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Authors

Zanella, Fabian
Sheikh, Farah

Publication Date

2014

DOI

10.1007/7651_2014_172

Peer reviewed



Published in final edited form as:

Methods Mol Biol. 2016 ; 1353: 147–162. doi:10.1007/7651_2014_172.

Patient-Specific Induced Pluripotent Stem Cell Models: Generation and Characterization of Cardiac Cells

Fabian Zanella and Farah Sheikh

Abstract

The generation of human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes has been of utmost interest for the study of cardiac development, cardiac disease modeling, and evaluation of cardiotoxic effects of novel candidate drugs. Several protocols have been developed to guide human stem cells toward the cardiogenic path. Pioneering work used serum to promote cardiogenesis; however, low cardiogenic throughputs, lack of chemical definition, and batch-to-batch variability of serum lots constituted a considerable impediment to the implementation of those protocols to large-scale cell biology. Further work focused on the manipulation of pathways that mouse genetics indicated to be fundamental in cardiac development to promote cardiac differentiation in stem cells. Although extremely elegant, those serum-free protocols involved the use of human recombinant cytokines that tend to be quite costly and which can also be variable between lots. The latest generation of cardiogenic protocols aimed for a more cost-effective and reproducible definition of the conditions driving cardiac differentiation, using small molecules to manipulate cardiogenic pathways overriding the need for cytokines. This chapter details methods based on currently available cardiac differentiation protocols for the generation and characterization of robust numbers of hiPSC-derived cardiomyocytes under chemically defined conditions.

Keywords

Human induced pluripotent stem cells; Cardiomyocyte; Cardiac differentiation; Cardiac assays; Small molecule; Wnt signaling pathway

1 Introduction

Since their establishment, human induced pluripotent stem cells (hiPSCs) have been considered a new gold standard for modeling human genetic-based diseases. Similar to human embryonic stem cells, hiPSCs have the potential to differentiate into virtually any cell type in the human body, offering enticing possibilities to study organ- and system-specific disorders, including diseases affecting the heart (1). Additionally, hiPSC-derived cardiomyocytes constitute a robust platform for the assessment of cardiotoxicity, a major contributor to the failure of potential novel drugs in later stages of clinical trials (2). Several genetic diseases bearing cardiac phenotypes have been modeled with hiPSCs including LEOPARD syndrome (3), long QT syndrome (4–7), Timothy syndrome (8), catecholaminergic polymorphic ventricular tachycardia (9–12), familial dilated (13) and

hypertrophic cardiomyopathies (14, 15), arrhythmogenic right ventricular cardiomyopathy (16–18), as well as an overlapping syndrome of a cardiac Na⁺ channel disease (19). As the field has evolved, several differentiation protocols have been developed for the differentiation of hiPSCs toward the cardiac lineages (1). Currently, the most popular protocols rely on small-molecule-mediated temporal modulation of the Wnt pathway (20, 21). Here we present an adapted version of a small-molecule-based protocol, which has been used successfully across many independent hiPSC control and diseased lines.

2 Materials

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| 2.1 Commercially Available Reagents. | See Table 1 |
| 2.2 Preparation of Extracellular Matrices (ECMs) | Add the corresponding volume of diluted ECMs to coat different tissue culture plates according to Table 2. |
| 2.2.1 Growth Factor-Reduced Matrigel | Thaw a 10 ml vial of growth factor-reduced matrigel on ice for 1 hour (h) or overnight at 4 °C. Add 10 ml of cold DMEM/F12 medium and mix quickly and thoroughly. Quickly prepare 600 µl aliquots, to be stored at –80 °C. |
| 2.2.2 Diluted Growth Factor-Reduced Matrigel | Thaw a 600 µl aliquot of growth factor-reduced matrigel on ice for 1 h or overnight at 4 °C. Add 50 ml of cold DMEM/F12 medium to a 50 ml falcon tube. Dilute the matrigel aliquot into DMEM/ F12 and quickly mix thoroughly by inverting the falcon tube several times. Cell culture plates are subsequently incubated with matrigel solution to coat surfaces. Coated plates may be stored at 37 °C for up to 5 days prior to cell seeding. |
| 2.2.3 Laminin | A 1 ml vial of laminin is thawed on ice and 50 µl aliquots are prepared for storage at –80 °C. For laminin coating, a 50 µl laminin aliquot is thawed on ice and diluted into a 5 ml solution of PBS+, mixed thoroughly and incubated on tissue culture dish surfaces overnight at 37 °C. Coated dishes may be stored at 4 °C for up to 7 days prior to cell seeding. |
| 2.3 Cell Culture Medium | hiPSCs are maintained in Essential 8 (E8) medium (22). Prepare stock solutions and make aliquots as indicated in Table 3. See Note 1. |
| 2.3.1 hiPSC Culture Medium | Prepare a 500 ml bottle of E8 medium by adding supplements according to Table 4. See Note 2. |
| 2.3.2 Differentiation Initiation Medium: RPMI Medium with B27 Supplement Without Insulin (RB–) | Take a 500 ml bottle of RPMI and add one 10 ml vial of B27 without insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at –20 °C. |
| 2.3.3 Differentiation Medium: RPMI Medium with B27 Containing Insulin (RB+) | Take a 500 ml bottle of RPMI and add one 10 ml vial of B27 with insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at –20 °C. |
| 2.3.4 Maturation Medium: DMEM/M199 Medium with B27 Containing Insulin (D/199B+) | Take a fresh bottle of DMEM and remove 140 ml of the medium. Add 125 ml of M199. Add one 10 ml vial of B27 with insulin. Add one 5 ml aliquot of P/S. Mix well. If the medium will be used within 1–10 days, then the bottle can be kept at 4 °C and 50 ml aliquots can be generated to be warmed when needed. Otherwise, 50 ml aliquots should be prepared for storage at –20 °C. |
| 2.4 Small Molecules | For a 10 mM (2,000×) solution, re-suspend 10 mg vial in 3.122 ml of DMSO. Mix well, vortex, and make 25–50 µl aliquots, to be stored at –20 °C. Aliquots may be reused up to three times. |
| 2.4.1 Rock Inhibitor/ Y27632: Rock_i | |

¹The solvent for all stock solutions is tissue culture-grade water, except for TGFβ, for which 10 mM citric acid with pH = 3 should be used. When preparing the ascorbic acid stock solution, pre-warm, pre-warm tissue culture-grade water to 37 °C before slowly dissolving with constant agitation and vortexing. Crystals may take several hours to dissolve completely. Store all aliquots of stock solutions at –20 °C.

²The cytokines in E8 medium are not stable at 37 °C for long periods of time. Therefore it is preferable to warm the medium to room temperature instead of 37 °C.

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| <i>2.4.2 CHIR99021: CH</i> | For a 12 mM (2,000×) solution, re-suspend 25 mg vial in 4.152 ml of DMSO. Mix well, vortex, and make 25–50 µl aliquots, to be stored at –20 °C. Aliquots may be reused up to three times. |
| <i>2.4.3 IWP-2</i> | For a 5 mM (1,000×) solution, re-suspend 10 mg vial in 4.286 ml of pre-warmed DMSO. Mix well, vortex, and make 25–50 µl aliquots, to be stored at –20 °C. Aliquots may be reused up to three times. |
| 2.5 Dissociation Reagents | Add 500 µl of 0.5 M EDTA to a bottle of PBS–. |
| <i>2.5.1 hiPSC Dissociation Solution: 0.5 mM EDTA</i> | |
| <i>2.5.2 Collagenase Type II Solution</i> | Cardiomyocytes are dissociated in a solution containing 200 units/ml of HBSS–. Estimate the required volume of collagenase II solution, and calculate and weigh the mass necessary to reach the target concentration in units/ml. Dissolve into HBSS– and filter sterilize. The solution can be kept at 4 °C for up to 1 week. |
| <i>2.5.3 Cardiomyocyte Dissociation Solution</i> | Prepare cardiomyocyte dissociation solution immediately before use. For 1 ml cardiomyocyte dissociation solution, prepare the stock solutions in tissue culture-grade water as described in Table 5. |
| 2.6 Buffers | In order to prepare 50 ml of immunofluorescence permeabilization buffer, dilute 100 µl of Triton-X in 50 ml of PBS+, for a final concentration of 0.2 % Triton-X. |
| <i>2.6.1 Immunofluorescence Permeabilization Buffer</i> | |
| <i>2.6.2 Immunofluorescence Dilution Buffer</i> | In order to prepare 50 ml of immunofluorescence buffer, prepare the stock solutions listed in Table 6 and add the appropriate volumes for the final desired concentrations. |
| <i>2.6.3 Immunofluorescence Blocking Buffer</i> | In order to prepare 10 ml of immunofluorescence blocking buffer, take 9.5 ml of immunofluorescence dilution buffer and add 500 µl of donkey serum for a final concentration of 5 %. |
| <i>2.6.4 FACS Dilution Buffer</i> | BD Perm/Wash buffer is sold as a 10× concentrate that must be diluted in PBS– before use. For 10 ml of 1× ready-to-use BD Perm/Wash buffer, add 1 ml of 10× concentrate BD Perm/Wash buffer to 9 ml of PBS–. |
| <i>2.6.5 FACS Blocking Buffer</i> | For 10 ml of FACS blocking buffer, take 9.5 ml of 1× ready-to-use BD Perm/Wash buffer and add 500 µl of donkey serum for a final concentration of 5 %. |

3 Methods

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| 3.1 hiPSC Culture | hiPSCs are grown as confluent monolayers on growth factor-reduced matrigel-coated 6 cm ² plates. Passaging is performed at a ratio of 1:4–1:10 every 4–6 days and achieved as outlined below: <ol style="list-style-type: none"> 1 Wash cells once with warm PBS–. 2 Add 2 ml of warm 0.5 mM EDTA in PBS–. 3 Incubate for 5 min at 37 °C. 4 Aspirate the 0.5 mM EDTA and re-suspend cells in 4 ml of E8 medium. If cells do not detach completely, use a cell lifter to gently scrap them and break larger clusters by pipetting 6–10 times. 5 Split cells as required and add Rock_i to a final concentration of 5 µM. |
| 3.2 hiPSC Seeding for Cardiac Differentiation | Cardiac differentiation is typically performed in a 12-well plate format, although other formats can be used with the appropriate adjustments to cell growth surfaces. Prior to seeding, coat a 12-well plate with diluted growth factor-reduced matrigel as described in Sections 2.2 and 2.2.1. <ol style="list-style-type: none"> 1 When hiPSCs (6 cm plate) reach 80–90 % confluence, aspirate E8 medium and wash cells once with 4 ml of warm PBS–. 2 Add 1.5 ml of warm Accutase, and move the plate in perpendicular directions to spread the enzyme evenly. Remove 1 ml of the Accutase and move the plate in perpendicular directions again. 3 Incubate hiPSC for 3–5 min at room temperature, until cells are singularized. See Note 3. |

- 4 Remove Accutase quickly and add 4 ml of maintenance medium. Pipette up and down 6–10 times to ensure that cells are singularized or reduced to very small clusters. Transfer cells to a 15 ml Falcon tube. Add 5 μ M of Rock_i (2.5 μ l of a 10 mM aliquot).
- 5 Count cells in the suspension.
- 6 Prepare 12.5 ml of a second suspension containing 5 μ M Rock_i in which the cell concentration is adequate for the desired number of cells to be plated in each well. *See Note 4.*
- 7 Plate 1 ml of the cell suspension prepared in step 4 in each well of the 12-well plate. Move the plate vigorously in perpendicular directions to ensure that cells are evenly distributed.

3.3 Cardiac Differentiation

The protocol described herein is an adaptation of a previously described small-molecule-based method (20, 23). The key differences are the following:

- 1 Washing hiPSCs before cardiac differentiation/induction. The purpose of this step is to remove traces of FGF at the beginning of differentiation.
- 2 Use of insulin during CH treatment. We have observed that cell death can significantly impact cardiac differentiation. Insulin, acting as a pro-survival cytokine, facilitates survival during the first 48 h of the cardiac differentiation protocol.
- 3 Washing hiPSCs after cardiac differentiation/induction. Insulin has been proposed to have a detrimental effect on cardiac differentiation after 72–96 h of induction (24). Therefore, cultures are washed with RPMI before the addition of RB⁻.
- 4 Switching to D/199B⁺ after day 30 of cardiac differentiation. After cardiomyocytes are differentiated, it is recommended that they are maintained in culture for an additional time to allow for further maturation. The nutrient composition of RPMI medium is relatively conservative; thus, cardiomyocyte death can occur also at later stages of differentiation/maturation due to stringent culture conditions. Thus, an adaptation of the medium used for neonate mouse cardiomyocyte cultures has been incorporated in this protocol to promote an environment richer in nutrients, allowing for enhanced cardiomyocyte health and survival during maturation.

3.3.1 Day 0: Induction

- 1 When hiPSCs plated on the 12-well plate reach 100 % confluence (within 2–5 days of plating), warm an aliquot of RPMI and one of RB⁺ medium.
- 2 Take 24.5 ml of RB⁺ and add CH to a final concentration of 6 μ M. Mix well. *See Note 5.*
- 3 Wash cells once with warm RPMI medium (1 ml per well).
- 4 Add 2 ml of RB⁺ with CH to each well. Record the time at which CH is added.

3.3.2 Day 2: Wash and Switch to RB⁻

After CH treatment, mild to considerable cell death is expected. Proceed with the protocol as outlined below:

- 1 Warm an aliquot of RPMI and RB⁻ medium.
- 2 48 h after adding CH to the cells:

³Since Accutase is an aggressive enzyme, cell dissociation should be monitored frequently as some cell lines will singularize and even detach at a shorter time. If it is noted that cells start to peel off, remove the enzyme immediately. If all cells become detached, add 3 ml of maintenance medium, pipette up and down to collect all cells, and transfer to a 15 ml Falcon tube. Add 5 μ M of Rock_i (2.5 μ l of a 10 mM aliquot) and spin at 800 rpm for 5 min. Re-suspend cells in 4 ml of maintenance medium, add 5 μ M of Rock_i, and proceed to next step (4).

⁴Seed 1×10^5 – 5×10^5 cells per well in matrigel-coated 12-well plates. Cell density should be optimized for each cell line, so confluence is reached within 2–5 days after plating. Ideally, cells should reach confluency within wells at the same approximate time frame that it took to reach confluency in 6 cm plates.

⁵CH aliquots are prepared at 12 mM (2,000 \times), so for 24.5 ml of RB⁺, 12.25 μ l of a CH aliquot is required. Also note that 6 μ M CH for 48 h seems to be optimal for most cell lines; however, specific lines may have different requirements. It is recommended that a range of 6–14 μ M is tested at treatment durations of 24 h (12–14 μ M) and 48 h (6–8 μ M).

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- a. Wash cells once with warm RPMI medium (2 ml per well).
- b. Add 2 ml of RB- to each well.
- 3.3.3 Day 4: IWP-2 in Conditioned Medium
- 1 Warm an aliquot of RB- medium.
 - 2 Collect 1 ml of conditioned medium from each well of the differentiation plate at 96 h after adding CH, plus an excess of 0.5 ml, and transfer to a 50 ml Falcon tube. The total volume of conditioned medium collected must be 12.5 ml.
 - 3 Add equal volumes (12.5 ml) of RB-. Add IWP-2 to the conditioned medium to a final concentration of 5 μ M. Mix well. *See* Note 6.
 - 4 Aspirate the remaining conditioned medium from all wells of the differentiation plate.
 - 5 Add 2 ml of combined medium with IWP-2 to each well.
- 3.3.4 Day 6: Medium Change *See* Note 7.
- 1 Warm an aliquot of RB- medium.
 - 2 144 h after adding CH to the cells: remove medium and add 2 ml of fresh RB- to each well.
- 3.3.5 Day 8: Medium Change
- 1 Warm an aliquot of RB- medium.
 - 2 192 h after adding CH to the cells: remove medium and add 2 ml of fresh RB- to each well.
- 3.3.6 Day 10: Medium Change (Switch to RB+) *See* Note 8.
- 1 Warm an aliquot of RB+ medium.
 - 2 240 h after adding CH to the cells: remove medium and add 2 ml of fresh RB+ to each well.
- 3.3.7 Day 11–30: Differentiation and Maintenance Change medium every 2–3 days with RB+ until day 30. *See* Note 9.
- 3.3.8 Day 31–45+: Maturation Switch medium to D/199B+:
- 1 Warm an aliquot of D/199B+ medium.
 - 2 Remove medium from cells and add 2 ml of fresh D/199B+ to each well.
 - 3 Change medium every 2–3 days with D/199B+ until cardiomyocytes have achieved desired time points.
- See* Note 10.
- 3.4 Characterization of hiPSC-Derived Cardiomyocytes** Bona fide hiPSC-derived cardiomyocytes express appreciable levels of the contractile protein sarcomeric α -actinin and the transcription factor NKX2.5. Co-staining of cells with this

⁶IWP-2 aliquots are prepared at 5 mM (1,000 \times), so if a whole 12-well plate is used, add 25 μ l of IWP to the 25 ml of combined medium prepared in step 3.

⁷From this time point onward, cells may display a type of growth pattern that resembles epithelial-to-mesenchymal transition (EMT). As a result, instead of growing flat and spreading out horizontally, they may grow vertically and pile up in the form of foci in the well. This type of growth is indicative of successful mesoderm and precardiac mesoderm formation.

⁸If medium is changed every 2 days, add 2 ml of medium per well. If medium is changed every 3 days, add 3 ml of medium per well.

⁹At this time point, there should be sheets and/or clusters of beating cells that can be readily visualized by light microscopy. Beating is first observed between days 7 and 11 for most of cell lines.

¹⁰If medium is changed every 2 days, add 2 ml of medium per well. If medium is changed every 3 days, add 3 ml of medium per well.

3.4.1 Cardiomyocyte Dissociation

combination of cardiac markers robustly identifies hiPSC-derived cardiomyocytes.

Beating clusters or sheets of hiPSC-derived cardiomyocytes can be microdissected, dissociated, and replated for specific downstream applications. *See* Note 11.

- 1 Wash cells once with warm HBSS–.
- 2 Add 1 ml of collagenase II solution. Add 10 μ M Rock_i and incubate for 30 min at 37 °C, swirling the plate or vortexing the microdissected cells every 5 min.
- 3 Pipette cells repeatedly 5–10 times to break larger clusters and transfer cells to a 2 ml Eppendorf tube.
- 4 Add 1 ml of dissociation solution dropwise. Mix gently.
- 5 Pass cells through a 20 G syringe needle 3–6 times until larger clumps can no longer be observed by the eye.
- 6 Spin cells for 5 min at 130 $\times g$ and re-suspend cells in 1 ml of D/199B+. Add 10 μ M Rock_i.
- 7 Count cells and replate at an appropriate density. For a 3.5 cm plate, a minimum of 2×10^5 cells/plate should be added. Add 10 μ M Rock_i.

See Note 12.

3.4.2 Flow Cytometry

Flow cytometry allows for robust, quantitative, and rapid estimation of cardiomyocyte throughputs in a given differentiation experiment. The staining protocol outlined below relies on co-staining of undifferentiated hiPSCs (negative control) and hiPSC-derived cardiomyocytes resulting from a differentiation experiment with sarcomeric α -actinin and NKX2.5. *See* Note 13.

- 1 Dissociate cardiomyocytes, and following resuspension, strain cells through a 100 μ M nylon mesh.
- 2 Count cells and prepare a suspension of 1×10^6 cells/ml.
- 3 Take 2×10^5 cells (200 μ l of suspension at 1×10^6 cells/ml) and transfer to an Eppendorf tube.
- 4 Centrifuge the cell suspension at $200 \times g$ for 2 min. Discard supernatant by carefully aspirating with a micropipette to avoid losing cells.
- 5 Re-suspend cells in 100 μ l of 1 \times BD Perm/Wash buffer and incubate for 10 min at room temperature (RT).
- 6 Centrifuge the cell suspension at $200 \times g$ for 2 min. Discard supernatant.
- 7 Re-suspend cells in 100 μ l of FACS blocking solution and incubate for 15 min at RT.
- 8 Centrifuge the cell suspension at $200 \times g$ for 2 min. Discard supernatant.
- 9 Re-suspend cells in 100 μ l of primary antibody mastermix that contains mouse anti-sarcomeric α -actinin (1:100) and rabbit anti-NKX2.5

¹¹24 h before dissociation, tissue culture dishes should be coated with 10 μ g/ml laminin in PBS+, overnight at 37 °C.

¹²Dissociated cardiomyocytes may take up to 3–5 days to start beating again. Change medium every 2–3 days.

¹³Cardiomyocytes can display considerable autofluorescence within the 488 nm wavelengths; thus, secondary antibodies with emissions within the yellow and far-red regions of the light spectrum are preferable.

(1:100) in blocking solution. Incubate for 30 min at RT.

- 10 Centrifuge the cell suspension at $200 \times g$ for 2 min. Discard supernatant.
- 11 Wash once with 100 μ l of 1 \times BD Perm/Wash buffer. Spin at 800 rpm for 2 min. Discard supernatant.
- 12 Re-suspend in 100 μ l of secondary antibody mastermix that contains phycoerythrin-conjugated donkey anti-mouse (1:100) and PerCP-conjugated donkey anti-rabbit (1:100) in FACS blocking solution. Incubate for 30 min at RT in the dark.
- 13 Centrifuge the cell suspension at $200 \times g$ for 2 min. Discard supernatant.
- 14 Wash once with 100 μ l of 1 \times BD Perm/Wash buffer.
- 15 Centrifuge the cell suspension at $200 \times g$ for 2 min. Discard supernatant.
- 16 Re-suspend cells in 200 μ l of PBS-. Keep cells on ice in the dark until ready to analyze.

Expected Results

Figure 1 exemplifies FACS analysis of NKX2.5 and sarcomeric α -actinin results from hiPSC before and after cardiac differentiation. Cellular debris and cell doublets were eliminated, as well as individual signals for both antibodies were analyzed through gates not shown here. Gate P4 was set after analysis of stained undifferentiated hiPSC and adjustment of the negative control signals to the first three decades of each axis.

3.4.3 Immunofluorescence Microscopy

Cardiomyocytes may be dissociated and replated on laminin-coated dishes that are appropriate for cell imaging such as chamber slides, glass coverslips, and black-walled optic plates for immunofluorescence-based microscopy analysis.

- 1 Wash cells once with PBS+ solution.
- 2 Fix cells with 4 % PFA for 30 min at RT.
- 3 Wash cells once with PBS+ solution.
- 4 Permeabilize cells with immunofluorescence permeabilization buffer for 10 min at RT.
- 5 Block cells with immunofluorescence blocking buffer for 30 min at room temperature.
- 6 Prepare primary antibodies in immunofluorescence blocking solution:
 Mouse anti-sarcomeric α -actinin (1:100).
 Rabbit anti-NKX 2.5 (1:100).
 Aspirate blocking solution and add primary antibody mixture. Incubate overnight at 4 °C in a humidified chamber.
- 7 Wash cells three times for 5 min at RT with PBS +.
- 8 Prepare secondary antibodies and DNA stain in immunofluorescence blocking solution:
 DyLight-488-conjugated donkey anti-mouse (1:100).
 DyLight-549-conjugated donkey anti-rabbit (1:100).
 Hoechst 33342: 1 μ g/ml.

- Aspirate PBS+ and add secondary antibody mixture. Incubate for 2 h in the dark at RT in a humidified chamber.
- 9 Wash cells three times for 5 min at RT with PBS +.
 - 10 Mount cells and maintain at 4 °C in the dark until ready to image by using confocal microscopy.

Expected Results

Figure 2 exemplifies results obtained from immunofluorescence staining analysis of sarcomeric α -actinin and NKX2.5 in hiPSC following cardiac differentiation. Cells are considered positive for sarcomeric α -actinin when a clear striated pattern can be visualized. Cardiomyocytes are also defined by expression of NKX2.5, which is found confined to the cell nucleus.

Acknowledgments

F.Z. is a current recipient of the American Heart Association Postdoctoral Fellowship. F.S. is supported by grants from the National Institutes of Health, American Heart Association and Saving tiny Heart Society grants.

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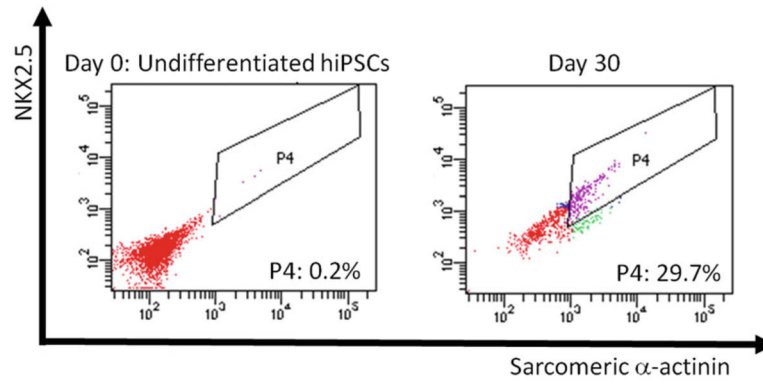


Fig. 1. An example of FACS analysis of sarcomeric α -actinin and NKX2.5 coexpression as an indicator of cardiomyocyte throughput. Gate P4 highlights double-positive cells in undifferentiated cells on day 0 and hiPSC-derived cardiomyocytes on day 30

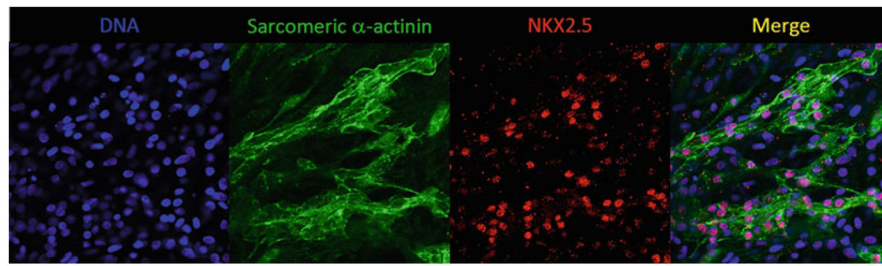


Fig. 2.
An example of immunofluorescence analysis of sarcomeric α -actinin and NKX2.5 coexpression on day 30 of differentiation as an indicator of cardiomyocyte throughput

Table 1

Commercially available reagents

| Description | Manufacturer | Catalog number |
|---|------------------------------|----------------|
| Cell growth matrices | | |
| Growth factor-reduced matrigel | Corning | 354230 |
| Laminin | Life Technologies | 23017-015 |
| Cell culture media | | |
| DMEM/F12 with glutamine and HEPES | Life Technologies | 11330-032 |
| RPMI 1640 with L-glutamine | Life Technologies | 11875093 |
| DMEM with L-glutamine and without sodium pyruvate | Corning | 10-017 |
| M199 with L-glutamine | Corning | 10-060 |
| Medium supplements | | |
| L-Ascorbic acid 2-phosphate | Sigma-Aldrich | A8960 |
| Insulin | Life Technologies | 12585-014 |
| Transferrin | Sigma-Aldrich | T3705 |
| Sodium selenite | Sigma-Aldrich | S5261 |
| B-27 with insulin | Life Technologies | 17504044 |
| B-27 without insulin | Life Technologies | A1895601 |
| Penicillin/streptomycin antibiotics (P/S) | Corning | 30-002 |
| Cytokines | | |
| FGF2 | PeptoTech | 100-18B |
| TGFβ | PeptoTech | 100-21 |
| Buffers | | |
| PBS without Ca ²⁺ and Mg ²⁺ (PBS ⁻) | Corning | 21-031 |
| PBS with Ca ²⁺ and Mg ²⁺ (PBS ⁺) | Corning | 21-030 |
| HBSS without Ca ²⁺ and Mg ²⁺ (HBSS ⁻) | Corning | 21-022 |
| 7.5 % sodium bicarbonate | Life Technologies | 25080-094 |
| 0.5 M EDTA | Life Technologies | 15575-020 |
| BD Perm/Wash buffer | BD Biosciences | 554723 |
| Cell dissociation reagents | | |
| Accutase | Innovative Cell Technologies | AT 104 |
| Collagenase type II | Worthington | LS004174 |
| Taurine | Sigma-Aldrich | T8691-100G |
| EGTA | Sigma-Aldrich | E-4378-25G |
| 25 % BSA | Life Technologies | A10008-01 |
| Small molecules | | |
| Y27632/Rock inhibitor (Rock ₁) | Selleckchem | S1049 |
| CHIR99021 (CH) | Selleckchem | S1263 |
| IWP-2 | Cayman Chemical | 13951 |

| Description | Manufacturer | Catalog number |
|--|------------------------|----------------|
| Antibodies and staining reagents | | |
| Donkey serum | Jackson ImmunoResearch | 017-000-121 |
| Mouse anti-sarcomeric alpha actinin | Sigma-Aldrich | A7811 |
| Rabbit anti-NKX2.5 | Santa Cruz | sc-14033 |
| DyLight-488-conjugated donkey anti-mouse | Jackson ImmunoResearch | 715-485-150 |
| DyLight-549-conjugated donkey anti-rabbit | Jackson ImmunoResearch | 711-505-152 |
| R-phycoerythrin-conjugated donkey anti-mouse | Jackson ImmunoResearch | 715-116-150 |
| PerCP-conjugated donkey anti-rabbit | Jackson ImmunoResearch | 711-126-152 |
| Hoechst 33342 nuclear DNA stain | Life Technologies | H1399 |

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Table 2

Tissue culture plate formats and coating volumes

| Tissue culture plate | ECM coating volume |
|---------------------------|--------------------|
| 6 cm ² plate | 3 ml |
| 3.5 cm ² plate | 1.5 ml |
| 12-well plate | 0.5 ml/well |
| 96-well plate | 50 µl/well |

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Table 3

Stock solutions and aliquot volumes

| Stock solution | Desired concentration | MW | Quantity needed | Resuspension volume | Aliquot volume (µl) |
|-----------------------------|-----------------------|--------|-----------------|---------------------|---------------------|
| L-Ascorbic acid 2-phosphate | 64 mg/ml | 289.54 | 5 g | 78.125 ml | 500 |
| Transferrin | 53.5 mg/ml | N/A | 1 g | 18.692 ml | 100 |
| Sodium selenite | 700 µg/ml | 172.94 | 35 mg | 50 ml | 10 |
| FGF2 | 100 µg/ml | N/A | 1 mg | 10 ml | 500 |
| TGFβ1 | 100 µg/ml | N/A | 100 µg | 1 ml | 10 |

Table 4

Preparation of E8 medium

| Component | Stock solution | Take from stock | Final concentration |
|-----------------------------------|----------------|-----------------|---------------------|
| DMEM/F12 with glutamine and HEPES | 1× | 500 ml | 1× |
| Sodium bicarbonate | 75 mg/ml | 3.62 ml | 543 µg/ml |
| L-Ascorbic acid 2-phosphate | 64 mg/ml | 500 µl | 64 µg/ml |
| Insulin | 4 mg/ml | 2.5 µl | 20 µg/ml |
| Transferrin | 53.5 mg/ml | 100 µl | 10.7 µg/ml |
| Sodium selenite | 700 µg/ml | 10 µl | 14 ng/ml |
| FGF2 | 100 µg/ml | 500 µl | 100 ng/ml |
| TGFβ | 100 µg/ml | 10 µl | 2 ng/ml |
| Pen/strep | 100× | 5 ml | 1× |

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Table 5

Preparation of cardiomyocyte dissociation solution

| Component | Stock solution | Take from stock (μ l) | Final concentration |
|-------------------------|----------------|----------------------------|---------------------|
| Collagenase II solution | 200 units/ml | 880 | 200 units/ml |
| Taurine | 200 mM | 100 | 1 mM |
| EGTA | 0.2 mM | 10 | 0.1 mM |
| BSA | 250 mg/ml | 8 | 1 mg/ml |

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Table 6

Preparation of immunofluorescence dilution buffer

| Component | Stock solution | Take from stock | Final concentration |
|-------------------|----------------|-----------------|---------------------|
| Milli-Q water | N/A | 42.13 ml | N/A |
| Tris base, pH 7.5 | 1 M | 1 ml | 20 mM |
| NaCl | 2 M | 3.87 ml | 155 mM |
| EGTA | 50 mM | 2 ml | 2 mM |
| MgCl ₂ | 100 mM | 1 ml | 100 mM |

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