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Developing 3D Organized Human Cardiac Tissue within a Microfluidic Platform

Jaimeson Veldhuizen[1], **Mehdi Nikkhah**[1,2]

[1] School of Biological and Health Systems Engineering, Arizona State University [2] Biodesign Virginia G. Piper Center for Personalized Diagnostics, Arizona State University

Corresponding Author

Mehdi Nikkhah
mnikkhah@asu.edu

Citation

Veldhuizen, J., Nikkhah, M. Developing 3D Organized Human Cardiac Tissue within a Microfluidic Platform. *J. Vis. Exp.* (172), e62539, doi:10.3791/62539 (2021).

Date Published

June 15, 2021

DOI

10.3791/62539

URL

jove.com/video/62539

Abstract

The leading cause of death worldwide persists as cardiovascular disease (CVD). However, modeling the physiological and biological complexity of the heart muscle, the myocardium, is notoriously difficult to accomplish *in vitro.* Mainly, obstacles lie in the need for human cardiomyocytes (CMs) that are either adult or exhibit adult-like phenotypes and can successfully replicate the myocardium's cellular complexity and intricate 3D architecture. Unfortunately, due to ethical concerns and lack of available primary patient-derived human cardiac tissue, combined with the minimal proliferation of CMs, the sourcing of viable human CMs has been a limiting step for cardiac tissue engineering. To this end, most research has transitioned toward cardiac differentiation of human induced pluripotent stem cells (hiPSCs) as the primary source of human CMs, resulting in the wide incorporation of hiPSC-CMs within *in vitro* assays for cardiac tissue modeling.

Here in this work, we demonstrate a protocol for developing a 3D mature stem cell-derived human cardiac tissue within a microfluidic device. We specifically explain and visually demonstrate the production of a 3D *in vitro* anisotropic cardiac tissue-on-a-chip model from hiPSC-derived CMs. We primarily describe a purification protocol to select for CMs, the co-culture of cells with a defined ratio via mixing CMs with human CFs (hCFs), and suspension of this co-culture within the collagen-based hydrogel. We further demonstrate the injection of the cell-laden hydrogel within our well-defined microfluidic device, embedded with staggered elliptical microposts that serve as surface topography to induce a high degree of alignment of the surrounding cells and the hydrogel matrix, mimicking the architecture of the native myocardium. We envision that the proposed 3D anisotropic cardiac tissue-on-chip model is suitable for fundamental biology studies, disease modeling, and, through its use as a screening tool, pharmaceutical testing.

Introduction

Tissue engineering approaches have been widely explored, in recent years, to accompany *in vivo* clinical findings in regenerative medicine and disease modeling[1,2]. Significant emphasis has been particularly placed on *in vitro* cardiac tissue modeling due to the inherent difficulties in sourcing human primary cardiac tissue and producing physiologically relevant *in vitro* surrogates, limiting the fundamental understanding of the complex mechanisms of cardiovascular diseases (CVDs)[1,3]. Traditional models have often involved 2D monolayer culture assays. However, the importance of culturing cardiac cells within a 3D environment to mimic both the native landscape of the myocardium and complex cellular interactions has been extensively characterized[4,5]. Additionally, most models produced thus far have included a mono-culture of CMs differentiated from stem cells. However, the heart is comprised of multiple cell types[6] within a complex 3D architecture[7], warranting the critical need to improve the complexity of the tissue composition within 3D *in vitro* models to better mimic cellular constituents of the native myocardium.

To date, many different approaches have been explored to produce biomimetic 3D models of the myocardium[8]. These approaches range from experimental setups that allow for the real-time calculation of generated force, from mono-culture CMs seeded on thin films (deemed muscular thin films (MTFs))[9], to co-culture cardiac cells in 3D hydrogel matrices suspended among free-standing cantilevers (deemed engineered heart tissues (EHTs))[10]. Other approaches have focused on implementing micromolding techniques to mimic myocardial anisotropy, from mono-culture CMs in a 3D hydrogel suspended among protruding microposts in a tissue patch[11], to mono-culture CMs seeded among indented microgrooves[12,13]. There are inherent advantages and disadvantages to each of these methods, therefore, it is pertinent to utilize the technique that aligns with the intended application and the corresponding biological question.

The ability to enhance the maturation of stem cell-derived CMs is essential for the successful *in vitro* engineering of adult-like myocardial tissue and translation of subsequent findings to clinical interpretations. To this end, methods to mature CMs have been widely explored, both in 2D and 3D[14,15,16]. For example, electrical stimulation incorporated in EHTs, forced alignment of CMs with surface topography, signaling cues, growth factors from co-culture, and/or 3D hydrogel conditions, etc., all lead to a change in favor of CM maturation in at least one of the following: cell morphology, calcium handling, sarcomeric structure, gene expression, or contractile force.

Of these models, the approaches that utilize microfluidic platforms retain certain advantages in nature, such as control of gradients, limited cell input, and minimal necessary reagents. Furthermore, many biological replicates can be generated at once using microfluidic platforms, serving to better dissect the biological mechanism of interest and increase the experimental sample size in favor of statistical power[17,18,19]. Additionally, using photolithography in the microfluidic device fabrication process enables the creation of precise features (e.g., topographies) at the micro- and nano-level, which serve as mesoscopic cues to enhance the surrounding cellular structure and macro-level tissue architecture[18,20,21,22] for different applications in tissue regeneration and disease modeling.

We previously demonstrated the development of a novel 3D cardiac tissue on-chip model that incorporates surface topography, in the form of innate elliptical microposts, to align hydrogel-encapsulated co-cultured cardiac cells into an interconnected, anisotropic tissue[20]. After 14 days of culture, the tissues formed within the microfluidic device are more mature in their phenotype, gene expression profile, calcium handling characteristics, and pharmaceutical response when compared to monolayer and 3D isotropic controls[23]. The protocol described herein outlines the method for creating this 3D co-cultured, aligned (i.e., anisotropic) human cardiac tissue within the microfluidic device using hiPSC-derived CMs. Specifically, we explain the methods to differentiate and purify hiPSCs towards CMs, supplementation of hCFs with CMs to produce an established co-culture population, insertion of the cell population encapsulated within the collagen hydrogel into the microfluidic devices, and subsequent analysis of the 3D constructed tissues through contractile and immunofluorescent assays. The resultant 3D engineered micro-tissues are suitable for various applications, including fundamental biology studies, CVD modeling, and pharmaceutical testing.

Protocol

Perform all cell handling and reagent preparation within a Biosafety Cabinet. Ensure all surfaces, materials, and equipment that come into contact with cells are sterile (i.e., spray down with 70% ethanol). Cells should be cultured in a humidified 37 °C, 5% CO_2 incubator. All hiPSC culture and differentiation is performed in 6-well plates.

1. Microfluidic device creation (approximate duration: 1 week)

1. **Photolithography**

NOTE: The mask, designed using the CAD file (provided as **Supplementary File 1**), contains the design of the microfluidic channel. Print the design on a transparent mask. Then, perform standard photolithography with the negative photoresist SU8 2075 on a 4-inch silicon wafer within a cleanroom.

1. Clean a silicon wafer with isopropyl alcohol (IPA) and dry with nitrogen. Bake at 200 °C for 5 min for dehydration.
   NOTE: Handle the wafer with wafer tweezers.
2. Place the wafer in the spin-coater. Deposit 3-4 mL of SU8 in the center of the wafer, then spin to form a layer of 200 µm (i.e., ramp up 15 s to 500 rpm, spin for 10 s, ramp up 5 s to 1,200 rpm, and spin for 30 s, then downspin for 15 s until stopped).
3. Remove wafer and soft bake for 7 min at 65 °C, then 45 min at 95 °C.
4. Move the wafer to a mask aligner and place the transparent mask in the mask holder with a UV filter. Expose the wafer to 2 cycles of 230 mJ/cm^2, with 30 s delay, for a total exposure of 460 mJ/cm^2.
5. Perform a post-exposure bake on the exposed wafer in a 50 °C oven overnight.
6. Turn off the oven the next morning, and after the wafer has cooled to room temperature, submerge it in SU8-developer. Remove the wafer from the developer every 5 min and wash with IPA, then place it back in the developer.
7. After around 20 min, or when the IPA runs clear, dry the wafer with air nitrogen and hard bake in an oven set to 150 °C; as soon as the oven reaches 150 °C, turn it off but do not open. Leave the wafer

in the oven until it has reached room temperature, then remove the wafer.

8. Confirm the height of SU8 with a profilometer and the optical features with a light microscope. Once confirmed, tape the wafer inside a 150 mm plastic Petri dish.

2. **Soft lithography**

NOTE: The wafer, taped to the Petri dish, needs to be silanized to prevent adherence of the PDMS to the SU8 features.

1. Invert the wafer (tapped to a plastic Petri dish) over a glass Petri dish of the same size containing 0.4 mL of methyltrichlorosilane (MTCS) and expose the wafer to the vapors for 4 min. Turn the wafer upright and place the lid on the Petri dish.
2. Mix 30 g of silicone elastomer base to the curing agent at a 10:1 ratio. Take the lid off the Petri dish and pour the polydimethylsiloxane (PDMS) on the wafer, then degas within a desiccator.
3. Once all bubbles are gone, place the wafer at 80 °C for 1.5 h to cure the PDMS.
4. Carefully peel off the PDMS, and punch inlets and outlets for the tissue ports and media channels with a 1 mm and 1.5 mm biopsy punch, respectively.
5. Clean the PDMS channels with tape to remove dust. Next, soak the coverslips (18 x 18 mm, No.1) in 70% ethanol for at least 15 min. Then, dry these off with tissue wipes.
6. Subject both the coverslips and PDMS channels (feature side exposed) to the plasma (setting on high) for 1 min, then quickly bond together and place in an 80 °C oven overnight to secure the bond.

NOTE: During bonding, it is essential to apply mild pressure on the edges of the PDMS channels to ensure a good seal between the PDMS and glass while avoiding the channel itself to prevent channel collapse.

3. **Device preparation**

1. Submerge the bonded PDMS devices in deionized water (DI H_2O) and autoclave with the liquid cycle. Next, aspirate the liquid from the devices and autoclave again with the gravity cycle. Then, dehydrate the sterilized devices overnight at 80 °C.

2. Stem cell culture (approximate duration: 1-2 months)

1. **hiPSC culture and maintenance**

NOTE: The hiPSCs need to be cultured for three consecutive passages after thawing *in vitro* before cryopreservation or differentiation. hiPSCs are cultured in either E8 or mTeSR1 medium, depending on the cell line, on the basement membrane matrix-coated plates[24].

1. To coat plates with hESC-quality basement membrane matrix, thaw one aliquot of the matrix medium (lot-dependent volume, generally 200-300 µL; stored at -80 °C) by adding it to 25 mL of DMEM/F-12K on ice. Dispense 1 mL of this suspension into each well of a 6-well plate. Leave the plate in the incubator at 37 °C for at least 1 h.
2. Upon thawing, modify the E8 media for hiPSC culture by adding 5 µM of Y-27632[25] (E8+RI). Use this media for 24 h afterward, then change the media to fresh E8.

NOTE: For routine media changes, unmodified E8 media is used for hiPSC culture. For regular

maintenance, E8 media must be changed every day, approximately 24 h after the previous media change.

3. Passage cells at Day 3 or Day 4, aspirate media, then wash each well with 1 mL of 1x Dulbecco's phosphate-buffered solution (DPBS).
   NOTE: Ensure that the cells are around 70% confluent. Do not let them grow beyond 70% confluency.
4. Aspirate the DPBS, then add 1 mL of 0.5 mM EDTA to each well and incubate at room temperature for 6-7 min.
5. Carefully aspirate EDTA, add 1 mL of E8+RI into each well and blast against the surface with a 1 mL pipette (~5-10 times to collect all of the cells). Collect the cell suspension in a 15 mL microcentrifuge tube.
6. Count the cell suspension and passage at the desired cell density (i.e., ~200 K per well) in E8+RI.
7. Change media to E8 (without RI) 24 h after. Do not leave the cells with RI for more than 24 h.
   NOTE: E8 should not be heated to 37 °C. Always leave it at room temperature for warming before cell culture.

2. **Cardiomyocyte (CM) directed differentiation**
   NOTE: It is important to note the existence of heterogeneity among different lines of the hiPSCs[26,27], so the following steps may need to be optimized for each cell line. Follow the steps below for CM differentiation.
   1. Prepare RPMI + B27 - insulin by adding 10 mL of B27 minus insulin and 5 mL of penicillin/ streptomycin (pen/strep) to 500 mL of RPMI 1640.
   2. Prepare RPMI + B27 + insulin by adding 10 mL of B27 and 5 mL of pen/strep to 500 mL of RPMI 1640.
   3. Prepare RPMI minus glucose + B27 + insulin by adding 10 mL of B27, 5 mL of pen/strep, and 4 mM sodium lactate to 500 mL of RPMI 1640 without glucose.
   4. Once the hiPSCs reach 85% confluency, begin differentiation (Day 0) by replacing the old medium with 4 mL of the RPMI + B27 - insulin medium containing 10 µM CHIR99021 to each well of a 6-well plate (i.e., add 25 µL of 10 mM CHIR99021 into 25 mL of RPMI + B27 - insulin and then immediately add 4 mL per well).
      NOTE: CHIR99021 is a GSK inhibitor and leads to Wnt activation. The optimal concentration of CHIR99021 and initial confluency varies for each cell line[28]. Always check a concentration gradient of 6-12 µM CHIR99021 and a series of seeding densities before the actual experiment to determine optimal conditions for initiating differentiation.
   5. Exactly 24 h later (Day 1), aspirate the medium and replace with 5 mL of prewarmed RPMI + B27 - insulin to each well.
   6. Exactly 72 h after CHIR99021 addition (Day 3), collect 2.5 mL of the spent medium from each well of the 6-well plate, totaling 15 mL of the spent medium in a tube.
   7. To this, add 15 mL of fresh RPMI + B27 - insulin medium. Add IWP2 to a concentration of 5 µM to the combined medium tube (i.e., 1 µL of IWP2 at 5 mM per 1 mL of combined medium or 30 µL of IWP2 into 30 total mL media).
   8. Remove ~1.5 mL of the remaining medium per well of the plate so that 1 mL of the medium remains. Swirl the plate vigorously to ensure adequate

removal of cell debris. Then, aspirate the rest of the old medium and add 5 mL of the combined medium containing IWP2 per well of the plate.

NOTE: The addition of IWP2 to the cells leads to Wnt inhibition.

9. On Day 5, aspirate the medium from each well and replace it with 5 mL of prewarmed RPMI + B27 - insulin.

10. CM Maturation: On Day 7, Day 9, and Day 11, aspirate the medium from each well and replace it with 5 mL of prewarmed RPMI + B27 + insulin. Spontaneous beating should be observed around these days.

11. CM Purification: On Day 13 and Day 16, start glucose starvation by aspirating the medium from each well, washing each well with 1 mL of 1x DPBS, and then adding 5 mL of prewarmed RPMI minus glucose + B27 + insulin, supplemented with 4 mM sodium lactate.

12. On Day 19, aspirate the spent medium and replace it with 5 mL of prewarmed RPMI + B27 + insulin to each well to allow for cell recovery after purification.

13. On Day 21, replate cells, following the below described CM dissociation protocol (step 3.3). Aim to plate 1.5-2 x 10^6 cells per well in a 6-well plate. For example, if it is a highly efficient differentiation, generally expanding the 6 wells into 9 wells is good.

14. From Day 21 on, aspirate the medium from each well and replace it with 4 mL of RPMI + B27 + insulin every 2-3 days.

NOTE: The hiPSC-CMs are ready for experimental use after Day 23.

3. Creation of 3D cardiac tissue within the microfluidic device: (Approximate duration: 2-3 h)

1. **hCF culture**

    1. Culture human ventricular cardiac fibroblasts (hCFs; obtained commercially from Lonza) in T75 flasks (at 250K cells per flask) in Fibroblast Growth Media-3 (FGM3). Change the media every other day, and passage when at 70% confluent. Use the hCFs before passage 10, as they may start to differentiate to myofibroblasts at high passages[29].

2. **hCF dissociation**

    1. To dissociate the hCFs, first take out the flask from the incubator. Put the flask inside the biosafety cabinet and begin aspirating the spent media from the flask. Then, wash the T75 flask with 3 mL of 1x DPBS. Close the cap and swirl the flask.

    2. Aspirate the DPBS. Take 3 mL of prewarmed 1x Trypsin-EDTA (0.05%) and add it to the flask. Tilt the flask and swirl to coat the bottom. Leave it in a 37 °C incubator for 4-6 min, checking the flask under a microscope to ensure cells are detaching, as evidenced through the round cell shape and floating cells. If not, then put the flask back in the incubator for another minute.

    3. Neutralize the trypsin action by adding 3 mL of prewarmed FGM3 to the flask. Then, pipette the solution up and down against the bottom of the flask to dislodge the CFs.

    4. Collect the cell suspension in a 15 mL microcentrifuge tube. Take 10 µL of the cell suspension and dispense it in a hemocytometer to count the cells with a microscope.

5. Centrifuge the cell suspension at 200 x *g* for 4 min. Aspirate the supernatant being careful not to disturb the cell pellet.

6. Resuspend the pellet in fresh FGM3, to make a desired 75 x 10^6 cells/mL. Either passage a portion (250K cells per T75 flask) or follow the below protocol to generate 3D cardiac tissue.

3. **CM dissociation**

NOTE: After differentiation and purification, prepare the CMs for use in the injection into microfluidic devices.

1. Take the plate of CMs out of the incubator and aspirate the media. Then wash the wells with 1 mL of 1x DPBS per well of a 6-well plate. Take 6 mL of DPBS and pipette 1 mL per well.

2. Aspirate the DPBS being careful not to disturb the cells attached to the plate. Pipette 6 mL of warm cell detachment solution (e.g., TrypLE express) and add 1 mL per well. Incubate the cells in a 37 °C incubator for 10 min.

3. Neutralize the enzyme with an equal volume of RPMI + B27+ insulin (i.e., 1 mL per well) and mechanically dissociate the cells by pipetting up and down against the culture vessel with a 1 mL pipette.

4. Collect the CMs in a 15 mL centrifuge tube. Centrifuge at 300 x *g* for 3 min.

5. Aspirate the supernatant. Resuspend the cell pellet in 5 mL of RPMI + B27 + insulin. Pipette the solution up and down with a 1 mL pipette to ensure proper mixing. Take 10 µL of the cell suspension and dispense it in a hemocytometer to determine the total cell number.

6. Centrifuge the cells again at 300 x *g* for 3 min (to ensure complete removal of TrypLE), and aspirate the supernatant. Then, add an appropriate volume of RPMI + B27 + insulin to achieve 75 x 10^6 cells/mL.

NOTE: If cardiac differentiation/selection does not result in high CM% (i.e., >80%), as evidenced through immunostaining or flow cytometry for CM-specific proteins like cTnT, do not consider cells as suitable for the tissue formation. The differentiation process should be optimized when this happens via adjustment of CHIR99021 concentrations and initial starting density. If CM purification needs improvement, other methods can be utilized, such as sorting for CMs with either fluorescence-activated cell sorting (FACS) or magnetic-activated cell sorting (MACS)[30,31,32].

4. **Collagen preparation**

NOTE: Prepare collagen from the high concentration of collagen stock (ranging from 8-11 mg/mL). The collagen used to create the cell:hydrogel mixture is at 6 mg/mL, and the final concentration is 2 mg/mL. Depending on the number of devices to inject, the volume of collagen solution to be made needs to be back calculated.

1. Keep all the required reagents on ice inside a biosafety hood.

NOTE: Collagen is a thermoresponsive hydrogel. Therefore, the temperature needs to remain low to prevent premature polymerization.

2. Take 75 µL of stock collagen (8 mg/mL) and dispense it in a microcentrifuge tube on ice. Collagen solution is very viscous, so slowly aspirate it with a pipette.

3. Take 13.85 µL of media (i.e., RPMI + B27 + insulin) and dispense it in the same tube.
4. Then take 10 µL of phenol red and add to the mixture and resuspend.
5. Lastly, take 1.15 µL of 1N NaOH and add to the suspension.
6. Using a 200 µL pipette tip, resuspend the suspension.

   NOTE: The stock collagen has an acidic pH, necessitating the addition of NaOH to neutralize before using it to encapsulate the cardiac cells. Phenol red acts as a pH indicator; therefore, add this before the NaOH addition. At this point, the collagen solution will be yellow, denoting its acidity. After the addition of NaOH, the solution should turn orange to light pink, indicating its neutralization.

5. **Hydrogel mixture and cell preparation**

   NOTE: In this step, the encapsulation of the cells within the collagen-based hydrogel is done. The cells, as well as all hydrogel precursors, should be placed on ice during the next steps.

   1. At this point, if the CFs have not yet been trypsinized, store the CM suspension in a 15 mL centrifuge tube, with the lid unscrewed to allow gas flow, within a 37 °C incubator. In parallel, dissociate the CFs and collect at a density of 75 x 10^6 cells/mL for device loading.
   2. Mix the suspended CMs with CFs at a 4:1 ratio. Take an aliquot of 8 µL of CMs and add to a fresh centrifuge tube on ice. Then take 2 µL of CFs and add to the cell suspension in the centrifuge tube.
   3. Resuspend the cell suspension, grab 5.6 µL of the cell suspension, and put it in a fresh microcentrifuge tube.
   4. Take 4 µL of the collagen just prepared in the above steps and add to a 4:1 CM:CF mixture. Add 2.4 µL of Growth Factor Reduced (GFR) basement membrane matrix, making the final cell density as 35 x 10^6 cells/mL for the device injection. Pipette the mixture up and down to ensure that the cell suspension is homogenous.

6. **Device insertion**

   NOTE: Once the cell:hydrogel mixture is prepared, it needs to be inserted into the devices.

   1. Take autoclaved microfluidic devices out of the 80 °C oven and set them in the Biosafety Cabinet for at least 1 h before the cell suspension insertion to allow the devices to cool to room temperature while maintaining sterility.
   2. Place the devices in 60 x 15 mm Petri dishes, at 3-4 devices per dish. Fill a 150 x 15 mm Petri dish with a thin layer DI H_2O to hold 3 of the 60 x 15 mm Petri dishes. This step creates a humidified environment surrounding the microfluidic devices.
   3. Use a new tip and resuspend the cell:hydrogel mixture thoroughly by pipetting the suspension while the tube remains on ice.
   4. Insert the tip into the injection port of the device and slowly and steadily inject 3 µL of the cell:hydrogel suspension into the tissue region inlet of a microfluidic device using a 20 µL pipette tip. Once the port is filled, stop the injection and remove the tip.

Repeat for all the devices, or the entirety of prepared hydrogel suspension.

NOTE: The small volume of the cell:hydrogel suspension heats up/cools down very quickly, so it is pertinent to keep the suspension on ice for as long as possible. When inserting, pipette the solution off the ice and insert into devices as quickly as possible, as the hydrogel may start to polymerize in the pipette tip. It is important to create small volumes of the cell:hydrogel suspension at a time, so if many devices are to be injected, the cell:hydrogel suspension will have to be made fresh for each set of 4 devices.

5. Flip the devices within their Petri dishes with tweezers and place them inside the large Petri dish with water. Incubate in a 37 °C incubator for 9 min for hydrogel polymerization.

6. Take the devices out of the incubator, flip the devices upright, and incubate at 37 °C for 9 min to complete hydrogel polymerization.

7. Inject RPMI + B27 + insulin into the flanking media channels (~20 µL per device). Place the devices back in the incubator at 37 °C. Change the media within the media channels with fresh RPMI + B27 + insulin every day. The devices have been demonstrated to be cultured from 14-21 days[20].

NOTE: Due to the small volume of media per chip, to prevent evaporation of media, it is important to maintain the devices within a large Petri dish filled with DI H_2O, which serves as a humidified chamber. Additionally, small droplets of excess RPMI + B27 + insulin can be pipetted on the top of the channel inlets/outlets during routine media changes.

4. Tissue analysis

1. **Live Imaging**

NOTE: All live imaging should be performed with a stage incubator to maintain 37 °C and 5% CO_2.

1. Place devices in an environmentally-controlled stage incubator. Record 30 s videos of multiple spots within each device at the maximal frame rate.
2. To assess tissue contractility after extracting the beating signals, use the supplementary custom-written MATLAB code to extract peaks (**Supplementary File 2**) for calculating inter-beat interval variability.
3. Change media in devices in the cell culture hood, then place back in cell culture incubator.

2. **Immunofluorescent staining**

1. Prepare PBS-Glycine: Dissolve 100 mM glycine in PBS and add 0.02% NaN_3 for long-term storage. Adjust pH to 7.4.
2. Prepare PBS-Tween-20: Add 0.05% Tween-20 to PBS and add 0.02% NaN_3 for long-term storage. Adjust pH to 7.4.
3. Prepare IF buffer: Add 0.2% Triton X-100, 0.1% BSA, and 0.05% Tween-20 to PBS, and add 0.02% NaN_3 for long-term storage. Adjust pH to 7.4.
4. Prepare 10% Goat serum: Resuspend the lyophilized goat serum in 2 mL of PBS to make 100% goat serum. Then, dilute the 2 mL with 18 mL of PBS to make 10% goat serum.
5. Fix the samples by adding 4% paraformaldehyde (PFA) to the tissue channels and incubating at 37 °C for 20 min.

6. Wash the cells by adding PBS-Glycine to the tissue channels 2x for 10 min incubation at room temperature.
7. Wash the cells by adding PBS -Tween-20 for 10 min at room temperature.
8. Permeabilize the cells by adding IF buffer to the tissue channels for 30 min at room temperature.
9. Block the cells by adding 10% goat serum solution to the tissue channels for 1 h at room temperature.
10. Dilute the non-conjugated primary antibodies in 10% goat serum at desired concentrations (refer to **Supplementary File 3**), add to the tissue channels, and incubate the samples at 4 °C overnight.
11. The following day, wash the samples by adding PBS-Tween-20 to tissue channels 3x for 20 min each at room temperature.
    NOTE: From step 4.2.12 on, perform all tasks in the dark, so the samples are protected from light.
12. Dilute the secondary antibodies in PBS-Tween-20 at desired concentrations, centrifuge at 10,000 x *g* for 10 min to collect any precipitates, then add to the tissue channels.
13. After 30 min-1 h, wash the samples with PBS-Tween-20 3x for 10 min each at room temperature.
14. Add anti-fade or desired mounting medium to the tissue channels. Then, the samples can be imaged using fluorescence microscopy or with a confocal microscope, if higher magnification is desired. To visualize the entire 3D tissue, images at different z-planes can be stacked and reconstructed to form representative 3D images.

Representative Results

To obtain a highly purified population of CMs from hiPSCs, a modified version involving a combination of the Lian differentiation protocol[33] and Tohyama purification steps[34] is used (refer to **Figure 1A** for experimental timeline). The hiPSCs need to be colony-like, ~85% confluent, and evenly spread throughout the culture well 3-4 days after passage, at the onset of CM differentiation (**Figure 1B**). Specifically, on Day 0, hiPSC colonies should have a high expression of pluripotency transcription factors, including SOX2 and Nanog (**Figure 1C**). Based on this protocol, evidence of a successful stem cell differentiation and purification process is demonstrated in **Figure 1D**, with dense colonies of CMs expressing sarcomeric α-actinin, with minimal surrounding non-CMs. Additionally, the hCFs must maintain a fibroblast morphology with high vimentin expression (**Figure 1E**), so they are to be used before P10 as they may start to differentiate to myofibroblasts at higher passages[29].

To maintain the robust nature of this protocol, it is pertinent to implement replating of the hiPSC-CMs after metabolic purification. Due to the presence of dead cells and debris that occur from glucose starvation, the CMs need a further purification step to maximize CM purity and health before use in the experiment; therefore, replating is used as it helps loosen the debris/ dead non-CMs (**Figures 2A-B, Videos 1-2**). **Video 1** shows an example population of CMs before replating, presenting with a high purity of CMs in multiple layers, however with much debris present on the cells and floating in the media due to the implemented purification. Correspondingly, **Video 2** shows the same population of CMs immediately after replating, presenting the CMs in a monolayer with significantly less debris, demonstrating the

effects of tissue digestion and single-cell dissociation that occur during replating.

Upon insertion of the cell-embedded hydrogel into the microfluidic device (i.e., chip; inset image in **Figure 3**), the cells are dense and evenly spaced throughout the posts. The cells start to spread at Day 1 (**Figure 3A**), then by Day 7, they resume beating and become more synchronous in their contractile patterns (**Figure 3B**). Additionally, the 3D tissues condense around the posts to form repeating elliptical pores, and the cells elongate. By Day 14, the matured tissues exhibit a high degree of anisotropy (i.e., directional organization), composed of cells with elongated shape (**Figures 3C, 4A**), striated, aligned sarcomeres, and localized gap junctions (**Figure 4B**). Furthermore, the spontaneous contractile patterns of these tissues are highly synchronous (**Figure 4C, Video 3**) due to the interconnected, aligned nature of the cardiac cells.

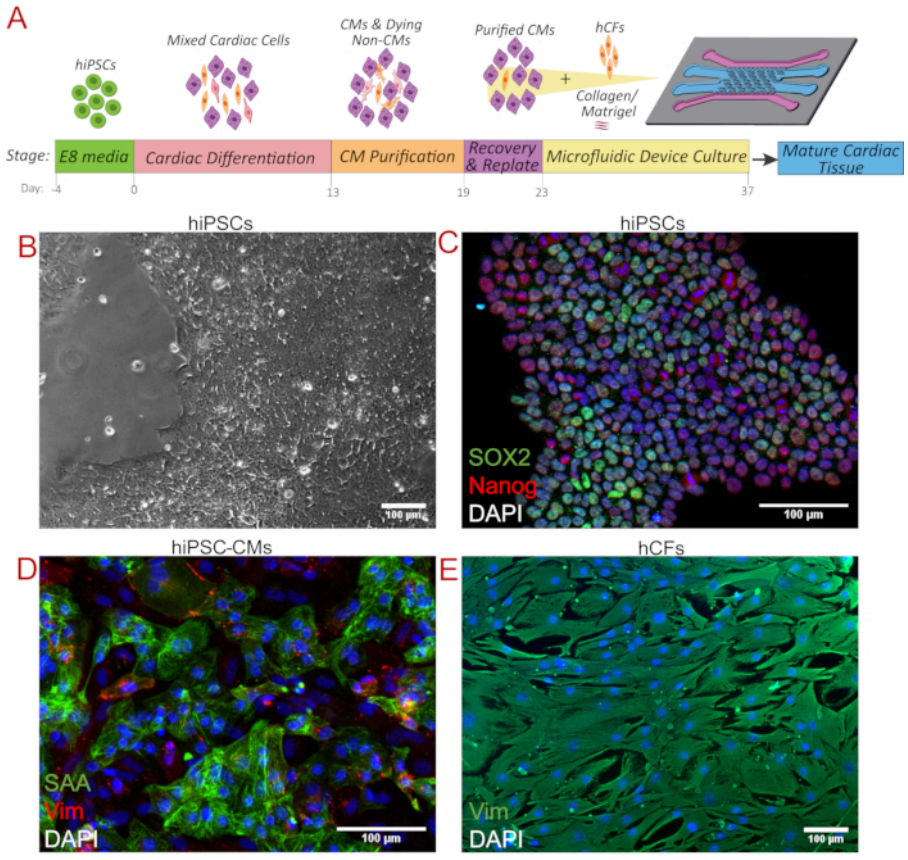

Figure 1: Experimental schematic and representative images of cellular morphology during preparation for device injection: Schematic of the protocol, describing steps after microfluidic device fabrication, from hiPSC culture to microfluidic cardiac tissue formation (**A**). hiPSCs should maintain a colony-like morphology (**B**) and high expression of pluripotency markers (SOX2, green; Nanog, red) (**C**) on the onset of differentiation. After hiPSC-CM differentiation, there should be abundant, dense patches of CMs, as stained with sarcomeric alpha-actinin (SAA, green), with minimal surrounding non-CMs, as evidenced by vimentin staining (vim, red) (**D**). hCFs should present with high levels of vimentin expression and maintain fibroblast morphology (**E**). Please click here to view a larger version of this figure.

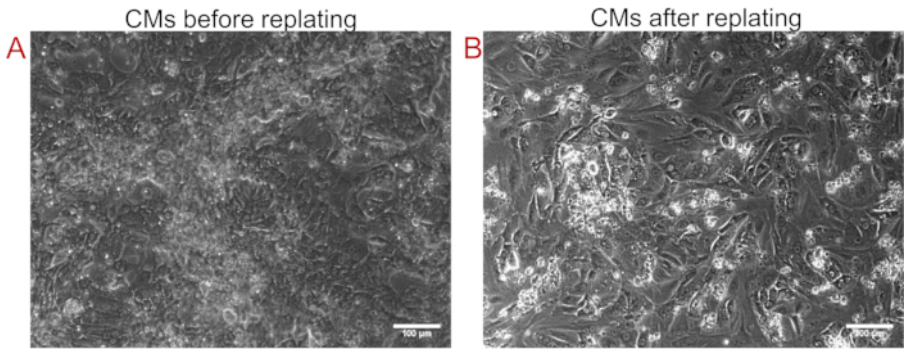

Figure 2: hiPSC-CM populations during the latter stages of differentiation: The purification process causes the death of non-CMs, producing debris in the cell culture, present before CM replating (**A**). After replating (**B**), the debris and non-CMs are dislodged, serving to further purify the CM population. Please click here to view a larger version of this figure.

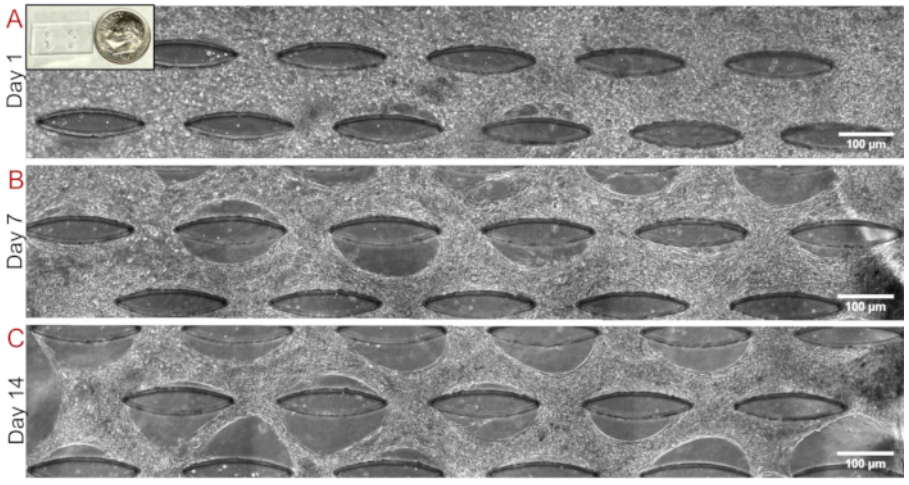

Figure 3: Human cardiac 3D tissue formation within the microfluidic device: The day following injection into the device (shown in the upper left inset, next to US dime for scale), the cells will begin to spread and will be dense and homogeneously distributed throughout the device (**A**). After a week of culture, the cells will resume spontaneously beating and form condensed, aligned tissues (**B**). By two weeks of culture, the cells form condensed, aligned tissues around the elliptical posts (**C**). Please click here to view a larger version of this figure.

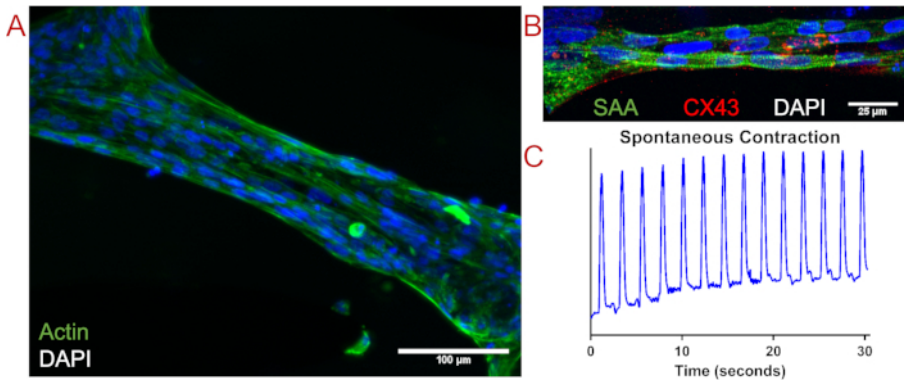

Figure 4: Representative characteristics of human cardiac tissue after culture for 14 days in the microfluidic device: The cells have formed elongated, highly aligned tissues, as denoted by actin staining (**A**). The sarcomeres are parallel and striated, and there is the localization of gap junctions as evidenced through staining for sarcomeric α-actinin (SAA) and connexin 43 (CX43), respectively (**B**). The spontaneous contraction is synchronous (**C**). Please click here to view a larger version of this figure.

Video 1: Spontaneous contraction of hiPSC-CMs on Day 21 after lactate purification and before replating Please click here to download this Video.

Video 2: Spontaneous contraction of hiPSC-CMs on Day 23 after replating Please click here to download this Video.

Video 3: Synchronous, spontaneous contraction of human cardiac tissue within device for 14 days Please click here to download this Video.

Supplementary File 1: AutoCAD file for heart on-a-chip device Please click here to download this File.

Supplementary File 2: MATLAB program to extract peaks to determine inter-beat interval variability from beating signals Please click here to download this File.

Supplementary File 3: Table of primary and secondary antibodies Please click here to download this File.

Discussion

The formation of an *in vitro* human cardiac tissue model with enhanced cell-cell interactions and biomimetic 3D structure is imperative for basic cardiovascular research and corresponding clinical applications[1]. This outlined protocol explains the development of 3D human anisotropic cardiac tissue within a microfluidic device, using co-culture of stem cell-derived CMs with connective CFs encapsulated within a collagen hydrogel, serving to model the complex cell composition and structure of the native myocardium. This specific protocol is highly reproducible, as the particular structure of the device has been optimized and validated for 3D anisotropic cardiac tissue formation from both rat-derived cardiac cells, and human stem cell-differentiated CMs, from hESCs, and two types of hiPSCs (SCVI20 and IMR90-4) as demonstrated in our recent publication[20]. We, among many other groups, have found that the efficiency of CM-differentiation of stem cells varies amongst cell lines[35,36].

The implemented purification protocol aids in increasing the differentiation yield; however, the length of purification time is dependent on cell line and differentiation efficiency. Therefore, the resultant success in the formation of the microfluidic tissues may vary between cell lines.

To capture pertinent components of the myocardium, the cellular composition for the demonstrated tissues is mainly a mixture of CMs and CFs, as CMs compromise most of the volume while CFs retain most of the cell population within the heart[37]. Furthermore, the particular ratio of 4:1 CM:CFs was extensively validated in recent published work[20] to result in optimal structure and cardiac tissue formation within this platform. Future studies involving this described platform could be further advanced in their complexity by supplementation with other penitent cell types to better mimic the native myocardium. For example, it has been recently found that resident macrophages are integral in conduction processes within the heart[38], in addition to their well-documented role in immune response[39]. Therefore, macrophages could be incorporated into the cell mixture before hydrogel encapsulation to model resident cardiac macrophages. Alternatively, monocytes could be delivered through the media channels as a model of recruitment through the blood circulation, which may lead to a population of inflammatory macrophages within the heart tissue.

There are inherent advantages in using a microfluidic device as a platform to construct 3D tissue models. Particularly, precise diffusion-based experiments can be established through exact control of chemicals, molecules, or gases that enable concentration gradients across a device[18,20]. Additionally, diverse sets of cell types[40] and fluid flow can be incorporated to mimic dynamic culture conditions and provide shear stress on seeded cells[41]. The latter may be of particular use in the study of an incorporated vascular system within the chip, as endothelial cells can be seeded in the adjacent media channels (as we have previously demonstrated using this device to model astrocytes-on-a-chip[23]), and constant fluid flow to mimic capillaries can easily be incorporated via a vacuum-based or gravity-based pump.

Another benefit of the microfluidic device is the material (i.e., PDMS) used to fabricate the device channels. Specifically, PDMS is a transparent, cheap, and biocompatible polymer[42] with easily adjustable stiffness. The limiting step in the fabrication of these devices lies in the photolithography, as the technique requires access to a cleanroom and acquisition of the associated skill. However, once the wafer is fabricated, it can be used to make hundreds of devices through the straight-forward soft lithography process to create the PDMS channels and the simple act of plasma bonding to seal the channels to coverslips. In future studies, if the device were to be modified to include the capability to measure electrical properties of the tissue in real-time, an additional step of fitting the device with electrodes and conductive components would have to be incorporated in the fabrication process. The use of PDMS to fabricate the channel may retain limitations, particularly if used in constructs for drug-response studies, as PDMS has been found to adsorb small hydrophobic molecules[43,44,45]. Therefore, other materials, such as thermoplastics[46,47], could be investigated as alternatives to PDMS during the soft lithography process.

A critical step in this outlined protocol is step 3.6.4 detailing cell:hydrogel insertion into the microfluidic devices. A few key variables need to be controlled to ensure success, including temperature, time, and proper handling. If either the hydrogel stock solutions or the prepared cell:hydrogel solution reach room temperature, they are at risk for partial polymerizing,

which is irreversible, making the solution viscous and near impossible to inject into the device without leakage into media channels. On the other hand, the cell:hydrogel solution cannot freeze, as the cells will die; therefore the solution must be maintained within this narrow temperature window. Similarly, the amount of time elapsed between cell:hydrogel preparation and device injection directly relates to increasing temperature of the prepared solution. Specifically, as soon as the solution is made and the aliquot (i.e., 3 µL) is obtained, the pipette holding the aliquot has to be repositioned, so the tip is inside the device ports, and the aliquot is to be slowly and steadily inserted into the device. Throughout this transition, the small volume is within a pipette tip that is at room temperature, therefore, the solution is rapidly increasing temperature from that of the ice it was prepared on (i.e., -20 °C), requiring a rather swift injection process. If the temperature sensitivity of the collagen-based hydrogel becomes a key factor during device injection, incorporation of other hydrogels[48], such as photocrosslinkable hydrogels (i.e., GelMA)[47,49,50,51] or enzymatically-crosslinked hydrogels (i.e., fibrin)[11,52,53], could be explored.

The design of the microfluidic device included in this protocol allows for the establishment of an anisotropic tissue due to the presence of the staggered, protruding elliptical posts within the main tissue channel[20]. This feature is advantageous over other methods, such as ECM contact printing, because it does not require a handling step to create the topography that may lead to variation between samples due to stamp deformation or ink diffusion[54]. However, as stated earlier, there are often difficulties inherent in the injection of a cell:hydrogel suspension into a device channel, particularly in a channel with innate posts. To that end, the handling pressure of device insertion is rather sensitive. The injection process has to be steady, at a consistent and relatively low pressure to avoid any leakage into the media channels.

Additionally, bubbles cannot be introduced either during the preparation of the solution or during device insertion, as bubbles will cause leakage from the main tissue channel into the media channels. Thus, proper care is needed to control handling, temperature, and timing of the cell:hydrogel injection to ensure the success in the formation of 3D homogeneously distributed tissue. Therefore, practice in handling the microfluidic devices before cell culture experiments may be beneficial. Maintenance thereafter of tissues within microfluidic devices is quite straightforward, simply necessitating a daily media change of 20 µL volume, and the coverslip-base of the device renders convenient handling during real-time imaging.

In summary, the protocol described herein utilizes a combination of micromolding techniques, including photolithography and soft lithography, to create an intricate architecture within a microfluidic device that induces high levels of 3D tissue anisotropy, with robust biological techniques, including stem cell differentiation, primary human cell culture, and hydrogel-based biomaterials. The end result of the outlined protocol is an aligned, 3D co-cultured cardiac tissue within a microfluidic chip with a mature phenotype, that has been repeatedly validated for multiple different cell types and lines[20], rendering it suitable for disease modeling and downstream preclinical applications.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgments

We would like to thank NSF CAREER Award #1653193, Arizona Biomedical Research Commission (ABRC) New Investigator Award (ADHS18-198872), and the Flinn Foundation Award for providing funding sources for this project. The hiPSC line, SCVI20, was obtained from Joseph C. Wu, MD, PhD at the Stanford Cardiovascular Institute funded by NIH R24 HL117756. The hiPSC line, IMR90-4, was obtained from WiCell Research Institute[55,56].

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