Enhancing iPSC-CMs maturation using a Matrigel-coated micropatterned PDMS substrate

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ABSTRACT

Cardiac myocytes isolated from adult heart tissue have a rod-like shape with highly organized intracellular structures. Human pluripotent stem cell-derived cardiomyocytes (iPSC-CMs), on the other hand, exhibit disorganized structure and contractile mechanics, reflecting their pronounced immaturity. These characteristics hamper research using iPSC-CMs. The protocol described here enhances iPSC-CM maturity and function by controlling the cellular shape and environment of the cultured cells. Microstructured silicone membranes function as a cell culture substrate that promotes cellular alignment. iPSC-CMs cultured on micropatterned membranes display an *in vivo*-like rod-shaped morphology. This physiological cellular morphology along with the soft biocompatible

silicone substrate, which has similar stiffness to the native cardiac matrix, promotes maturation of contractile function, calcium handling, and electrophysiology. Incorporating this technique for enhanced iPSC-CM maturation will help bridge the gap between animal models and clinical care, and ultimately improve personalized medicine for cardiovascular diseases.

Basic Protocol 1: **Cardiomyocyte differentiation of iPSCs**

Basic Protocol 2: **Purification of differentiated hiPSC-CMs using MACs negative selection**

Basic Protocol 3: **Micropatterning on PDMS**

Keywords

iPSC-CMs, cardiomyocyte, micropatterning, micropatterned iPSC-CMs

INTRODUCTION

Animal models have aided in the understanding of a variety of cardiovascular diseases and, consequently, in the development of numerous cardiovascular therapies (Chorro, Such-Belenguer, & Lopez-Merino, 2009; Oh, Kho, Hajjar, & Ishikawa, 2019). However, animal models are not free of limitations. For example, the ion channel kinetics and functional effects of drug compounds in animals can vary widely from that observed in human pathophysiology.

Human induced pluripotent stem (iPS) cells remove the issues associated with inter-species comparisons. And because they also retain the genetic background of the individual, human iPS cells have ushered in a new era of patient-specific research (Takahashi et al., 2007; Takahashi & Yamanaka, 2006; Yu et al., 2007). A comprehensive understanding of the human cellular phenotype remains elusive due largely to a lack of adequate human cellular models. As a result, there is a great deal of interest in using iPSC-CMs to study the effects of mutations and their potential treatments in a patient- specific manner, as well as to supplement existing studies.

iPSC-derived cardiomyocytes have the potential to be excellent *in vitro* models of cardiac health and disease, but they differ from mature cardiomyocytes. They exhibit, for example, poorly organized excitation-contraction coupling machinery, they lack clear t-tubules, and they produce immature mitochondria (Gaspar et al., 2014; Li, Chen, & Li, 2013; Lieu et al., 2009; Rana, Anson, Engle, & Will, 2012). They also generally present a

circular and flat shape with isotropic filament organization, disorganized contractility patterns, as well as low expression of cytoskeletal proteins and ion channels (Ribeiro et al., 2015; van den Berg et al., 2015; Yang, Pabon, & Murry, 2014).

In 2016 it was demonstrated that culturing iPSC-CMs monolayers on soft PDMS membranes coated with Matrigel promotes maturation, resulting in cardiomyocyte hypertrophy and expression of key mature sarcolemma (SCN5A, Kir2.1, and Cx43) and myofilament markers (cardiac troponin I), matured action potential profiles, and faster conduction velocities (T. J. Herron et al., 2016). These PDMS-cultured cells still displayed many immature morphological characteristics. This is important because cell shape is known to have important effects on cardiomyocyte properties including cytoskeletal structure, growth, and differentiation (Chen, Mrksich, Huang, Whitesides, & Ingber, 1997, 1998; Dike et al., 1999). Cell shape is also critical in determining contractile performance of single iPSC-cardiomyocytes and neonatal cardiomyocytes by regulating intracellular structure, increasing contractile activity, and promoting a more mature electrophysiology phenotype (faster upstroke and conduction velocities) (Helms et al., 2020; Jimenez-Vazquez et al., 2022; Kuo et al., 2012; Ribeiro et al., 2015; Tsan et al., 2021).

A certain degree of iPSC-CM maturation is required for effective disease modeling or regenerative purposes. iPSC-CMs with characteristics that closely resemble those of adult cardiomyocytes are a more effective model of a patient's disease. Matured human iPSC-CMs more closely reproduce the electrophysiological and pharmacological properties of both healthy and diseased adult cardiomyocytes (R. P. Davis et al., 2012; Itzhaki et al., 2011; Ma et al., 2011; Novak et al., 2012; Sun et al., 2012). The following protocols describe the generation of matured iPSC-CMs and their subsequent seeding on a Matrigel-coated micropatterned PDMS membrane. This method generates a large number (~8,640 cells/stamp) of thick, cylindrical-shaped cardiomyocytes with well-defined sarcomeres. These rod-shaped iPSC-CMs can be used in a variety of techniques including immunostaining, stretch assays, optical mapping, western blotting, patch-clamping, etc. Furthermore, this protocol can be adapted to create a variety of cell patterns on either hard or elastic membranes.

1. BASIC PROTOCOL 1. Cardiomyocyte differentiation from iPSCs.

Years ago, Lian and colleagues developed a protocol for differentiating iPS cells into cardiomyocytes by modulating canonical Wnt signaling pathways with small molecules (Lian et al., 2013). Here, we have adapted that protocol from (Lian et al., 2013) and (T. J. Herron et al., 2016) to obtain differentiated cardiomyocytes from iPS cells (**Fig. 1**). The

following protocol yields mostly ventricular-like cardiomyocytes, with a small proportion of atrial and nodal-like cells as well (Fig. 1A). Minor variations in the protocol can allow differentiation of only ventricular-like or atrial- like cardiomyocytes (**Fig. 1 B,C**).

[*Insert figure 1 near here]

Materials

Laminar flow hood

37°C, 5% CO_2

incubator iPS cell

lines

Matrigel (Catalog #: 345277, Corning)

Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} added, $HBSS^{++}$ (Catalog #: 14025- 092, Gibco)

StemMACS iPS-Brew XF (w/supplement) (Catalog #: 130-107-086, Miltenyi Biotec) RPMI

media (w/L-glutamine) (Catalog #: 10-040-CV, Corning)

B27- supplement (Catalog #: A1895602,

Invitrogen) B27+ supplement (Catalog #:

17504004, Invitrogen) CHIR99021 (Catalog #: C-

6556, LC labs)

IWP-4 (Catalog #: 04-0036, Reprocell)

Rock inhibitor (Catalog #: Y-27632,

Gibco)

Protocol Steps

1. In a Matrigel-coated 6-well plate, culture ~ 5 million of iPS cells for 5–6 days in 2 mL of StemMACs iPSC Brew XF medium per well until confluent.

   - *Media is changed every other day until cells show high confluency.*

- *Depending on the iPS cell line, some iPSC colonies will exhibit morphological changes after ~6 days in culture, which may indicate spontaneous differentiation. It is worth noting that undifferentiated iPSC colonies are compact with well-defined edges. Furthermore, iPS cells have a large nucleus with little cytoplasm.*

- *To continue the culture of any iPS cell line, transfer a few colonies to an additional Matrigel-coated plate and wait until they reach 80-90 percent confluency. Repeat the process as needed.*

- *Depending on the iPS cell line, Rock Inhibitor may be required (e.g., when the cells do not form a stable attachment to the matrix substrate).*

2. Aspirate the media and then dissociate iPS cells using 1 mL/well of Versene solution at 37°C for 7 min and reseed as monolayers on Matrigel-coated (100 µg/mL) 12-well plates at a density of 8.5×10^6 cells/well in 2 mL StemMACs iPSC Brew XF medium supplemented with 5 µmol/L ROCK inhibitor.

   - *Replace medium every day.*

3. After 2 days, or when the monolayers reach 90–100% confluence (**Fig. 2 A**), change the medium to 3 mL of RPMI supplemented with B27 minus insulin containing 10 µmol/L CHIR99021.

   - *This day is labelled as differentiation day 0.*

   - *CHIR99021 is a WNT pathway inhibitor that promotes cardiomyocyte differentiation from human iPS cells. This small molecule induces iPSC differentiation to vascular progenitors in this protocol step.*

   - *A small percentage of cell death (~20%) is usually expected. If more than half of the cells die after CHIR treatment, discard the plate, and begin the differentiation process again (**Fig. 2 B, left**).*

   - *The concentration of CHIR may vary depending on the iPS cell line. It needs to be adjusted empirically until the appropriate concentration is identified. Usually, the start point is 3 µM.*

   [*Insert figure 2 near here]

4. Day 1: Remove CHIR99021 and add 2 mL of RPMI supplemented with B27 minus insulin.

5. Day 2: Do nothing.

- *Do not even handle the plate.*

6. Day 3: take 1.5 mL from each well and add 1.5 mL of RPMI supplemented with B27 minus insulin. Add 1 µL/mL of IWP4 (10 µmol/L), you will have a total volume of 3 mL per well.

  - *IWP4 promotes cardiomyocyte differentiation in iPS cells after treatment with CHIR99021.*

  - *A small percentage of cell death (~20%) is usually expected, although at a less extent compared to CHIR addition (**Fig. 2 B, right**). If more than half of the cells die after IWP4 treatment, discard the plate, and begin the differentiation process again.*

7. Day 4: Do nothing.

  - *Do not even handle the plate.*

8. Day 5: Aspirate the RPMI/IWP4 solution and add 2 mL of B27-/RPMI per well.

9. Day 6: Do nothing.

  - *Do not even handle the plate.*

10. Day 7: Aspirate the medium and add 2 mL of B27+/RPMI per well.

11. From day 8 onwards, change the medium every day until the cells are ready for purification.

  - *Cells typically begin beating between days 9 and 12. If no beating is observed by day 15, discard the plate.*

  - *The cells usually are ready for purification approximately 15–30 days after starting the differentiation protocol.*

  - *Unpurified beating monolayers can be cultured for up to 1 month at this stage (**Fig. 2C**).*

2. BASIC PROTOCOL 2. Post directed differentiation iPSC-CM purification using MACs negative selection.

The directed differentiation method used here, adapted from (T. Herron, Monteiro da Rocha, & Campbell, 2017) and (Pekkanen-Mattila et al., 2019), should generate a cell population that is 20–80% cardiomyocytes. For most downstream applications, the

cardiac monolayers need to be purified. Currently, the most widely used method for purifying iPSC-CMs is based on significant differences in glucose and lactate metabolism between CMs and non-CMs (Tohyama et al., 2013). Although this method of iPSC-CM purification has been widely adopted and is a highly efficient method for large-scale purification, this same media composition has also been used in the past to simulate ischemia preconditioning in cultured adult CMs (Diaz & Wilson, 2006). In other studies, a similar glucose-free solution was used as "injury solution" to cause localized ischemic injury in cardiac monolayers (Arutunyan, Webster, Swift, & Sarvazyan, 2001; Vanden Hoek et al., 1996). On the other hand, using the following magnetic bead purification protocol (**Fig. 3**), we obtain a large number of iPSC-CMs without an ischemic injury-like phenotype that is appropriate for variety of studies (Block et al., 2020; da Rocha, Creech, Thonn, Mironov, & Herron, 2020; J. Davis et al., 2021; T. Herron et al., 2017; Jimenez-Vazquez et al., 2022; Ponce-Balbuena et al., 2018). This method has been also described and validated for use in proarrhythmia cardiotoxicity assays (da Rocha et al., 2017).

[*insert figure 3 near here]

Materials

Laminar flow hood

37°C, 5% CO_2

incubator Benchtop

centrifuge

Ice bucket

Hemocytom

eter Trypan

Blue

PDMS (Catalog #: SM21045730, SMi)

Magnetic MACS separator (Catalog #: 130-090-976, Miltenyi biotech)

LS columns (Catalog #: 130-042-401, Miltenyi biotech)

MACS separation buffer (Catalog #: 130-091-221, Miltenyi biotech)

Cardiomyocyte purification Kit (includes non-cardiomyocyte depletion cocktail and anti-Biotin magnetic microbeads) (Catalog #: 130-110-188, Miltenyi biotech)

70-µm strainers (sterile) (Catalog #: 352350, Corning)

30-µm pre-separation filters (Catalog #: 130-041-407, Miltenyi biotech)

50-mL conical tube (sterile)

DMEM F12 media (Catalog #: 11320-033, Gibco)

RPMI media (w/L-Glutamine) (Catalog #: 10-040-CV, Corning) B27+

supplement (Catalog #: 17504004, Invitrogen)

0.25% Trypsin/EDTA (Catalog #: 25200-056, Gibco)

Hank's Balanced Salt Solution (HBSS) (Catalog #: 14175-095, Gibco)

Hank's Balanced Salt Solution with Ca^{2+} and Mg^{2+} added, $HBSS^{++}$ (Catalog #: 14025- 092, Gibco)

Non-Essential amino acids (Catalog #: 11140-050, Gibco)

L-Glutamine (Catalog #: 25030-081, Gibco)

Blebbistatin (Catalog #: 13186, Cayman Chemical)

Rock inhibitor (Catalog #: Y-27632, Cayman Chemical)

β-Mercaptoethanol (Catalog #: M6250, Sigma)

Protocol steps

1. Prepare a PDMS 6-well plate. For monolayer plating, pipette 500 µL of ice cold diluted Matrigel onto the PDMS in a large droplet. Allow the droplet to gel at room temperature for at least 30 minutes.

   - *Matrigel is diluted in DMEM/F12 media (1:100).*

- *Different dilutions of Matrigel may be required depending on the iPSC-CM line. Adjust the concentration to achieve the best iPSC-CM adhesion to the matrix substrate.*

2. After ~30 days in culture (see Basic Protocol 1, step 11), wash unpurified iPSC-CMs with HBSS and dissociate them using 1 mL of 0.25% Trypsin/EDTA per well with an incubation time of 3–5 min at 37°C/5% CO_2.

3. Add 2 mL of EB20 media per well of dissociated cells to inactivate the trypsin. Triturate each well up to 10 times with a 10-mL serological pipette and transfer the cells into a sterile 50-mL conical fitting with a 70-μm strainer.

   - *The EB20 media is composed of: 80% DMEM/F12, 0.1 mM Non-Essential Amino Acids, 1 mM ʟ-Glutamine, 0.1 mM β-mercaptoethanol, 20% FBS, 10 μM Blebbistatin, and 5 μM Rock Inhibitor.*

   - *Wash the strainer with 3 mL of EB20.*

   - *Count cells before continuing with the process. If the cell count is less or more than 5×10^6 cells, adapt the amount of the cardiomyocyte purification kit ingredients. Typically, 20 μL of either non-cardiomyocyte depletion cocktail or anti-Biotin magnetic microbeads is required for 5×10^6 cells. If there are fewer than a million cells on the plate, discard it and proceed to purify other cell monolayers.*

   - *To count the cells, transfer 5 μL of the trypsinized cell suspension to a 1 mL centrifuge tube and mix with 45 μL of Trypan Blue. Transfer 15–20 μL of the cell suspension between the hemocytometer and cover glass using a P-20 micropipettor. Count the number of cells in all four outer squares (**red, inset 1**) and divide by four (the mean number of cells/square). The number of cells per square $\times 10^4 \times$ dilution (10) = the number of cells/mL of suspension.*

4. Transfer cells into a 15-mL conical tube and centrifuge at 1000 RPM for 5 min at 4°C.

5. Remove the supernatant and add 1 mL of HBSS^{++} to wash the pellet.

6. Centrifuge the cells at 1000 RPM for 5 min at 4°C. Immediately after, aspirate the supernatant and add 100 μL of cold MACs Buffer per 5×10^6 cells to resuspend the pellet in the 15-mL conical tube.

7. Add 20 µL of non-cardiomyocyte depletion cocktail (-Biotin conjugated) per 5×10^6 cells, flick 5 times to mix and incubate on ice for 5 min.

    - The following protocol steps are based on the recommendations included in the Miltenyi Biotec cardiomyocyte purification kit.

8. After Biotin-conjugated cocktail incubation, add 1 mL of MACs Buffer per 15-mL conical. Triturate the cells gently and centrifuge at 1000 RPM for 5 min at 4°C.

9. Aspirate the supernatant and resuspend the pellet in 100 µL of cold MACs Buffer per 5×10^6 cells.

10. Add 20 µL of anti-Biotin magnetic microbeads per 5×10^6 cells, flick 5 times to mix and incubate on ice for 10 min.

    - *During the 10 min incubation, place the LS columns fitted with the 30 µm pre-separation filters on the Magnetic MACS Separator.*

    - *Place the appropriately labeled 15-mL conical tube positioned under each column and prime the column with 3 mL of MACs Buffer.*

11. After anti-Biotin magnetic microbeads incubation, mix the cells gently with 8 mL of MACs Buffer.

12. Add the cell suspension to the pre-separating filter on top of the LS flowing column while continuously collecting the total flow through.

13. Collect 11 mL of flow through and centrifuge it at 1000 RPM for 5 min at 4°C.

14. Discard the supernatant and triturate gently the iPSC-CM pellet with 1 mL of EB20 media.

    - *Before proceeding to the next step, count the purified iPSC-CMs.*

15. Resuspend the purified iPSC-CM fractions in EB20 media with 5 µM of ROCK inhibitor to 200k–300k cells/200–300 µL volume and plate as monolayers on Matrigel-coated 22 mm × 22 mm PDMS membranes adhered to the bottom of a 6-well culture dish (See Basic Protocol 2, step 1).

    - *The size of the PDMS membranes can be adjusted to provide enough plating area for the iPSC-CMs.*

    - *Immediately before plating the iPSC-CMs, aspirate the excess of Matrigel from the PDMS and add 200–300 µL of iPSC-CM cell suspension to the Matrigel-coated area.*

- *Plate enough iPSC-CMs to form a monolayer syncytium for better cell maturation (**Fig. 4 A**).*

[*insert figure 4 near here]

16. Transfer the plate to the incubator at 37°C and 5% CO_2 and leave it there for at least 4 hours to allow attachment of iPSC-CMs to the Matrigel.

17. After ≥4 hours, add 3 mL of EB20 media with ROCK inhibitor to each well.

18. After 2 days, wash the cells with 3 mL of HBSS^{++}, aspirate and replace with 3 mL of RPMI/B27+ media.

- *Change the media every 3 days.*

- *It is no longer necessary to wash the cells with HBSS++ every time the media is changed after this step.*

19. Culture the highly purified iPSC-CMs monolayer on Matrigel-PDMS for at least 7 days before re-plating onto micropatterned PDMS (**Fig. 4 A**).

- *Complete ALL cell work in a sterile biosafety cabinet. All triturations are performed a total of 5 times unless noted otherwise. Use one LS column with pre-separation filter for each well of iPSC-CMs dissociated at the start.*

3. BASIC PROTOCOL 3. Micropatterning on PDMS.

Standard cell culture techniques produce confluent but unstructured monolayers. Unlike native adult cardiomyocytes, iPSC-CMs do not retain many key structural and functional properties that are essential for many applications in cardiac research. The lack of myocyte alignment in iPSC-CMs is a significant deviation from the phenotype of adult cardiomyocytes *in vivo*, as evidenced by the irregular sarcomere alignment of iPSC-CMs plated on traditional planar substrates (**Fig. 4 B**). Importantly, there are several reports indicating that cell shape and substrate stiffness improve contractile activity and facilitate maturation of iPSC-CMs (Chen et al., 1998; da Rocha et al., 2017; Dike et al., 1999; T. J. Herron et al., 2016; Kuo et al., 2012; Ribeiro et al., 2015). Micropatterning provides a structural framework that promotes the formation of rod-shaped iPSC-CMs that more accurately reproduce adult cardiomyocyte structure and function (e.g. increased expression of structural genes, greater sarcomere organization, matured mitochondria function, binucleation, etc.). The following micropatterning platform (**Fig. 5**), adapted from (Kuo et al., 2012), produces contracting single cells (**Fig. 6**) using a reusable micropatterned

silicone stamp that is compatible with a variety of matrix protein solutions. The stamp will leave a patterned growth substrate on the PDMS membrane that will provide the spatial resolution and structure for appropriate iPSC-CM anisotropy. The cell seeding pattern is also easily adaptable using different types of micropatterned silicone stamps.

[*Insert figure 5 near here]

Materials

Laminar flow hood

37°C, 5% CO_2 incubator

UV/Ozone (UVO) Machine

Benchtop centrifuge

Gasket hole punch kit

Sonicator

Ice bucket

Scotch tape

Silicone stamps

Trypan Blue

50-mL conical tube (sterile)

70-μm strainers (sterile) (Catalog #: 352350, Corning)

Matrigel (Catalog #: 345277, Corning)

Phosphate-buffer saline (PBS)

PDMS (Catalog #: SM21045730, SMi)

0.25% trypsin/EDTA (Catalog #: 25200-056, Gibco)

Pluronic F-127 (Catalog #: P2443, Sigma)

B27+ supplement (Catalog #: 17504004, Invitrogen)

RPMI media (w/L-Glutamine) (Catalog #: 10-040-CV, Corning) Rock

inhibitor (Catalog #: Y-27632, Cayman chemical)

Penicillin-Streptomycin-Amphotericin B solution (PSA) (Catalog #: 15070063, Gibco)

Hemocytometer

Protocol steps

iPSC-CMs should be cultured as a monolayer for at least 7 days to induce maturation before re-plating the cardiomyocytes onto the micropatterned PDMS (See Basic Protocol 2, step 19).

1. Carefully clean the surface of the silicone stamps with scotch tape by placing it onto the stamp surface and slowly removing it (this is to remove residues from Matrigel used before). After that, sonicate the stamps in 70% ethanol/milli-Q water for at least 20 min.

   - *Avoid scratching the stamp surface.*

   - *Micropatterned area is 1 cm × 1 cm total (**inset 2**). Each micropattern is 50 μm height, 300 μm width, and are spaced apart from each other by 4 μm.*

2. Dry the stamps in a sterile hood before applying 250 μL of Matrigel diluted in di-water (1:100). Incubate the stamps in the diluted Matrigel at room temperature for at least 1 h.

3. Using a punch tool, cut 18 mm PDMS circles and sonicate them in 70% ethanol for 20 min.

4. Transfer the 18 mm PDMS circles to a 6-well plate after shaking excess EtOH off.

5. When ready for microprinting, treat the PDMS culture dish with UVO (UV light/Ozone) for 9 min with lid off.

   - *UVO treatment is necessary to reduce the hydrophobicity of PDMS and allow Matrigel micropatterns to be transferred from the stamps.*

- *This UVO incubation time is for PDMS with a stiffness of ~3 MPa.*

6. While UVO is performed on PDMS circles, aspirate the Matrigel solution from the PDMS stamps.

7. After UVO is completed, invert the dried stamps onto each PDMS circle, and remove one by one after ~2 min.

   - *Let the plate cool down for ~20 seconds before placing the dried stamps onto each PDMS membrane.*

   - *Dry the silicone stamps for no more than 10 min.*

8. Incubate the micropatterned PDMS plate with 1% pluronic-F127 overnight at room temperature.

   - *Pluronic-F127 is a non-ionic surfactant polyol that is used in this application as a passivating agent to block unpatterned regions, making them resistant to protein absorption and thus cell adhesion.*

   - *Failure to add Pluronic-F127 results in micropattern fouling.*

9. Before re-plating iPSC-CMs, clean the micropatterned plates for 1 h with 3× PSA diluted in PBS, aspirate the PSA, and expose them to UV light for 15 min.

10. Dissociate the iPSC-CMs monolayers using 1 mL of 0.25% trypsin with EDTA and incubate the cardiomyocytes for 8–10 min at 37°C and 5% CO_2.

    - *Spontaneous contraction in monolayers should always be observed before re-plating on the micropatterned PDMS. If monolayers are not contracting wait additional days for up to five days.*

11. After dissociation, add 1 mL of RPMI media containing 10% FBS to inactivate the trypsin.

12. Transfer dissociated iPSC-CMs through a 70-µm filter into a 50-mL conical tube.

13. Collect the iPSC-CMs into a 15-mL conical tube and centrifuge at 700 RPM for 3 min at room temperature.

14. Resuspend the iPSC-CMs in 1 mL of warm RPMI/B27+ media supplemented with 2% FBS and 5 µM of ROCK inhibitor (re-plating media).

15. Count and plate ~30k iPSC-CMs in 350 µL of re-plating media in the center of the micropatterned area.

16. After ~5 h, gently add 2 mL of re-plating media.

- *The use of re-plating media for the next three days is <u>absolutely critical</u> for cell survival after re-plating iPSC-CMs onto the Matrigel-coated micropatterned PDMS.*

17. Return the plate to the incubator

18. Change the media at day 1 and 3 after re-plating.

- *iPSC-CMs must be on micropatterns for at least 4 days before being used in an experiment.*

- *Matrigel-coated micropatterned PDMS plates can be kept for up to 3 days at 4°C before plating iPSC-cardiomyocytes.*

COMMENTARY

Background information

Animal models have been and will continue to be useful in defining mechanisms of cardiovascular development, physiology, and disease (Chorro et al., 2009; Houser et al., 2012; Oh et al., 2019). However, differences between humans and animals often limit the translation of findings in animal models into human therapies (Doncheva et al., 2021). Patient-specific *in vitro* models can help to fill the gap between animal models and the clinic to facilitate the development of novel therapies (Kim, 2014). Human-based models are especially important for cardiovascular research because the cardiac physiology of many animal models differs dramatically from humans. These differences include beating rates, calcium handling, as well as ion channel types and their expression levels (Liu, Laksman, & Backx, 2016; Milani-Nejad & Janssen, 2014; Zhao et al., 2018). And, while the physiological differences in large animal models are less than those observed in murine models (Dixon & Spinale, 2009), there is still a need for a human model that can replicate the cellular and genetic environment of the human cardiomyocyte.

Models that recapitulate individual patient disease at the molecular and cellular levels are obvious candidates for improving the understanding of disease pathogenesis and progression and predicting individual patient responses to specific treatments. The development of iPS cell technology (Takahashi et al., 2007; Takahashi & Yamanaka, 2006), and the improved ability to differentiate iPS cells into disease-relevant cell types such as cardiomyocytes (Burridge, Keller, Gold, & Wu, 2012) has created an extraordinary

opportunity for the generation of human patient-specific cell lines for use in disease modeling, personalized drug screening, and regenerative approaches to precision medicine (Kim, 2014; Liu et al., 2016; Matsa, Burridge, & Wu, 2014; Moretti et al., 2010).

Many barriers remain in the iPS cell differentiation field. Even though human iPS cell technology has advanced rapidly since 2007, iPS cell clones can exhibit differences in differentiation efficiency and in phenotype, including between clones derived from the same person. Thus, line-to-line variation often complicates data interpretation (Shi, Inoue, Wu, & Yamanaka, 2017). Another barrier is the variability in differentiated cell maturation across lines (Ivashchenko et al., 2013; Zhu, Santana, & Laflamme, 2009). Modeling diseases with iPS cells has been further hindered by the fetal-like properties of iPS cell-derived cell lines (Studer, Vera, & Cornacchia, 2015). Standardized human iPS cell protocols reduce technical variability and enable more reliable identification of true biological phenotypes. Concerning the variations in maturation, the micropatterning technique has been shown to be useful in controlling cell size, shape, and maturation state (Helms et al., 2020; Jimenez-Vazquez et al., 2022; Kuo et al., 2012; Ribeiro et al., 2015; Tsan et al., 2021). Plating myocytes or other cell types (e.g., BHK cells, MDCK cells, etc.) in controlled two-dimensional arrangements has previously been shown to also improve cell differentiation, functionality, and longevity (Clark, Connolly, Curtis, Dow, & Wilkinson, 1991; Motlagh, Hartman, Desai, & Russell, 2003; Motlagh, Senyo, Desai, & Russell, 2003).

A variety of cell patterns can be generated using the Matrigel-based micropatterning protocol. They can be used with a variety of techniques and studies, including optical mapping, immunostaining, high-throughput compound testing, stretching analysis, and patch-clamping. This method creates a culture environment that combines a flexible platform with a microenvironment that promotes iPSC-CM cell alignment, growth, connection, and maturation.

This protocol cannot reproduce the three-dimensional multilayer structure of the native myocardium. This is significant because the electromechanical function in the heart is closely related to the three-dimensional spatiotemporal gradients that are essential to cardiac structure and function (Burton et al., 2006; Vadakkumpadan et al., 2010). As a result, while this protocol produces iPSC-CMs with morphologies that are similar to adult cardiomyocytes, it does not accurately reflect the *in-situ* environment. Despite its limitations, seeding iPSC-CMs on micropatterned surfaces clearly mimics important structural and functional aspects of native ventricular myocardium.

Critical parameters and troubleshooting

The micropatterning technique is a valuable tool for improving several functional aspects of iPSC-CMs. However, its success is dependent upon several critical parameters, and an understanding of the limitations associated with this technology. Table 1 discusses the main troubleshooting strategies.

Critical Steps:

- The patterned region of the silicone stamp should never be touched by anything other than the matrix protein solution, and the scotch tape when cleaning it.

- When preparing the PDMS plates, make sure all air bubbles are removed. Otherwise, they will be incorporated into the final micropattern, resulting in micropattern defects.

- Place the silicone stamp covered in Matrigel carefully onto the PDMS to avoid smearing the transferred micropatterns.

- Pluronic F-127 is cytotoxic. Make sure to wash it carefully at least 5 times with PBS before plating the iPSC-CMs.

- Do not expose the micropatterned PDMS plate to UV light for more than 20 minutes. This may damage the micropatterns.

- Before plating the iPSC-CMs, make sure there are no clumps of cells to avoid forming small monolayers or cell clusters.

- Rinse the PSA from the micropatterned PDMS plate thoroughly because antibiotics like streptomycin block stretch-activated ion channels in isolated cells (Belus & White, 2003; Shen, Chou, & Chiu, 2003), and may affect the responses of the iPSC-CMs to stretch.

- Depending on the stiffness of the PDMS, the UVO incubation time may need to be adjusted.

Table 1. Main troubleshooting strategies for Matrigel-based micropatterning technique.

Possible problems	Troubleshooting strategies

No single cells on the micropatterns.	Pipette the iPSC-CM monolayers up and down more times to better dissociate them.
	Plate no more than 50k iPSC-CMs per micropatterned area.
Micropatterns are not being transferred from the stamp to the PDMS sheet.	Incubate the PDMS plate under UVO for a longer time. Stiffness of the PDMS is important. Thicker PDMS usually needs longer exposition time to UVO.
	Before transferring the micropatterns to the PDMS, remove any excess Matrigel from the stamp.
When transferring the Matrigel micropatterns, there is only smearing.	You are pressing the silicone stamp too firmly against the PDMS sheet. When transferring the micropattern to the PDMS, apply minimal pressure to the stamp.
Micropatterns do not last long enough on the PDMS sheets, or iPSC-CMs adhere poorly to the Matrigel micropatterns.	Matrigel degrades quickly. Use a proper dilution of not less than 1:100.
	Do not leave the micropatterned PDMS plate under UV light for more than 20 min, as it will damage the Matrigel micropatterns.

iPSC-CMs do not survive after re-plating on micropatterns	When dissociating the iPSC-CM monolayers, be gentle. Allow the cells to incubate with trypsin for no more than 10 minutes.
	Because Pluronic F-127 is cytotoxic, rinse the Matrigel-coated micropatterned PDMS e thoroughly after incubation.

Anticipated results

[*Insert figure 6 near here]

This protocol describes the methods used to create *in vitro* models of iPSC-CMs that mimic important structural and mechanical parameters of adult cardiomyocytes. iPSC-CMs grown on Matrigel-coated micropatterned PDMS membranes develop *in vivo*-like cell morphology, including organized sarcomeres and hyperpolarized resting membrane potentials (**Fig. 6 A–D**). Other silicone stamps can be used to produce strands of aligned iPSC-CMs that can be plated with fibroblasts to mimic the cellular spatial distribution of the native myocardium (**Fig. 6 E**).

In addition to increased maturation, these micropatterns can be used to study mechanical, biophysical, and biochemical events from iPSC-CMs derived from healthy and diseased individuals. Importantly, the application of this technique is not limited to cardiomyocytes; controlling shape and size may be significant in a wide range of cells, including fibroblasts, smooth muscle cells, endothelial cells, and others.

Time considerations

The generation of iPSC-cardiomyocytes, as described in Basic Protocol 1, will take at least 15 days after starting the differentiation process, which may vary depending on the iPS cell line. The purification protocol (Basic Protocol 2) takes only a few hours to complete, depending on the number of cells to be purified. However, the purified iPSC-CMs must mature as monolayers for an additional 7 days before being seeded on micropatterns. The micropatterning process (Basic Protocol 3) is typically carried out in two steps: one day to prepare the Matrigel-coated micropatterned PDMS plates, and another day to perform the cell re-plating. Before any type of analysis, iPSC-CMs must be on micropatterns for at least 3 days. As a result, the entire process, from iPSC differentiation to their use in the desired assay following micropatterning, takes about a month. The

processing and acquisition of the data following iPSC-CM micropatterning will determined by the type of study to be conducted.

FIGURE LEGENDS

Fig. 1. Schematic of the cardiac differentiation protocols. A) Mixed CM Population Protocol: After iPSC monolayers reach 90–100% confluence, the cardiomyocyte induction begins on Day 0 with the addition of CHIR and concludes on Day 8 with the addition of media RPMI B27+ to the cells. **B)** Ventricular Population Protocol: Using media containing L-ascorbic acid and human albumin from Day 0 to Day 7 produces ventricular- like iPSC-cardiomyocytes. **C)** Atrial Population Protocol: Basal media, like the ventricular protocol, is used to induce chamber-specific iPSC-CMs. Here, retinoic acid is also added from Day 3 to Day 6 to induce atrial-like iPSC-cardiomyocytes. RPMI B27- media is not used in either the ventricular or atrial specific protocols. All of the protocols displayed follow similar steps to induce iPSC-CM differentiation.

Fig. 2. iPSC differentiation into cardiomyocytes. A) iPSC monolayer at ~90% confluency stained for the pluripotency marker Nanog (red), and DAPI (blue). **B)** View of a small area of an iPSC monolayer after CHIR (left) and IWP4 (right) addition. During this differentiation steps, a small number of iPS cells will die, leaving empty spaces in the monolayer (white arrows). **C)** Unpurified iPSC-CM monolayer stained with F-actin (green) and DAPI (blue). Approximately 80% of the cells are either F-actin or cTnI positive. Non-cardiomyocytes cells will be removed following the negative selection purification process.

Fig. 3. Magnetic purification of iPSC-CMs using negative selection. Briefly, **(1)** Matrigel is plated on PDMS membranes. Then, **(2)** dissociated and unpurified iPSC-CMs are collected and incubated with a non-cardiomyocyte depletion cocktail (biotin- conjugated). **(3)** Non-target cells are then magnetically labeled and depleted with magnetic anti-biotin microbeads. **(4)** Unlabeled iPSC-CMs are collected in the flow- through fraction during the cell separation, while unwanted cell types are retained in the magnetic column. **(5)** Isolated iPSC-CMs are plated as monolayers on Matrigel coated wells from step 1. After at least 1 week of monolayer culture to induce maturation, iPSC-CMs can then be plated onto micropatterned PDMS.

Fig. 4. Purified iPSC-cardiomyocytes. A) Purified iPSC-CMs plated at a density of 300 k cells in 300 μL of media to form a monolayer syncytium. **B)** iPSC-cardiomyocytes plated at a low density to allow single cell pattern formation (left). Individual iPSC-CM show flat shape and irregular sarcomere alignment (right). F-actin is displayed in green and DAPI in blue.

Fig. 5. Schematic representation of the PDMS micropatterning process. **(1)** A diluted Matrigel solution is first incubated for 1 hour on the microstructure face of the sonicated silicone stamp. Then, **(2)** during the PDMS UVO treatment, the Matrigel-covered surface of the stamp is gently dried by aspirating the Matrigel and incubated at room temperature.

(3) When the PDMS UVO treatment is complete, the stamp's Matrigel- covered surface is carefully placed onto the PDMS for 2 min. **(4)** The stamp is then gently removed from the PDMS. **(5)** The micropatterned PDMS is passivated overnight at room temperature with Pluronic F-127 to block non-patterned areas and to protect the micropatterns until iPSC-CMs are ready to be plated. Finally, **(6)** the micropatterned Matrigel-coated PDMS is washed with PBS several times to remove the Pluronic F-127, and ~50k iPSC-cardiomyocytes are seeded per well.

Fig. 6. Purified micropatterned iPSC-CMs. A) Single iPSC-CMs zoom (right) seeded on a Matrigel-coated micropatterned PDMS membrane (left). **B)** Micropatterned iPSC- CMs stained for Troponin I (red) and DAPI (blue). **C)** Single iPSC-CMs stained for Troponin I (left) and F-actin (right). Nuclei are stained with DAPI (blue). **D)** Representative action potentials recorded from a single micropatterned ventricular-like iPSC-CM using the patch clamp technique in current clamp mode. **E)** Zoomed in view of iPSC-CMs plated as strands on a Matrigel-coated micropatterned PDMS membrane.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

DATA AVAILABILITY

All data generated or analyzed in this study are included in this manuscript.

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