localization of antigens and/or proteins. Several labeling methods are available depending on the biological sample, cell preparation, and antibodies against the target. The protocol presented here has demonstrated utility in labeling cardiac troponin T, myosin light chain, sarcomeric alpha-actinin, connexin-43, and N-cadherin in iCell® Cardiomyocytes. This protocol should serve as a guide for immunofluorescent labeling other cardiac proteins.

Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Cardiomyocytes User's Guide.

Item	Vendor	Catalog Number
Equipment		
Fluorescent Microscope with Digital Camera	Multiple Vendors	
Consumables		
iCell Cardiomyocytes Kit	Cellular Dynamics International (CDI)	CMC-100-110-001 CMC-100-110-005 CMC-100-010-001 CMC-100-010-005
12-well Cell Culture Plates	Multiple Vendors	
15 mm Glass Coverslips	Warner Instruments	64-0703
Dulbecco's Phosphate Buffered Saline without Ca^{2+} and Mg^{2+} (D-PBS)	Invitrogen	14190
Fibronectin	Roche Applied Science	11051407001
Formaldehyde (37%)	Multiple Vendors	
Hoechst 33342	Invitrogen	H3570
Mounting Solution	Multiple Vendors	
Nonfat Dry Milk	Multiple Vendors	
Triton X-100	Sigma	X100

Recommended Antibodies

The following table of primary and secondary antibodies provides the dilution factor to use for labeling iCell Cardiomyocytes. Select the appropriate combination of primary and secondary antibodies.

Item	Vendor	Catalog Number	Dilution Factor
Primary Antibodies			
Mouse Anti-cardiac Troponin T	NeoMarkers	MS-295	1:200
Mouse Anti-sarcomeric Alpha-actinin	Abcam	ab9465	1:200
Rabbit Anti-cardiac Troponin T	Abcam	ab45932	1:200
Rabbit Anti-connexin 43	Abcam	ab11370	1:1000
Rabbit Anti-myosin Light Chain 2, Ventricular Isoform	ProteinTech	10906-1-AP	1:200
Rabbit Anti-N-cadherin	Abcam	ab76057	1:200
Secondary Antibodies			
Donkey Anti-mouse -647	Invitrogen	A-31571	1:500
Donkey Anti-rabbit -488	Invitrogen	A-21206	1:500

Methods

Culturing iCell Cardiomyocytes

1. Place a sterile coverslip in each well of a 12-well cell culture plate.
2. Dilute 1 mg/ml of fibronectin solution in sterile D-PBS to a final concentration of 5 µg/ml immediately before use.

   Note: *Reconstitute fibronectin in sterile water at 1 mg/ml according to the manufacturer's instructions. Aliquot and store at -20°C.*
3. Add 1 ml/well of 5 µg/ml fibronectin solution to the 12-well cell culture plate.
4. Incubate in a cell culture incubator at 37°C overnight.
5. Thaw iCell Cardiomyocytes according to the iCell Cardiomyocytes User's Guide.
6. Dilute the iCell Cardiomyocytes cell suspension in iCell Cardiomyocytes Plating Medium to 420,000 viable cells/ml. See the iCell Cardiomyocytes User's Guide for instructions to calculate the *Viable Cell Density*.
7. Aspirate the fibronectin solution from the 12-well cell culture plate. Immediately add 1.2 ml/well of the cell suspension (500,000 viable cells/well).
8. Culture iCell Cardiomyocytes in a cell culture incubator at 37°C, 7% CO_2.
9. Maintain the cardiomyocytes according to the User's Guide until ready to perform immunofluorescent labeling.

Notes

Notes

Labeling iCell Cardiomyocytes: Day 1 - Fixation, Permeabilization, and Primary Antibody Incubation

The following procedure details labeling iCell Cardiomyocytes cultured in 12-well cell culture plates. Scale volumes appropriately for other cell culture vessel formats.

1. Dilute 37% formaldehyde solution in D-PBS to a final concentration of 4%.
2. Wash the cardiomyocytes once with 1 ml/well of D-PBS .
3. Fix the cardiomyocytes with 1 ml/well of 4% formaldehyde solution at room temperature for 15 minutes.
4. Wash the cardiomyocytes twice with 1 ml/well of D-PBS for 5 minutes each wash.
5. Prepare the Blocking Buffer by diluting nonfat dry milk to 3% (w/v) and Triton X-100 to 0.1% (v/v) in D-PBS.
6. Incubate the cardiomyocytes with 1 ml/well of Blocking Buffer at room temperature for 15 minutes.
7. Dilute the primary antibody in Blocking Buffer. Use the dilution factor specified in the above table.
8. Aspirate the Blocking Buffer. Incubate the cardiomyocytes with 0.5 ml/well of diluted primary antibody rocking at 4°C overnight.

Labeling iCell Cardiomyocytes: Day 2 - Secondary Antibody Incubation and Nuclei Staining

1. Wash the cardiomyocytes twice with 1 ml/well of D-PBS for 5 minutes each wash.
2. Dilute the appropriate secondary antibody in D-PBS. Use the dilution factor specified in the above table.
3. Incubate the cardiomyocytes with 0.5 ml/well of diluted secondary antibody at room temperature for 30 minutes.
4. Wash the cardiomyocytes once with 1 ml/well of D-PBS for 1 minute.
5. Dilute Hoechst 33342 in D-PBS to 1:10000.
6. Incubate the cardiomyocytes with 0.5 ml/well of diluted Hoechst 33342 at room temperature for 15 minutes.
7. Wash the cardiomyocytes 3 times with 1 ml of D-PBS for 5 minutes each wash.
8. Mount the coverslips with mounting solution and take images using the fluorescent microscope.

   Note: *If necessary, store the slides with labeled cardiomyocytes at 4°C for up to 1 month, protecting from light and properly sealing to prevent evaporation.*

Notes

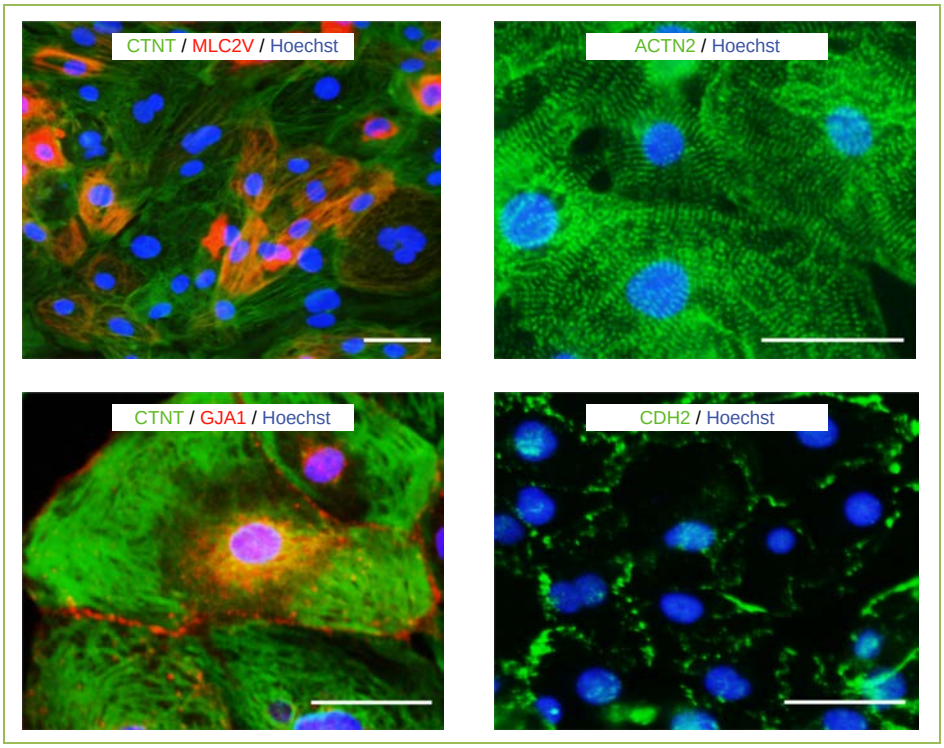

Figure 1: Immunofluorescent-labeled iCell Cardiomyocytes
These images show iCell Cardiomyocytes labeled for these proteins: cardiac troponin T (CTNT), ventricular myosin light chain 2 (MLC2V), sarcomeric alpha-actinin (ACTN2), connexin-43 (GJA1), and N-cadherin (CDH2). Nuclei were stained with Hoechst 33342. Scale bar = 25 µm.

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