Standard Operating Procedure: Flow Cytometry-Based Assessment of Troponin Positivity within hPSC-CM Cultures

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1 DOCUMENT INFORMATION

1.1 Purpose

This standard operating protocol (SOP) document is the result of evaluating the fit-for-purpose of a flow cytometry protocol for assessing the cardiomyocyte content in human pluripotent stem cell-derived cardiomyocyte (hPSC-CM) differentiation cultures. In an effort to respond to recent calls for increased scientific rigor in antibody use, we provide instructions and suggestions for the purpose of transparency, to minimize the need for duplication of the method development efforts, and to provide an easy-to-implement protocol that non-experts can implement with high success.

1.2 Scope

We expect that the information contained herein will be valuable for investigators new to this technique and will be consistent with the observations of laboratories with established expertise. Although the SOP has been successfully implemented in three laboratories, we encourage users to independently validate this protocol in their own laboratory.

1.3 Outline

The document is organized into the following sections:

> ***General Suggestions*:** a list of general observations, suggestions, and considerations for flow cytometry experiments
>
> ***Protocol for Routine Assessment of hPSC-CM*:** a streamlined, validated, and replicable protocol for assessing troponin-positive cells in hPSC-CM
>
> ***Fit-for-Purpose Study Design:*** details to be considered and strategies for evaluating the fit-for-purpose of a flow cytometry protocol
>
> ***Flow Cytometry Experiment Record***: a template for recording and reporting the details required to replicate a flow cytometry experiment

1.4 Abbreviations

The following abbreviations are used in this document:

- hPSC human pluripotent stem cells
- hPSC-CM human pluripotent stem cell-derived cardiomyocytes
- DPBS-/- phosphate buffered saline
- BSA bovine serum albumin
- w/v weight / volume
- DMEM/F12 Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
- DAPI 4',6-Diamidino-2-Phenylindole, Dihydrochloride)

2 GENERAL SUGGESTIONS

2.1 On the timing of experiments:

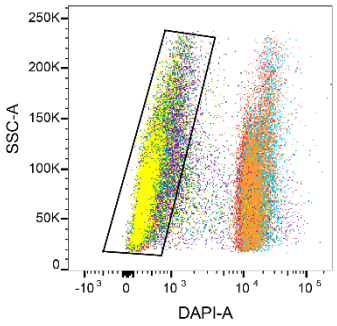
It is most desirable to prepare ALL buffers and analyze cells on the day of experiment for the cleanest flow cytometry data. In some cases, this is not possible or not favorable for experimental design such as those that include multiple time points. Some delay is tolerable between the fixation, staining, and analysis of cells – however freshly prepared buffers are always suggested.

- Cells can be fixed and then stained/analyzed at a future date (up to one week).
  - Store cells at 4°C in DPBS-/- w/0.5% (w/v) BSA.
  - Some cell types tolerate being stored in 0.01% formaldehyde in DPBS-/-.
- Cells can be fixed/stained and then analyzed at a future date (up to 72 hours).
  - Store cells at 4°C in DPBS-/- w/0.5% (w/v) BSA.
  - If stained with a fluorophore, keep cells in the dark.
  - Wait until the day of analysis to process cells through a filter-cap flow tube.

2.2 On the collection of cells:

Flow cytometry requires single cell suspensions. Cell dissociation solutions/enzymes (e.g. trypsin, collagenase, chelating agents), incubation times, and collection buffer may need to be altered for each cell type or antibody of interest.

- In general, longer incubation in enzyme solutions is favorable to trituration for facilitating dispersion.
- Trituration can affect various cell types differently. We have found hPSC-CM to be very sensitive to trituration, whereas hPSC and cardiac fibroblasts are more resilient.
  - Though avoiding trituration altogether is not advisable, our general rule for triturating hPSC-CM is ‘as little and as slowly as necessary’.
  - Trituration with a serological pipet or tapping on plates are more tolerated (though less effective) means of cell dispersion than using a P1000 pipette tip.
- Tapping plates with your palm can assist dispersion without compromising viability.
- Routinely assess viability post-collection by Trypan Blue exclusion.
- A more quantitative, flow cytometry-based, assessment of cell viability is suggested.
- Example data from a flow cytometry experiment using DAPI (BD Pharmigen 564907) to assess viability post-collection of hPSC-CM, according to manufacturer’s instructions. Using the protocol here, viability –as measured by DAPI exclusion – was ~94%. The side-scatter of events is plotted against the DAPI signal. Live cells are those which excluded DAPI, shown with the gate. The percentage of cells that within the gate are shown in the table on the right. Permeabilized cells are included as a positive control to demonstrate that DAPI could enter cells that were not intact.



Sample Name	% Live
DAPI, fix	96.9
DAPI, fix	94.7
DAPI, fix	91.4
DAPI, live	94.4
DAPI, live	94.3
DAPI, live	95.5
Fix, perm, DAPI	1.28
Fix, perm, DAPI	1.34
Fix, perm, DAPI	1.47

2.3 On the fixation and permeabilization of cells:

For each new antibody it is advisable to test different fixation and permeabilization methods. A suggested heuristic is to perform the minimal sample manipulation necessary to detect an epitope. For example, fixation and/or permeabilization may be avoided if using an antibody directed toward an extracellular epitope (e.g. the extracellular domain of a cell surface protein).

- Stabilized formaldehyde solution (*e.g.* stabilized with methanol) can affect flow cytometry results for some targets. We suggest that if using formaldehyde as a fixing agent, use a methanol-free formaldehyde stock.
    - Prepare fixation solution fresh on day of use.
    - Concentrated formaldehyde solutions (*e.g.* 16%) can be kept for up to a week (in the dark).
- Permeabilization agents are available in different concentrations so this should be noted when selecting stock reagents and recording experimental details.
    - Strong detergents (*e.g.* Triton or NP-40) permeabilize the nuclear membrane to a greater degree than mild detergent (e.g. saponin, digitonin), which are thought to primarily permeabilize the cell membrane. Depending on the localization of the desired target, a stronger permeabilization agent may be desirable.
    - In our experience, permeabilization by methanol renders cells difficult to work with (*i.e.* loose, clear pellet of cells).

2.4 On how many events to collect:

The ability to make conclusions about the reliability and accuracy of a flow cytometry observation has more to do with the inclusion of and comparison to proper controls than about the number of events.

- How to think about the various sources of error in measurement of population frequency by flow cytometry has explained exceptionally well in a recent publication [1].
    - "*The precision of a subset frequency is easily defined. The standard deviation for relatively rare populations is simply* $n^{1/2}$ *where "n" is the number of events comprising the subset. For a gate with a single event, the relative precision on its frequency is ±100%; for a gate with 1,000 events, it is ±3%. However, assay variation (biology, experimental, operator, etc.) is typically greater than 30%. Thus, once the number of events in a gate exceeds 10, the precision of the frequency measurement is dominated by assay errors, not the paucity of events analyzed."*
- Though likely excessive, statistically speaking, we default to collecting 10,000 events (with typically >70% of events being single cells). This keeps the precision of the measurement to ~1%.
    - With the flow rate on modern flow cytometers, the measurement of each sample can take less than 10 seconds.
    - The collection of more events can sometimes help clarify where distinct populations are clustering.

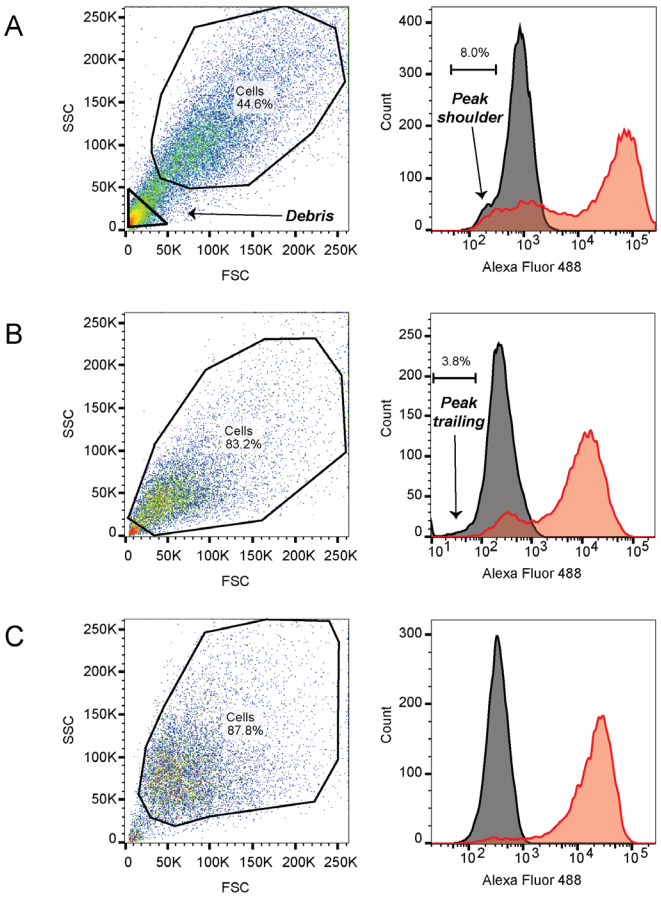
2.5 On interpreting the scatterplot:

Differences in scatterplots can provide information about the sample collection and buffer quality.

- Particulates in sample buffer can complicate data interpretation by interfering with the ability to distinguish cells from debris using forward and side scatter. We find increased particulate matter when serum is used or when the albumin solution is not fresh. This can be partially mitigated by filtering (0.2-0.4µm) flow buffer before use.
- Generally, >70% of the events should be single cells. A diminished single cell population may be due to excessive debris or low cell recovery. Assess cell quality and quantity by microscopy. Low cell recovery after staining may indicate loss of cells during wash steps, poor cell integrity/viability at collection, or sub-optimal fixation/permeabilization methods.
    - If the cluster of cells is not separated from debris or dead cells, histograms may trail off to the left or have a left shoulder (Section 2.6A-B). This can have a negative impact on the ability to interpret population frequencies (percent positive).
- Insufficient cell dispersion can result in doublets or cell clusters, causing a significant population to be off-axis of the scatterplot. When analyzing hPSC-CM, this can often be corrected by extending the incubation with Liberase-TH or other collagenases.

2.6 Examples of scatterplots:

As supporting information to Section 2.5, the following examples of scatterplots and the resulting histograms are provided. (A) Poor cell viability in collection causes a low percentage of events to be cells – due to increased debris – resulting in a significant left shoulder. (B) Forward and side scatter laser power set too low cause cells to cluster near debris resulting in left trailing of histogram. The same shape of histogram can be observed with old resuspension buffer. (C) Fresh buffer, proper cell collection, and appropriate laser result in high percentage of events being cells that cluster away from debris and off-axis.



3 PROTOCOL FOR ROUTINE ASSESSMENT OF hPSC-CM

3.1 Reagent List

Item	*Vendor, Catalog #*	*Item*	*Vendor, Catalog #*
Phosphate Buffered Saline (1X DPBS-/-)	Sigma #D8537	BSA	Sigma #A7906
RPMI	Thermofisher #11875-093	Saponin	Sigma #47036
Liberase-TH	Sigma #5401135001	Trypan Blue	Thermofisher #15250-061
DNase I	Sigma #10104159001	TrypLE	Thermofisher #12605-010
Accutase	Innovative Cell Tech. #AT104	Round bottom tubes	Fisher #14-961-10A
16% Formaldehyde Solution	ThermoFisher #28906	Filter top round bottom tubes	Fisher #352235
Solutions			
Liberase/DNase solution – 0.5U/mL Liberase-TH, 50U/mL DNase I in RPMI			
Fixation solution – 2% formaldehyde in DPBS-/-			
Flow buffer – 0.5% w/v BSA, 0.5% w/v Saponin in DPBS-/-			
Antibodies			
	Item	*Vendor, Catalog #*	
Primary Antibody (Select one)	Anti-TNNI3 clone 2Q1100-PE	US Biological #T8665-13F	
	Anti-TNNI3 clone C5	Fitzgerald #10R-T123k	
	Anti-TNNT2 clone 1C11-FITC	Abcam #ab105439	
Isotype control (Select based on primary)	IC mIgG2b	eBioscience # 14-4732-85	
	IC mIgG2b-PE	Millipore #MABF-1795	
	IC mIgG1-FITC	eBiosciences #11-4714	
Secondary Antibody	Anti-mIgG2b alexa488	Thermofisher # A21141	

3.2 Protocol

3.2.1 Important Notes

- All steps are performed at room temperature, unless otherwise specified.
- Experimental details are for collection of cells from 1 well of 6 well plate.
- Wash steps are performed by addition of 3 mL DPBS-/-, centrifugation at 200xg for 3 min, and aspiration of supernatant.

3.2.2 Cell Collection

3.2.2.1 hPSC-CM

1. Wash cells 1x with 2 mL of DPBS-/-
2. Add 1 mL of Liberase/DNase solution
3. Incubate cells for 30 min at 37°C
4. Add 1mL of TrypLE
5. Incubate for 5 min at 37°C, gently triturating with P1000 at 3 and 5 min
6. Collect cells with into 8mL of growth media in 15 mL conical tube, centrifuge at 200xg for 5 min
7. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4mL of DPBS-/-, perform cell count with trypan blue exclusion
    - If low cell recovery: ensure gentle trituration, collection performed with cell growth media, and viability should be >90%.

3.2.2.2 hPSC

1. Wash cells 1x with 2 mL of DPBS-/-
2. Add 1 mL of Accutase solution
3. Incubate cells for 4-6 min at room temperature
4. Collect cells by gentle trituration into 1mL of basal media in 15 mL conical tube, centrifuge at 200xg for 5 min
5. Resuspend the pellet by finger-flicking the tube, then slowly triturate in 4mL of DPBS-/-, perform cell count with trypan blue exclusion
    - If low cell recovery: ensure sufficient incubation in Accutase, gentle trituration, and viability should be >90%.

3.2.3 Cell Labeling and Preparation for Flow Cytometry

3.2.3.1 Fixation and Permeabilization

1. Place 1e6 cells in each 5 mL round bottom tube, centrifuge and remove supernatant, and resuspend the cell pellets in 100uL of *2% formaldehyde in DPBS-/-*
    - We have observed that many antibodies directed at cardiomyocyte contraction machinery will display false positive signal in negative cell type controls with methanol-based fixation.
2. For gentle agitation, place on cell rocker for 20 min
3. Wash 2x
4. Resuspend pellets in 100µL of *flow buffer*, incubate for 15 min to begin permeabilization and blocking

3.2.3.2 Antibody Labeling

1. Add appropriate amount of primary antibody to each tube
    - The antibody amounts listed below are based on our own titration experiments for the specific lot numbers we have used. However, users should titrate their own lots for confirmation
        - Anti-TNNI3 clone 2Q1100 – 0.5µg
        - Anti-TNNI3 clone C5 – 0.1µg
        - Anti-TNNT2 clone 1C11-FITC – 0.5µg
2. Incubate for 45 min
3. Wash 2x
4. *Optional: Secondary Antibody*. Resuspend pellets in 100µL of flow buffer, add secondary antibody, incubate 30 min, followed by wash 2x.
    - For ThermoFisher AlexaFluor antibodies, we typically find 0.6 µg secondary antibody per $1x10^6$ cells to be a suitable amount

3.2.3.3 Resuspension of Cells for Cytometer

1. Resuspend pellets in 450µL of DPBS-/- w/ 0.5% BSA
2. Wet 35 µm mesh filter cap on round bottom tube by placing 40 µL DPBS-/- with 0.5% BSA on top of the filter, then gently pass cell suspension through cap by holding the tip of the pipette ~2mm above the mesh and allowing gravity to pull through droplets.

3.2.4 Acquire data using a Flow Cytometer

1. While collecting data, adjust Forward and Side Scatter laser settings to manipulate the distribution of cells and debris within the scatterplot. Greater than 70% of events should

be within cell gate. If cytometer has an adjustable flow rate, perform this step on a slower setting to avoid using up sample before data acquisition.
    - As the ability to interpret collected data relies on the capacity to gate on single cells, much care should be taken in selecting the correct laser settings.
2. Fluorophore laser settings should be based on the fluorophore signal from the cell gate of the Isotype Control Sample – the signal should be centered within the log values of $1x10^2$ to $1x10^3$
3. Acquire 10,000 events for population of interest per experimental sample.

4 FIT-FOR-PURPOSE STUDY DESIGN

4.1 Marker Selection

Detection of a marker is only useful if it can be used to distinguish between cell types of interest. As such, selecting a marker that accurately reflects cell-type identity is critical.

- Proteins may not be completely unique to a cell type of interest. Therefore, a protein that serves as a marker of cell-type identity in one context may not be specific to that cell type in another context. **Markers are always context-dependent.**
- Literature is the most economical means of marker selection. However, previous publication does not guarantee that it's capacity to serve as marker was fully justified or validated. Also, consider how the experimental differences between published literature and your study may change the cell-type specificity.
- Although cell surface markers offer the significant advantage of being amenable to detection on live cells, thereby enabling live cell sorting, detection of cell surface proteins can be complicated because of their potential sensitivity to the enzymatic conditions sometimes employed to prepare single-cell suspensions for flow cytometry. In addition, the binding of antibodies to critical cell surface proteins can sometimes lead to altered biological states or to cell death.
- Markers can be informative in different ways.
    - A positive marker is present on the cell type of interest.
    - A negative marker is absent on the cell type of interest.
- Using antibody-independent approaches (*e.g.* mass spectrometry) to confirm the presence of the selected marker at the protein level is highly recommended.

4.2 Antibody Clone Selection

There are often many different antibodies available for detecting a protein of interest. Selecting an antibody clone deserves careful consideration of how the antibody was generated and the reported epitope.

- If available, testing more than one clone is highly recommended.
- For quantitative measurements, such as flow cytometry, monoclonal antibodies are essential.
- Antibodies can be identified using screens or can be generated by injection of peptides or protein (truncated or full-length) into an animal.
    - For techniques that detect the protein in its native form, it may be desirable to use an antibody that was generated against the full-length protein.
- The epitope of an antibody can have implications for its capacity to specifically detect the target. Though some features of the epitope may not preclude its use, they may affect interpretation of results.
    - If the epitope contains a site of post-translational modification (*e.g.* phosphorylation, acetylation), the biology of these modifications should be considered and how they affect epitope recognition by the antibody may not be predictable.
    - The sequence identity or homology of an epitope to other proteins should be investigated.

- Often, the same antibody clone is available from different vendors. The format (conjugated, unconjugated to fluorophore), concentration, and purification scheme can be relevant differences for selecting a particular formulation or vendor.
- Another important consideration is the isotype of the available antibodies as this factor can affect the ability to perform co-immunodetection experiments.

4.3 Antibody Screen and Protocol Selection

Each new antibody clone should be tested with multiple protocols using positive and negative controls. Establishing how sample preparation reagents affect antibody binding is an important aspect of data interpretation.

- An **essential** control for antibody testing is the negative cell-type control. This can be a cell type that does not contain the protein naturally, or a cell that has been manipulated (genetically or otherwise) to deplete the protein of interest.
    - Using an antibody-independent method to confirm the absence of a protein in the negative cell type control is suggested.
- Though the application of an ideal amount of antibody (determined by titration) is necessary to fairly judge antibody performance, the results of the screen may indicate clones, sample preparation strategies, or a combination of that are not worth testing further (*e.g.* when signal on the negative cell-type control is stronger than signal on the positive control).

4.4 Titration

Titration is performed to determine the antibody-to-cell ratio that produces the maximum intensity difference between negative and positive cells.

- Ideal amounts of antibody can range drastically, use published data or manufacturer's recommendations as a starting point.
    - We typically test 0.25x, 0.5x, 1x, 1.5-2x of manufacturer's recommendations
- Titration should be performed on negative and positive control cells and should test at least four amounts of antibody. The resulting histograms from the titration should span from insufficient signal to overstaining.
    - Insufficient signal – not enough antibody to saturate signal on the positive population
    - Overstaining – signal is relatively saturated on positive population, signal continues to increase on the negative population
- Titration should be performed on each new lot of antibody.
- In our experience, titrating primary antibodies seems to have a larger effect than titrating secondary antibodies.
    - If trying a new secondary, testing multiple concentration is advised.
    - Titrate the secondary antibody after the amount of primary has been titrated.
- Results are best when the total amount antibody for all samples is prepared as a stock such that no less than 1.5µL is added to an individual sample.

4.5 Verifying Specificity

To verify the specificity of an antibody, there are a variety of experiments that provide different types of evidence and each has their own caveats. The suggestions and experiments we outline here are those that we have found most informative and are consistent with several of the conceptual pillars for validation of antibodies proposed by the International Working Group for Antibody Validation [2].

4.5.1 Epitope Competition

A competition assay can be designed to test the specificity of an antibody for its epitope (if the epitope is reported) in which signal from naïve antibody is compared to antibody pre-incubated with peptide antigen. In this manner, a diminution or ablation of signal caused by incubation with the peptide antigen can be indicative of specificity for the reported epitope.

- A response-curve (*i.e.* testing multiple peptide-to-antibody ratios) provides further evidence of a specific relationship between antibody and epitope.
- The inclusion of additional peptide controls should be considered if the epitope is modified by post-translational modification or has high sequence conservation with other proteins that are present.
- The use of a negative control (*i.e.* a different peptide sequence) is recommended.
- A major caveat of this approach is that it uses peptides which may lack the secondary or tertiary structure of the native epitope. Though successful blocking is indicative of specificity, the failure to block should not be considered strong evidence that an antibody lacks specificity.

4.5.2 Immunofluorescent Imaging

Immunofluorescent microscopy offers the ability to visualize the localization of bound antibody. If the localization of a protein target is known, antibody binding in the expected localization pattern provides additional evidence of antibody specificity.

- Do not rely on database annotations for subcellular localization information if they lack experimental verification. Recognize that subcellular localization of a protein can be cell-type or context-dependent.
- If using immunofluorescence to help judge whether an antibody is specific, the same sample preparation strategy (*e.g*. fixation and permeabilization) used for flow cytometry should be applied.
- The quantitation of the percent of a population that is positive for a protein by microscopy is challenging, though possible.
    - Due to the high degree of cellular density in a well, passaging of cells should be considered. However, the degree to which passaged populations can change the relative proportion of subpopulations is not well-defined.
    - We suggest using imaging as a reference, without the expectation that percentages measured by flow and imaging will completely overlap.
- The importance of negative cell type controls still applies. However, in contrast to an isotype control, the use of a secondary-only control (*i.e.* secondary without primary) is more apropos for immunofluorescent imaging.

4.5.3 Co-immunodetection

A co-immunodetection experiment can be used to compare the subpopulations marked by two or more antibodies and the extent to which the marked populations overlap. Though a powerful technique to tease out the relationship between two clones or two subpopulations, co-immunodetection experiments are technically more challenging to plan and perform than routine single-color experiments.

- The antibodies included in a co-immunodetection experiment can be against different proteins or can be multiple clones against the same protein.
- Co-immunodetection experiments require careful consideration of the isotypes of antibodies and the secondary antibodies used for detection.
    - The brighter fluorophore is typically used for detecting the protein that is less abundant.
    - The use of conjugated primary antibodies can simplify experimental design.
- The use of multiple fluorophores requires compensation controls to deal with the potential issue of overlapping fluorescent spectra.

4.5.4 Mixed Population Experiment

Mixed population experiments demonstrate the capacity of the antibodies and protocol to discriminate between positive and negative cells within the same experimental sample, which is the ultimate test of suitability for assessing population heterogeneity. To perform this experiment, populations of cells are mixed together in known ratios immediately following their collection. The remainder of steps are performed in parallel on the set of mixed samples.

- The percent positivity measured can be compared to the expected positivity. The expected positivity should be calculated based on the percentages of the pure, unmixed populations.
- By mixing positive and negative cells in various ratios, a working range for percent positivity can be defined within which it is possible to distinguish negative and positive cells.
- Included below is the cell collection and mixing plan for testing used in this publication (where ‘protocol’ refers to Section 3 of this document):

  At least 3e6 cells of hPSC-CM and hPSC are required for this experiment. Perform fixation, antibody labelling, and acquisition steps per the protocol.

  1. Perform hPSC-CM collection per protocol. During Liberase-TH digestion, proceed to hPSC collection.
  2. hPSC collection: wash cells 1x with DPBS-/-, incubate cells with 1mL Accutase for 5 min, collect into 1mL of DMEM/F12, centrifuge at 200xg for 5min, resuspend in 2mL of DPBS-/-, perform count by trypan blue exclusion.
  3. Prepare the following six tubes for the mixed-population experiment
     a. 1.00e6 hPSC-CM
     b. 0.75e6 hPSC-CM , 0.25e6 hPSC
     c. 0.50e6 hPSC-CM , 0.50e6 hPSC (for isotype control)
     d. 0.50e6 hPSC-CM , 0.50e6 hPSC
     e. 0.25e6 hPSC-CM , 0.75e6 hPSC
     f. 1.00e6 hPSC

4.5.5 Additional Antibody Validation Experiments

We include several additional experiments that can be useful for verifying antibody specificity for certain applications, but which can have significant limitations for flow cytometry.

- *Overexpression* – heterologous expression systems can be designed to overexpress the target protein of an antibody. The challenge in interpreting these experiments is the difference in endogenous and heterologous expression levels and the differences in background between the systems.
- *Knockdowns* – experimental technique by which the amount of transcript for a specific gene is reduced. Cells can often compensate for decreased transcript by increasing translation. As the signal in flow cytometry experiments is a log-scale, a two-fold decrease in the amount of protein may not significantly affect the observed histogram.
- *Immunoprecipitation* – the precipitation of an antigen out of a solution by an antibody whereby mass spectrometry is used as the method of detection. The sensitivity of modern mass spectrometers combined with the sticky nature of proteins and affinity enrichment stationary phases leads to the detection of many proteins in an immunoprecipitation experiment. Though quantitative approaches exist to assist interpretation, the recognition of the antigen by the antibody is still outside the context of its native state within the cell.
- *Crosslinking mass spectrometry* – analyzes protein-protein interactions by chemical crosslinking interacting complexes. The major drawback of these experiments is the significant amount of technical expertise required for experimental design, sample preparation, method design, and data analysis

5 FLOW CYTOMETRY EXPERIMENT RECORD

The inclusion of key sample processing or reagent details required for the interpretation or replication of flow cytometry experiments are often omitted from publication. Good record keeping starts with the recording of details for each experiment (Sections 5.1 and 5.2) which will facilitate the subsequent inclusion of these details into publications (Section 5.3). Examples of forms we use to summarize the large amount of information required for, and generated during, a flow cytometry experiment are provided in the hope that this will facilitate other laboratories to record and report a similar level of detail called for by us and other sources [3, 4].

5.1 Protocol Details – Blank

This table includes the information required to replicate a flow cytometry experiment. This level of detail should be recorded for every flow cytometry experiment.

PROTOCOL DETAILS	
1. Fixation	
2. Wash	
3. Permeabilization	
4. Wash	
5. Block	
6. 1° Antibody	
7. Wash	
8. 2° Antibody (if applicable)	
9. Wash (if applicable)	
10. Resuspension	

REAGENTS	
Fixation	
Permeabilization	
Blocking / Antibody Binding	
Resuspension	
Block Solution =	
Wash Solution =	

ANTIBODIES USED			
Name	Catalog	Company	Lot

INSTRUMENT CONFIGURATION	
Instrument	
Laser line	
Emission filter	
Fluorochrome	

SAMPLES ANALYZED							
Tube #	Cell Type/ Notes	1 Antibody	Quantity (ug)	Antibody Volume (uL)	2 Antibody	Quantity (ug)	Volume Added (uL)
1							
2							
3							
4							

5.2 Protocol Details – Example

PROTOCOL DETAILS	
1. Fixation	20 minutes
2. Wash	two X 3mL
3. Permeabilization	Performed as one
4. Wash	15 minute
5. Block	incubation
6. 1° Antibody	45 minutes
7. Wash	two X 3mL
8. 2° Antibody (if applicable)	30 minutes
9. Wash (if applicable)	two X 3mL
10. Resuspension	500 µL

REAGENTS	
Fixation	2% Formaldehyde (w/v) in 1X DPBS-/-
Permeabilization	0.5% Saponin (w/v) in Block Solution
Blocking / Antibody Binding	0.5% Saponin (w/v) in Block Solution
Resuspension	Block Solution

Block Solution = 0.5% w/v BSA in DPBS-/-
Wash Solution = DPBS-/-

ANTIBODIES USED

Name	Catalog	Company	Lot
Anti-TNNI3 clone C5	10R-T123K	Fitzgerald	4326
IC mouse IgG2b	14-4732	eBiosciences	E04254-1634
Anti-mouse IgG2b - AlexaFluor 488	A21141	ThermoFisher	1723667

INSTRUMENT CONFIGURATION

Instrument	BD LSR II		
Laser line	488nm (50mw)	562nm (100mw)	640nm (100mw)
Emission filter	525/50	585/15	670/30
Fluorochrome	FITC / Alexa Fluor 488	PE	APC

SAMPLES ANALYZED

Tube #	Cell Type/ Notes	1 Antibody	Quantity (ug)	Antibody Volume (uL)	2 Antibody	Quantity (ug)	Volume Added (uL)
1	hPSC	IC mouse IgG2b	0.1	2uL of 1:9 dilution	Anti-mouse IgG2b -a488	0.6	0.3
2	hPSC	Anti-TNNI3 clone C5	0.1	2uL of 1:146 dilution	Anti-mouse IgG2b -a488	0.6	0.3
3	hPSC-CM	IC mouse IgG2b	0.1	2uL of 1:9 dilution	Anti-mouse IgG2b -a488	0.6	0.3
4	hPSC-CM	Anti-TNNI3 clone C5	0.1	2uL of 1:146 dilution	Anti-mouse IgG2b -a488	0.6	0.3

5.3 Flow Cytometry Details: Checklist for Publication

This checklist outlines experimental details that should be included when publishing flow cytometry data for the analysis of hPSC-CM. These suggestions are largely consistent with suggestions by MIFlowCyt[4], but are more relaxed for the purpose being approachable to users at all levels of expertise while still requiring the minimum information required for replication.

Sample Information

- ☐ Cell type and culture information
- ☐ Cell collection method
  - ☐ Enzymes
  - ☐ Solutions
  - ☐ Incubation time and temperature

Antibody Information

- ☐ Vendor
  - ☐ Primary antibody
  - ☐ Isotype control
  - ☐ Secondary antibody
- ☐ Catalog Number
  - ☐ Primary antibody
  - ☐ Isotype control
  - ☐ Secondary antibody
- ☐ Clone
  - ☐ Primary antibody
  - ☐ Isotype control
  - ☐ Secondary antibody
- ☐ Amount used
  - ☐ Primary antibody
  - ☐ Isotype control
  - ☐ Secondary antibody

Controls and antibody characterization

- ☐ Negative cell-type control information and results
- ☐ Titration information and results
- ☐ Any additional steps taken to ensure antibody specificity

Sample Preparation

- ☐ Solution composition (w/catalog numbers)
  - ☐ Fixation
  - ☐ Permeabilization
  - ☐ Blocking
  - ☐ Antibody Binding
  - ☐ Wash
- ☐ Incubation time and temperature
  - ☐ Fixation
  - ☐ Permeabilization
  - ☐ Blocking
  - ☐ Antibody Binding
  - ☐ Wash
- ☐ Centrifuge speed and time
- ☐ Resuspension buffer and use of filter

Data analysis

- ☐ Instrument information
  - ☐ Instrument name
  - ☐ Laser line for each fluorophore
  - ☐ Emission filter for each fluorophore
- ☐ Number of events collected
- ☐ Gating strategy for data analysis (e.g. exclude debris, singe cell discrimination, live/dead stain)
- ☐ Examples of scatterplots with gates drawn and percentages
- ☐ Compensation strategy and controls

6 REFERENCES

1. Roederer, M., *How many events is enough? Are you positive?* Cytometry A, 2008. **73**(5): p. 384-5.
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