Journal of Visualized Experiments

Developing 3D Organized Human Cardiac Tissue Within a Microfluidic Platform

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE62539R1
Full Title:	Developing 3D Organized Human Cardiac Tissue Within a Microfluidic Platform
Corresponding Author:	Mehdi Nikkhah UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	mnikkhah@asu.edu
Order of Authors:	Jaimeson Veldhuizen
	Mehdi Nikkhah
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$1200)
Please specify the section of the submitted manuscript.	Bioengineering
Please confirm that you have read and agree to the terms and conditions of the author license agreement that applies below:	I agree to the Author License Agreement
Please provide any comments to the journal here.	
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)

TITLE:

Developing 3D Organized Human Cardiac Tissue Within a Microfluidic Platform

AUTHORS AND AFFILIATIONS:

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

[1]School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ, USA

[2]Biodesign Virginia G. Piper Center for Personalized Diagnostics, Arizona State University, Tempe, AZ, USA

Email addresses of co-authors:

Jaimeson Veldhuizen (jveldhui@asu.edu)

*Corresponding author:

Mehdi Nikkhah (mnikkhah@asu.edu)

KEYWORDS:

stem cell, cardiac tissue, microenvironment, myocardium, microfluidic chips

SUMMARY:

The goal of this protocol is to explain and demonstrate the development of a three-dimensional (3D) microfluidic model of highly aligned human cardiac tissue, composed of stem cell-derived cardiomyocytes co-cultured with cardiac fibroblasts (CFs) within a biomimetic, collagen-based hydrogel, for applications in cardiac tissue engineering, drug screening, and disease modeling.

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-specific 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[14-16]. 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[17-19]. 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[18,20-22] 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)

1.1 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.

1.1.1 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.

1.1.2 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).

1.1.3 Remove wafer and soft bake for 7 min at 65 °C, then 45 min at 95 °C.

1.1.4 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.

1.1.5 Perform a post-exposure bake on the exposed wafer in a 50 °C oven overnight.

1.1.6 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.

1.1.7 After ~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.

1.1.8 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.

1.2 Soft lithography

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

1.2.1 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.

1.2.2 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.

1.2.3 Once all bubbles are gone, place the wafer at 80 °C for 1.5 h to cure the PDMS.

1.2.4 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.

1.2.5 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.

1.2.6 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.

1.3 Device preparation

1.3.1. 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)

2.1 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 E8 medium on the basement membrane matrix-coated plates[24].

2.1.1 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.

2.1.2. 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.

2.1.3. 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.

2.1.4. Aspirate the DPBS, then add 1 mL of 0.5 mM EDTA to each well and incubate at room temperature for 6-7 min.

2.1.5. 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.

2.1.6. Count the cell suspension and passage at the desired cell density (i.e., ~200 K per well) in E8+RI.

2.1.7. 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.

2.2 Cardiomyocyte (CM) directed differentiation.

NOTE: It is important to note the existence of heterogeneity among different lines of the human-induced pluripotent stem cells[26,27], so the following steps may need to be optimized for each cell line. Follow the steps below for CM differentiation.

2.2.1 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.

2.2.2 Prepare RPMI + B27 + insulin by adding 10 mL of B27 and 5 mL of pen/strep to 500 mL of RPMI 1640.

2.2.3 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.

2.2.4 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 minus 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.

2.2.5**.** Exactly 24 h later (Day 1), aspirate the medium and replace with 5 mL of prewarmed RPMI + B27 - insulin to each well.

2.2.6. 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.

2.2.7. 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).

2.2.8. 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.

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

2.2.10. 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.

2.2.11. 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.

2.2.12. 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.

2.2.13. 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.

2.2.14. 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)

3.1. hCF culture

3.1.1. 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].

3.2. hCF dissociation:

3.2.1. To dissociate the hCF, 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.

3.2.2. 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.

3.2.3. 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.

3.2.4. 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.

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

3.2.6. 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.

3.3. CM dissociation

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

3.3.1. 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.

3.3.2. 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.

3.3.3. 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.

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

3.3.5. 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.

3.3.6. 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)[30-32].

3.4. 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.

3.4.1. 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.

3.4.2. 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.

3.4.3. Take 13.85 µL of media (i.e., RPMI+B27 + insulin) and dispense it in the same tube.

3.4.4. Then take 10 µL of phenol red and add to the mixture and resuspend.

3.4.5. Lastly, take 1.15 µL of 1N NaOH and add to the suspension.

3.4.6. 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.

3.5. 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.

3.5.1. 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.

3.5.2. 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.

3.5.3. Resuspend the cell suspension, grab 5.6 µL of the cell suspension, and put it in a fresh microcentrifuge tube.

3.5.4. 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.

3.6. Device insertion

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

3.6.1. 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.

3.6.2. 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.

3.6.3. Use a new tip and resuspend the hydrogel cell mixture thoroughly by pipetting the suspension while the tube remains on ice.

3.6.4. Insert the tip into the injection port of the device and slowly and steadily inject 3 µL of the hydrogel cell 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.

3.6.5. 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.

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

3.6.7. 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

4.1 Live Imaging

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

4.1.1 Place devices in an environmentally controlled stage incubator. Record 30 s videos of multiple spots within each device at the maximal frame rate.

4.1.2. 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.

4.1.3. Change media in devices in the cell-culture hood, then place back in cell culture incubator.

4.2 Immunofluorescent staining

4.2.1. Prepare PBS-Glycine: Dissolve 100 mM glycine in PBS and add 0.02% NaN_3 for long-term storage. Adjust pH to 7.4.

4.2.2. 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.

4.2.3. 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.

4.2.4. 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.

4.2.5. Fix the samples by adding 4% paraformaldehyde (PFA) to the tissue channels and

incubating at 37 °C for 20 min.

4.2.6. Wash the cells by adding PBS-Glycine to the tissue channels 2x for 10 min incubation at room temperature.

4.2.7. Wash the cells by adding PBS -Tween-20 for 10 min at room temperature.

4.2.8. Permeabilize the cells by adding IF buffer to the tissue channels for 30 min at room temperature.

4.2.9. Block the cells by adding 10% goat serum solution to the tissue channels for 1 h at room temperature.

4.2.10. 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.

4.2.11. The following day, wash the samples by adding PBS-Tween-20 to tissue channels three times for 20 min each at room temperature.

NOTE: From step 4.2.8 on, perform all tasks in the dark, so the samples are protected from light.

4.2.12. Dilute the secondary antibodies in PBS-Tween-20 at desired concentrations, centrifuge at 1,000 x *g* for 10 min to collect any precipitates, then add to the tissue channels.

4.2.13. After 30 min-1 h, wash the samples with PBS-Tween-20 three times for 10 min each at room temperature.

4.2.14. 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 media does not leak into the main channel during media changes. 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 AND TABLE LEGENDS:

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**).

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.

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**).

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**).

Video 1: Spontaneous contraction of hiPSC-CMs on Day 21 after lactate purification and before replating

Video 2: Spontaneous contraction of hiPSC-CMs on Day 23 after replating

Video 3: Synchronous, spontaneous contraction of human cardiac tissue within device for 14 days

Supplementary File 1: AutoCAD file for heart on-a-chip device

Supplementary File 2: MATLAB program to extract peaks to determine inter-beat interval variability from beating signals

Supplementary File 3: Table of primary and secondary antibodies

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 2 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[43-45]. 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)[47,49-51] 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.

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].

DISCLOSURES:

The authors declare that they have no competing financial interests.

REFERENCES:

1 Savoji, H. et al. Cardiovascular disease models: A game changing paradigm in drug discovery and screening. *Biomaterials.* **198**, 3-26 (2019).

2 Patino-Guerrero, A., Veldhuizen, J., Zhu, W., Migrino, R. Q. Nikkhah, M. Three-dimensional scaffold-free microtissues engineered for cardiac repair. *Journal of Materials Chemistry B.* **8**, 7571-7590 (2020).

3 Breslin, S., O'Driscoll, L. Three-dimensional cell culture: the missing link in drug discovery. *Drug Discovery Today.* **18**, 240-249 (2013).

4 Pontes Soares, C. et al. 2D and 3D-organized cardiac cells shows differences in cellular morphology, adhesion junctions, presence of myofibrils and protein expression. *PLoS One.* **7**, e38147 (2012).

5 Jensen, C., Teng, Y. Is it time to start transitioning from 2d to 3d cell culture? *Frontiers in Molecular Biosciences.* **7**, e00033 (2020).

6 Pinto, A. R. et al. Revisiting cardiac cellular composition. *Circulation Research.* **118**, 400-409 (2016).

7 LeGrice, I., Pope, A., Smaill, B. *Interstitial Fibrosis in Heart Failure.* Vol. 253, Springer. Parts of the Developments in Cardiovascular Medicine book series. 3-21 (2005).

8 Veldhuizen, J., Migrino, R. Q., Nikkhah, M. Three-dimensional microengineered models of human cardiac diseases. *Journal of Biological Engineering* **13**, 29 (2019).

9 Agarwal, A., Goss, J. A., Cho, A., McCain, M. L., Parker, K. K. Microfluidic heart on a chip for higher throughput pharmacological studies. *Lab Chip* **13**, 3599-3608 (2013).

10 Schaaf, S. et al. Human engineered heart tissue as a versatile tool in basic research and preclinical toxicology. *PLoS One.* **6**, e26397 (2011).

11 Zhang, D. et al. Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials* **34**, 5813-5820 (2013).

12 Rao, C. et al. The effect of microgrooved culture substrates on calcium cycling of cardiac myocytes derived from human induced pluripotent stem cells. *Biomaterials* **34**, 2399-2411 (2013).

13 Navaei, A. et al. Electrically conductive hydrogel-based micro-topographies for the development of organized cardiac tissues. *RSC Advances.* **7**, 3302-3312 (2017).

14 Jiang, Y., Park, P., Hong, S. M., Ban, K. Maturation of cardiomyocytes derived from human pluripotent stem cells: Current strategies and limitations. *Molecules and Cells.* **41**, 613-621 (2018).

15 Yang, X., Pabon, L., Murry, C. E. Engineering adolescence maturation of human pluripotent stem cell–derived cardiomyocytes. *Circulation Research* **114**, 511-523 (2014).

16 Kharaziha, M., Memic, A., Akbari, M., Brafman, D. A., Nikkhah, M. Nano-enabled approaches for stem cell-based cardiac tissue engineering. *Advanced Healthcare Materials* **5**, 1533-1553 (2016).

17 Ellis, B. W., Acun, A., Can, U. I., Zorlutuna, P. Human iPSC-derived myocardium-on-chip with capillary-like flow for personalized medicine. *Biomicrofluidics* **11**, 024105 (2017).

18 Mathur, A. et al. Human iPSC-based cardiac microphysiological system for drug screening applications. *Scientific Reports.* **5**, 8883 (2015).

19 Mastikhina, O. et al. Human cardiac fibrosis-on-a-chip model recapitulates disease hallmarks and can serve as a platform for drug testing. *Biomaterials.* **233**, 119741 (2020).
20 Veldhuizen, J., Cutts, J., Brafman, D. A., Migrino, R. Q., Nikkhah, M. Engineering anisotropic human stem cell-derived three-dimensional cardiac tissue on-a-chip. *Biomaterials.* **256**, 120195 (2020).
21 Truong, D. et al. Human organotypic microfluidic tumor model permits investigation of the interplay between patient-derived fibroblasts and breast cancer cells. *Cancer Research* **7**, 3139-3151 (2019).
22 Benam, K. H. et al. Engineered in vitro disease models. *Annual Review of Pathology: Mechanisms of Disease* **10**, 195-262 (2015).
23 Karamanova, N. et al. Endothelial immune activation by medin: Potential role in cerebrovascular disease and reversal by monosialoganglioside-containing nanoliposomes. *Journal of the American Heart Association.* **9**, e014810 (2020).
24 Chen, G. et al. Chemically defined conditions for human iPSC derivation and culture. *Nature Methods.* **8**, 424-429 (2011).
25 Watanabe, K. et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology.* **25**, 681-686 (2007).
26 Narsinh, K. H. et al. Single cell transcriptional profiling reveals heterogeneity of human induced pluripotent stem cells. *Journal of Clinical Investigation.* **121**, 1217-1221 (2011).
27 Cahan, P., Daley, G. Q. Origins and implications of pluripotent stem cell variability and heterogeneity. *Nature Reviews Molecular Cell Biology.* **14**, 357-368 (2013).
28 Laco, F. et al. Unraveling the inconsistencies of cardiac differentiation efficiency induced by GSKB inhibitor CHIR99021 in human pluripotent stem cells. *Stem Cell Reports.* **10**, (2018).
29 Rupert, C. E., Kim, T. Y., Choi, B. R., Coulombe, K. L. K. Human cardiac fibroblast number and activation state modulate electromechanical function of hiPSC-cardiomyocytes in engineered myocardium. *Stem Cells International.* **2020**, 9363809 (2020).
30 Ban, K., Bae, S., Yoon, Y. S. Current strategies and challenges for purification of cardiomyocytes derived from human pluripotent stem cells. *Theranostics.* **7**, 2067-2077 (2017).
31 Uosaki, H. et al. Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. *PLoS One.* **6**, e23657 (2011).
32 Dubois, N. C. et al. SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nature Biotechnology.* **29**, 1011-U1082 (2011).
33 Lian, X. et al. Directed cardiomyocyte differentiation from human pluripotent stem cells by modulating Wnt/β-catenin signaling under fully defined conditions. *Nature Protocols.* **8**, (2013).
34 Tohyama, S. et al. Distinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell-derived cardiomyocytes. *Cell Stem Cell.* **12**, 127-137 (2013).
35 Burridge, P. W., Keller, G., Gold, J. D., Wu, J. C. Production of de novo cardiomyocytes: human pluripotent stem cell differentiation and direct reprogramming. *Cell Stem Cell.* **10**, 16-28 (2012).
36 Mummery, C. L. et al. Differentiation of human embryonic stem cells and induced pluripotent stem cells to cardiomyocytes: a methods overview. *Circulation Research.* **111**, 344-358 (2012).

37 Radisic, M. et al. Biomimetic approach to cardiac tissue engineering. *Philosophical Transactions of the Royal Society of London B Biological Science.* **362**, 1357-1368 (2007).
38 Hulsmans, M. et al. Macrophages facilitate electrical conduction in the heart. *Cell.* **169**, 510-522 e520 (2017).
39 Frantz, S., Nahrendorf, M. Cardiac macrophages and their role in ischaemic heart disease. *Cardiovascular Research.* **102**, ecvu025 (2014).
40 Truong, D. et al. A three-dimensional (3D) organotypic microfluidic model for glioma stem cells - Vascular interactions. *Biomaterials.* **198**, 63-77 (2019).
41 Shao, J. et al. Integrated microfluidic chip for endothelial cells culture and analysis exposed to a pulsatile and oscillatory shear stress. *Lab Chip.* **9**, 3118-3125 (2009).
42 Leclerc, E., Sakai, Y., Fujii, T. Cell culture in 3-dimensional microfluidic structure of PDMS (polydimethylsiloxane). *Biomedical Microdevices.* **5**, 109-114 (2003).
43 Mukhopadhyay, R. When PDMS isn't the best. What are its weaknesses, and which other polymers can researchers add to their toolboxes? *Analytical Chemistry.* **79**, 3248-3253 (2007).
44 van Meer, B. J. et al. Small molecule absorption by PDMS in the context of drug response bioassays. *Biochemical and Biophysical Research Communication.* **482**, 323-328 (2017).
45 Berthier, E., Young, E. W., Beebe, D. Engineers are from PDMS-land, Biologists are from Polystyrenia. *Lab Chip.* **12**, 1224-1237 (2012).
46 Chuchuy, J. et al. Integration of electrospun membranes into low-absorption thermoplastic organ-on-chip. *ACS Biomaterials Science & Engineering* (2021).
47 Soucy, J. R. et al. Reconfigurable microphysiological systems for modeling innervation and multitissue interactions. *Advanced Biosystems.* **4**, e2000133 (2020).
48 Cutts, J., Nikkhah, M., Brafman, D. A. Biomaterial approaches for stem cell-based myocardial tissue engineering. *Biomarker Insights.* **10**, 77-90 (2015).
49 Navaei, A. et al. Gold nanorod-incorporated gelatin-based conductive hydrogels for engineering cardiac tissue constructs. *Acta Biomaterialia.* **41**, 133-146 (2016).
50 Navaei, A. et al. The influence of electrically conductive and non-conductive nanocomposite scaffolds on the maturation and excitability of engineered cardiac tissues. *Biomaterial Sciences.* **7**, 585-595 (2019).
51 Saini, H., Navaei, A., Van Putten, A., Nikkhah, M. 3D cardiac microtissues encapsulated with the co-culture of cardiomyocytes and cardiac fibroblasts. *Advanced Healthcare Materials.* **4**, 1961-1971 (2015).
52 Shadrin, I. Y. et al. Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues. *Nature Communications.* **8**, 1825 (2017).
53 Ronaldson-Bouchard, K. et al. Advanced maturation of human cardiac tissue grown from pluripotent stem cells. *Nature.* **556**, 239-243 (2018).
54 Perl, A., Reinhoudt, D. N., Huskens, J. Microcontact Printing: Limitations and Achievements. *Advanced Materials.* **21**, 2257-2268 (2009).
55 Yu, J. et al. Human induced pluripotent stem cells free of vector and transgene sequences. *Science* **324**, 797-801 (2009).
56 Yu, J. et al. Induced pluripotent stem cell lines derived from human somatic cells. *Science* **318**, 1917-1920 (2007).

Figure 1

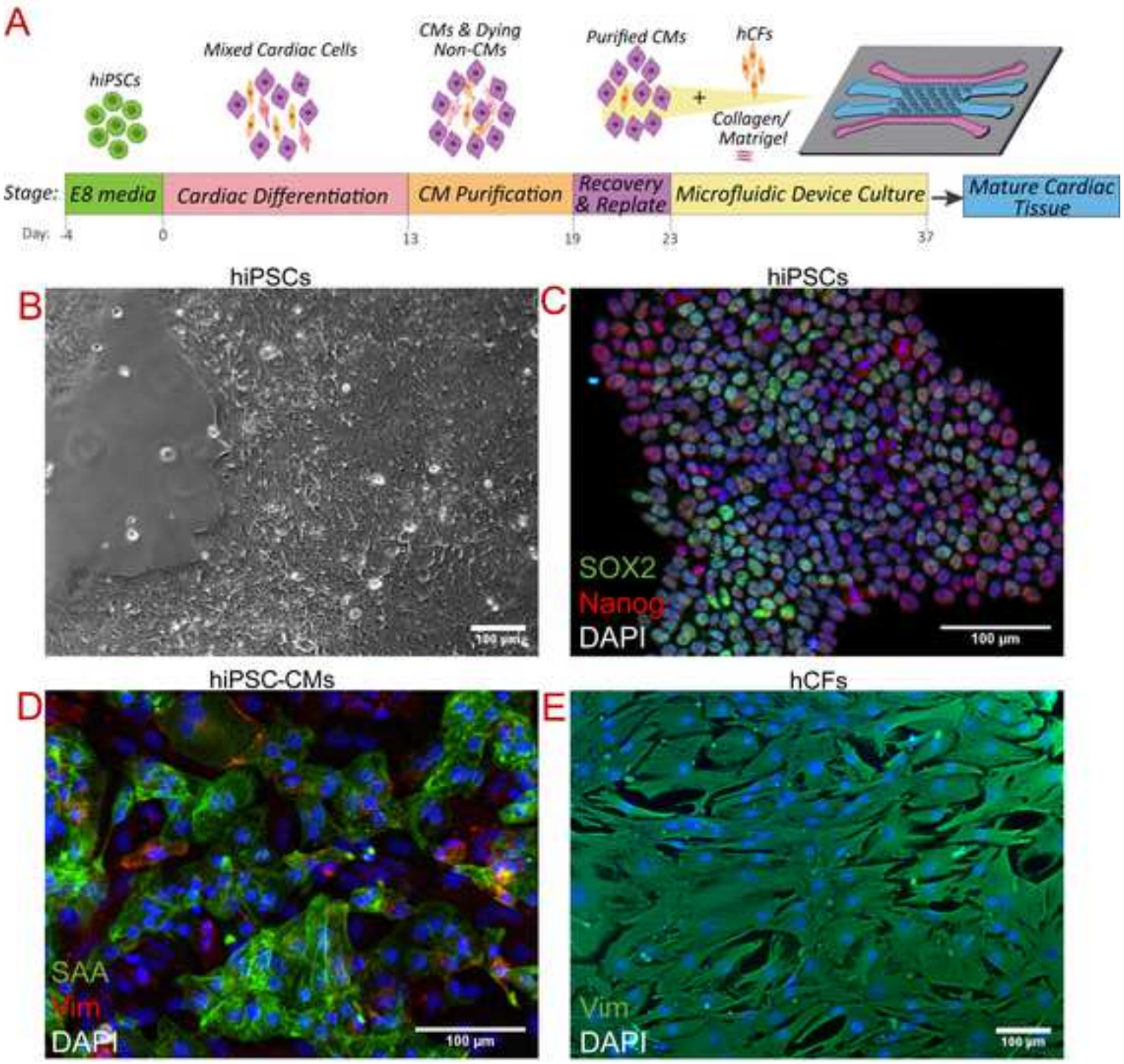


Figure 2

Click here to access/download;Figure;jove2_compressed.tiff

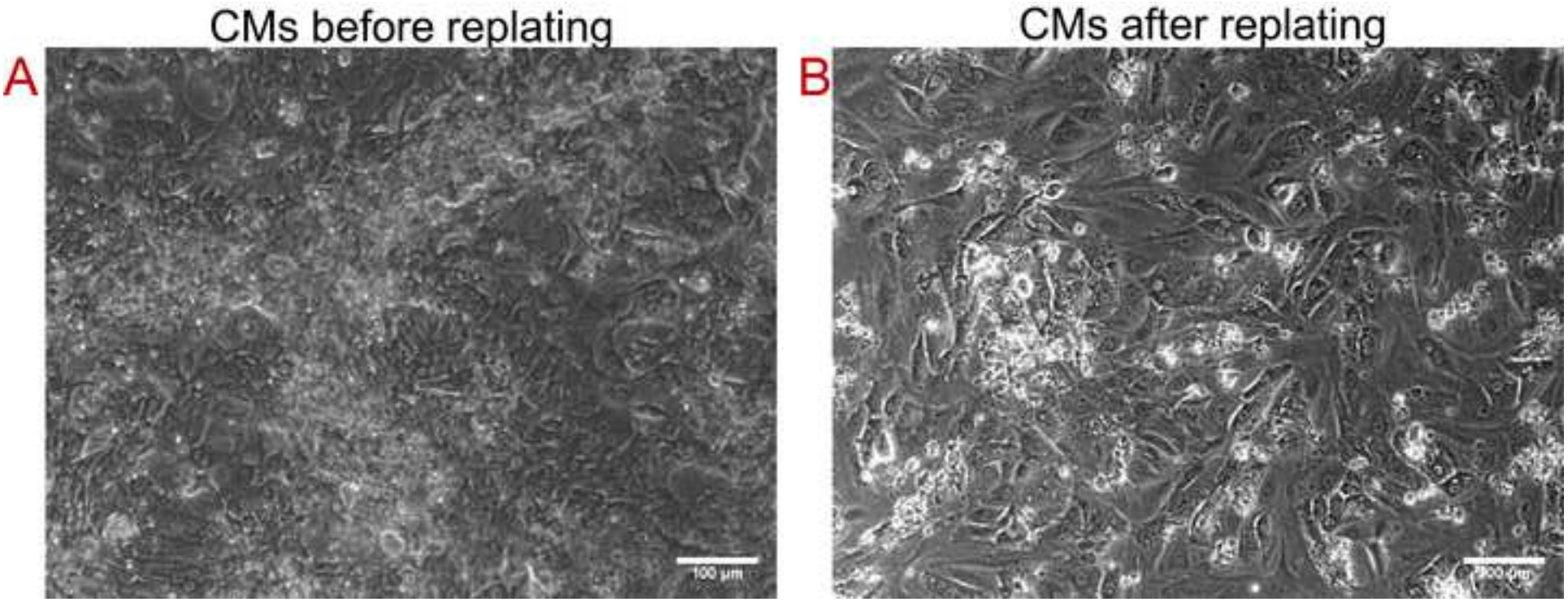


Figure 3

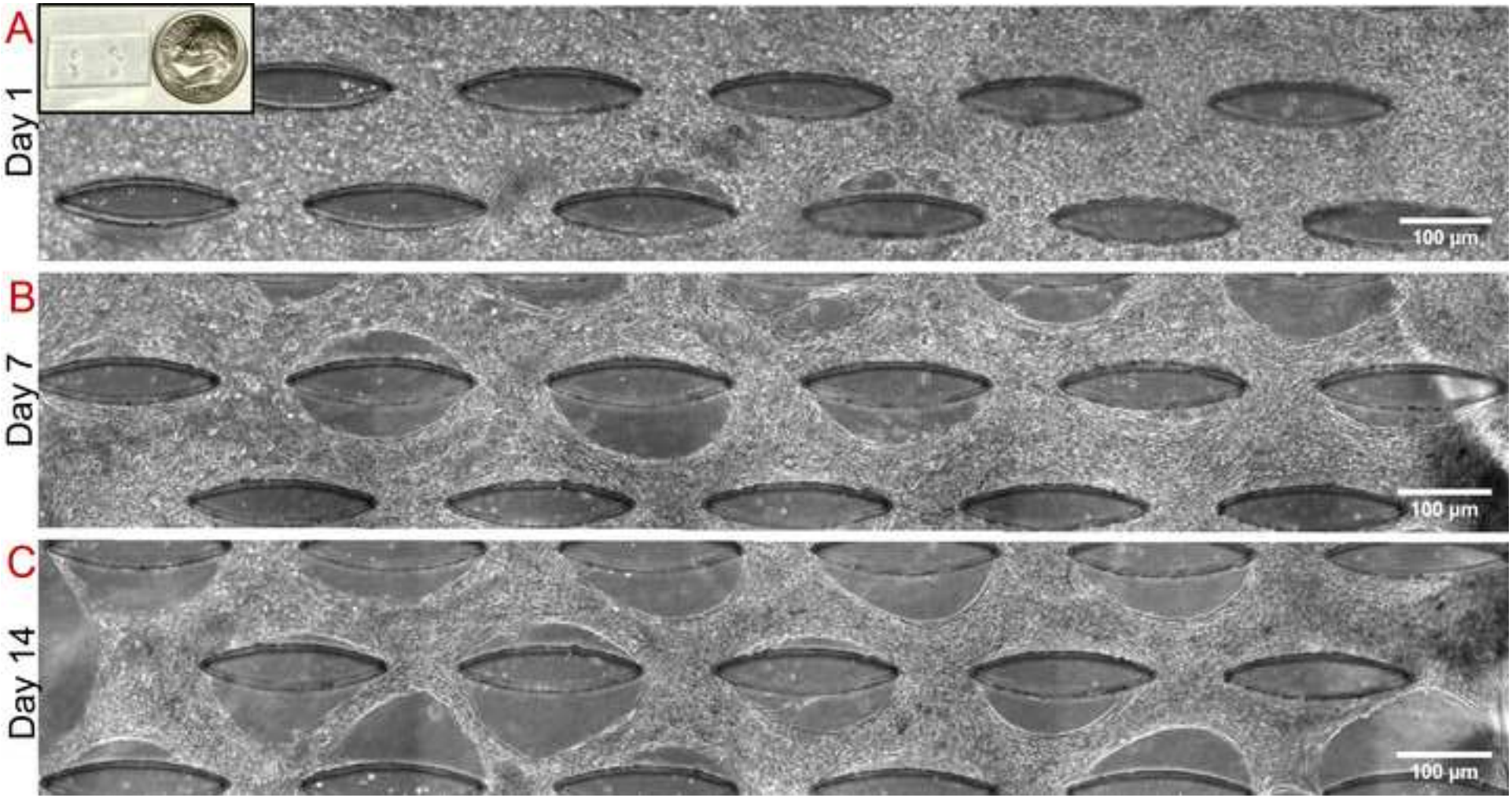
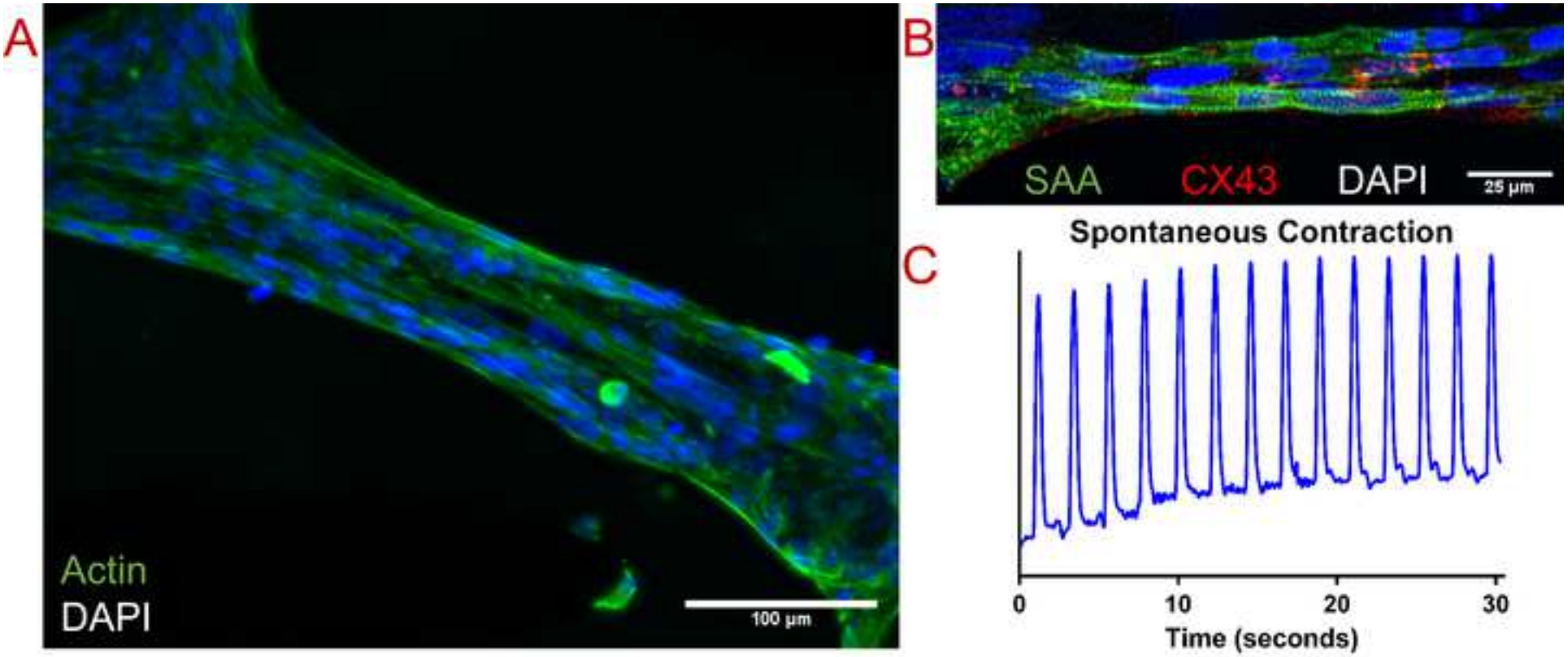


Figure 4



Video or Animated Figure 1

Click here to access/download

Video or Animated Figure

Video 1.avi

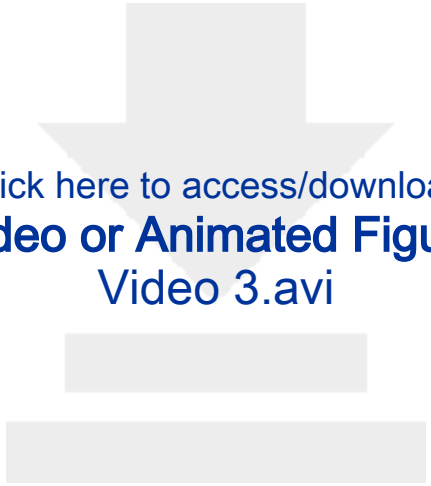
Video or Animated Figure 2

Click here to access/download

Video or Animated Figure

Video 2.avi

Video or Animated Figure 3



Click here to access/download

Video or Animated Figure

Video 3.avi

Manuscript Number: JoVE62539

Title: Method to Develop 3D Organized Human Cardiac Tissue Within a Microfluidic Platform

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

Response to Reviewers:

We would like to thank the editor and the reviewers for their valuable and constructive comments which have helped us to conduct additional experiments that substantially raised the quality of our manuscript. In the following point-by-point response letter, we have addressed in detail the reviewers' comments/suggestions, created new schematics, added relevant files, and incorporated additional details in our revised manuscript, as well as revised the video. Please note that the response portions to specific points and modified sections/Figures have been highlighted in green in the revised manuscript, as the sections that specifically correspond to the video remain highlighted in yellow. Please kindly note that we also revised text throughout the manuscript to address grammar issues and inconsistencies.

Response to Editorial and Production:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

2. Please revise the title for conciseness and reflect it in the video as well: Developing 3D Organized Human Cardiac Tissue Within a Microfluidic Platform

3. Please spell out the journal titles.

Changes to be made by the Author(s) regarding the written manuscript:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, the narration is a word for word reading of the written protocol.

2. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

3. Turn up the volume of the audio, it is too quiet

4. Title card doesn't show affiliations

5. Move the title card to the center of the screen

6. The video is very long, the length can be cut down by editing some of the required actions down. For example, at 2:29 the viewer doesn't need to see the cap being removed, the whole quantity of liquid being pippetted in, the cap being put back on, the cap of the flask being removed, and every time the liquid is pippetted around the flask. Also cutting down the aspirations will save a lot of time.

7. At 8:17 it shows the chapter title on one line, then cuts to it being on two lines. Keep the version of it on two lines

8. At 9:17 and 12:02 a frame of a different title flashes on screen, remove that

9. Take out the motion on the figures in the results section, it hides parts of the figures the speaker is talking about

Response: We thank the editors and production team for their comments. We have thoroughly re-read the manuscript, and added details, fixed grammar issues, revised the title to "Developing 3D Organized Human Cardiac Tissue Within a Microfluidic Platform", and fixed the journal titles in the citations. Additionally, for the video, we have added affiliations to the title card and moved the text to the center of the screen, increased homogeneity between the video and written manuscript, increased audio volume, trimmed length of video by removing unnecessary parts, revised the chapter titles for consistency, and removed the motions on the figures in the Results section of the video.

Response to Reviewer #1:

This Methods Article by Veldhuizen and Nikkhah presents an interesting protocol for development of 3D mature anisotropic human cardiac tissues from stem cells using an in vitro microfluidic platform. This visual protocol nicely demonstrates purification of hiPSC-CMs, their co-culture with human cardiac fibroblasts (hCFs) at predefined ratios, and 3D encapsulation of cardiac co-cultures within collagen-based hydrogels. The cellular hydrogel is subsequently injected in the designed microfluidic device that contains staggered elliptical microposts, serving as surface topography to induce CM alignment. The 3D anisotropic cardiac tissue chip introduced here can be used as a robust platform to various disease modeling and drug screening applications.

Response: We thank the reviewer for his/her valuable comments on our manuscript.

- How do the authors ensure the high CM differentiation yield from their instructed protocol? There are no technical steps listed on how one would assess/quantify CM differentiation yield in this protocol, those shall be added (e.g., FACS analysis, IHC, etc.). Authors mention "if cardiac differentiation/selection does not result in high CM% (i.e. >80%), then cells are not suitable for tissue formation, and the differentiation process should be optimized" - they should suggest what specific methods could be used here to further improve CM yield/purity. Also, add some published works related to that.

Response: We thank the reviewer for his/her helpful insight. We have added relevant discussion for methods to optimize cardiomyocyte (CM) differentiation and purification to the manuscript, as well as citations to relevant works. We have also included the added text below with relevant references to the revised manuscript:

Protocol (pg. 6):

"*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, then cells are not suitable for tissue formation, and the differentiation process should be optimized, such as through 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)[1-3]."

- The hiPSC-CMs shown in Figure 1C does not show a quite well differentiated CM population. The cells are lacking a healthy mature-like elongated morphology and also no evidence of sarcomeric striations.

Response: We thank the reviewer for his/her comment. We agree, the CMs are immature in the image shown in Figure 1C, as they are CMs immediately after differentiation from stem cells (hiPSCs), before insertion into the microfluidic device. As hPSC-CMs are notoriously immature immediately upon differentiation, it is expected that they have rounded shape and punctuated sarcomeres, as also demonstrated and discussed extensively in the literature[4-6]. However, notably, we have demonstrated that culture within our microfluidic device enhances the maturation state of the of hiSPCs-CMs[7]. To that end, we would like to kindly draw the attention of the reviewer to **Figure 4** of CMs after culture in the device, demonstrating clear, striated sarcomeres and elongated shape.

In regards to the CM purity of the population, we would like to kindly refer the reviewer to our recent published manuscript in *Biomaterials* (10.1016/j.biomaterials.2020.120195), in which we provide extensive characterization of both hESC and hiPSC-derived CMs, including both immunofluorescent images and flow cytometry, demonstrating CM purities averaging 85.5%[7].

- CMs before and after replating, shown in Figure 2, are not quite clear and informative. The bright field images provided here do not really inform the viewer of the major changes as a result of replating. These should be replaced with IHC images and proper staining to highlight the effect clearly.

Response: We thank the reviewer for his/her comment. We have further supplemented **Figure 2** with videos of the CMs before and after replating (**Video 1** and **Video 2**), which better highlight the differences in the populations. To complement these videos, we have also added a discussion that highlights the population differences in the representative results:

Representative Results (pgs. 9-10):

"**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."

- Figure 3: again, the bright field images are not quite clear and informative. Addition of some IHC images, particularly at higher mags, will be pretty helpful.

Response: We thank the reviewer for his/her comment. We agree that more IHC images may be helpful. We would like to point the reviewer's attention to the IHC images provided in **Figure 4**, of the end timepoint of Day 14 of the 3D tissue, to demonstrate the expected results after following our presented protocol herein. Furthermore, we have provided phase contrast images (**Figure 3**) and believe that these images also have the potential to aid the reader in replicating the results. In addition, we would like to also emphasize that this manuscript is mainly a method paper to demonstrate the protocol for the formation of an organized 3D cardiac tissue within our microfluidic chip. To that end, we also would like to kindly refer the reviewer to our recently published manuscript in *Biomaterials* (10.1016/j.biomaterials.2020.120195) that has extensive IHC images for the different CM:CF ratios, duration of culture, as well as for different cardiac cell types (rat, hESC, and hiPSC).

Minor Concerns:
- Collagen preparation: pH measurement and adjustment are not elaborated. Those are quite critical steps to ensure proper cell viability and function in such hydrogels.

Response: We thank the reviewer for his/her insightful comment. We have added relevant details to the revised manuscript to aid in the preparation of collagen, and have highlighted the passage and included below:

Protocol (pg. 7):

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

- Why a CM to CF ratio of 4:1 is suggested here. There is no justification provided. Citations needed as well.

Response: We thank the reviewer for his/her suggestions, and have included discussion about the chosen cellular composition of 4:1 CM:CF to the revised manuscript. We have also further added discussion about other cell types that could be incorporated within the system. We have included the paragraph below for reference:

Discussion (pg. 11):

"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 a majority of the cell population within the heart[35]. 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 our 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[36], in addition to their well-documented role in immune response[37]. Therefore, macrophages could be incorporated in 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, that may lead to a population of inflammatory macrophages within the heart tissue."

- Please make sure to define all the technical acronyms that are used in the manuscript. e.g., IPA, U8 2075 (need to describe what this exactly is), EVG620, PDMS, DI H2O, etc.. There are many incidences like this throughout the text.

Response: We thank the reviewer for his/her suggestions and have addressed his/her concern by defining all acronyms listed throughout the text.

- Avoid using uppercase words in unnecessary places, e.g. "turn off the oven, but DO NOT open the oven".

Response: We thank the reviewer for his/her suggestion and have addressed his/her concern by removing any unnecessary uppercase words.

- Authors should cite some seminal works related to iPSC culture and maintenance.

Response: We thank the reviewer for his/her suggestion. We have added citations for the seminal works of 10.1038/nmeth.1593, for culture of hiPSCs in E8, and of 10.1038/nbt1310, for the use of ROCK inhibitor to enhance survival of stem cells during single cell dissociation.

Response to Reviewer #2:

This manuscript by Veldhuizen and Nikkah describes their methods to establish an anisotropic cardiac microtissue from human iPSC-derived CMs and human cardiac fibroblasts within a PDMS microfluidic device as previously described in Veldhuizen et al. (2020). The authors describe 1) the fabrication of custom PDMS microfluidic devices via soft lithography, 2) the differentiation and maintenance of human stem cells towards cardiomyocytes, 3) the culture human cardiac fibroblast, 4) the preparation and handling of multiple cardiac cell types for 3D cell encapsulation within microfluidic devices, and 5) the quantification of contractility and immunofluorescence within these devices. Representative figures and the supplied video support these methods allowing the readers to replicate the authors' studies. A discussion is provided to describe the benefits of using this model system, and suggestions are given for future studies. Lastly, the authors describe many of the potential challenges associated with their protocol to help the readers avoid running into similar issues.

Response: We thank the reviewer for his/her valuable comments on our manuscript.

Major Concerns:

PDMS adsorbs small hydrophobic molecules making it not well suited for the drug screening applications suggested by the authors [10.1021/ac071903e, 10.1016/j.bbrc.2016.11.062, 10.1039/c2lc20982a]. This limitation needs to be made clear in the discussion, and the utility of these devices not overstated in the abstract and introduction. Furthermore, while PDMS is great for rapid prototyping, recent publications have established alternatives using thermoplastics [10.1021/acsbiomaterials.0c01062, 10.1002/adbi.202000133]. As an alternative to PDMS, could the authors comment on if their chips can be fabricated from PMMA?

Response: We thank the reviewer for his/her comment. To address the comment, we have added a discussion regarding the use of PDMS and its limitations in our manuscript, and have provided the added paragraph below to the revised manuscript. In the passage below, we have cited the important works that the reviewer has mentioned. Furthermore, we agree that PDMS has limitations, particularly if it is to be used in drug-testing studies, due to the mentioned adsorption of small hydrophobic molecules. As also stated by the reviewer, there are other promising materials, such as thermoplastics, that have potential for organ on-a-chip platforms. For example, PMMA has been used to construct microfluidic chips. However, for this application specifically, PMMA may not be suitable due to a few reasons. PMMA is rigid, which would make plasma bonding, the method used here to seal the microfluidic device, very difficult and challenging to accomplish. Therefore, the most common method to bond PMMA in literature to create a sealed device is through thermal bonding[8], which requires high temperatures (above 105°C). These necessary high temperatures can distort channel geometry[9], which may prove detrimental in this particular device that contains precise, micron-sized microposts pertinent for the formation of anisotropic tissue structure. Other methods have been demonstrated for PMMA bonding, such as through capillary-assisted adhesive that can be performed at room temperature[9]. However, this novel technique still retains a central challenge in homogenously distributing the adhesive without clogging the microchannels. We also would like to emphasize that our manuscript is centered on presenting a protocol for the formation of 3D organized human cardiac tissue, hence it is not focused on platform material optimization itself. Due to these reasons, we believe materials other than PDMS should be explored in drug-testing studies, however this may require extensive optimization steps and experiments, and/or development of new novel methods for fabrication and bonding. We have added the paragraph below to the revised manuscript text.

Discussion (pg. 12):

"The use of PDMS to fabricate microfluidic channels may retain limitations, particularly if used in constructs for drug-response studies, as PDMS has been found to adsorb small hydrophobic molecules[44-46]. Therefore, other materials, such as thermoplastics[47,48], could be also investigated as alternatives to PDMS during the soft lithography process."

The authors clearly describe the complications associated with using collagen, which is thermally crosslinked. Why not avoid all of these issues using a photocrosslinkable hydrogel system, which is compatible with both PDMS and thermoplastics [10.1002/elps.201400465, 10.1002/adbi.202000133]? Could the authors include this option as an alternative to collagen to reduce variability and enable easier handling?

Response: We thank the reviewer for his/her insightful comments. We agree that there may be inherent complications in working with a thermally-crosslinked hydrogel like collagen, particularly in small volumes such as those needed for microfluidic devices. However, the utility of this platform is in it's ability to form anisotropic structure around the elliptical microposts, which is highly dependent on the encapsulation hydrogel and how the cells, particularly the CFs, respond and remodel the hydrogel. We have found that the cardiac cells are very sensitive to hydrogel composition. We have demonstrated an optimal ratio of 85:15 fibrin:collagen for rat CMs culture, and an 80:20 collagen:Matrigel hydrogel (as described in this manuscript) ideal for human CMs culture[7]. We agree that other hydrogels could be explored within our platform, however such studies would necessitate some further optimization experiments. For example, fibrin, an enzymatically crosslinked hydrogel, is a popular hydrogel that is used in encapsulation of hiPSC-CMs, however it is generally supplemented with Matrigel[10,11] to aid in cell viability, rendering the hydrogel still thermally sensitive. Photo-crosslinkable hydrogels have their clear advantages, particularly those that are crosslinked with visible light over harmful UV light. However, there are also experimental limitations to such hydrogels, such as cell exposure to uncrosslinked photoinitiator or formation of residual unreacted double bonds that might cause negative reactions from cardiac cells[12] which would need to be further investigated. Altogether, we agree that future studies could include investigation into other types of hydrogels to aid in performance of this protocol. We have added a discussion about use of alternative hydrogels to the revised manuscript, in the following passage:

Discussion (pg. 12):

"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)[47,49-51] or enzymatically crosslinked hydrogels (i.e., fibrin)[11,52,53], could be explored."

As stated in the introduction, the heart is a complex multicellular organ [10.1161/CIRCRESAHA.115.307778]; could the authors provide some rationale as to why only cardiomyocytes and cardiac fibroblast were chosen for this model system? Additionally, in addition to endothelial cells, could the authors please add to their discussion how other critical cardiac cell populations, such as cardiac macrophages that play an essential role in cardiac electrical conduction [10.1016/j.cell.2017.03.050], may be incorporated into this model?

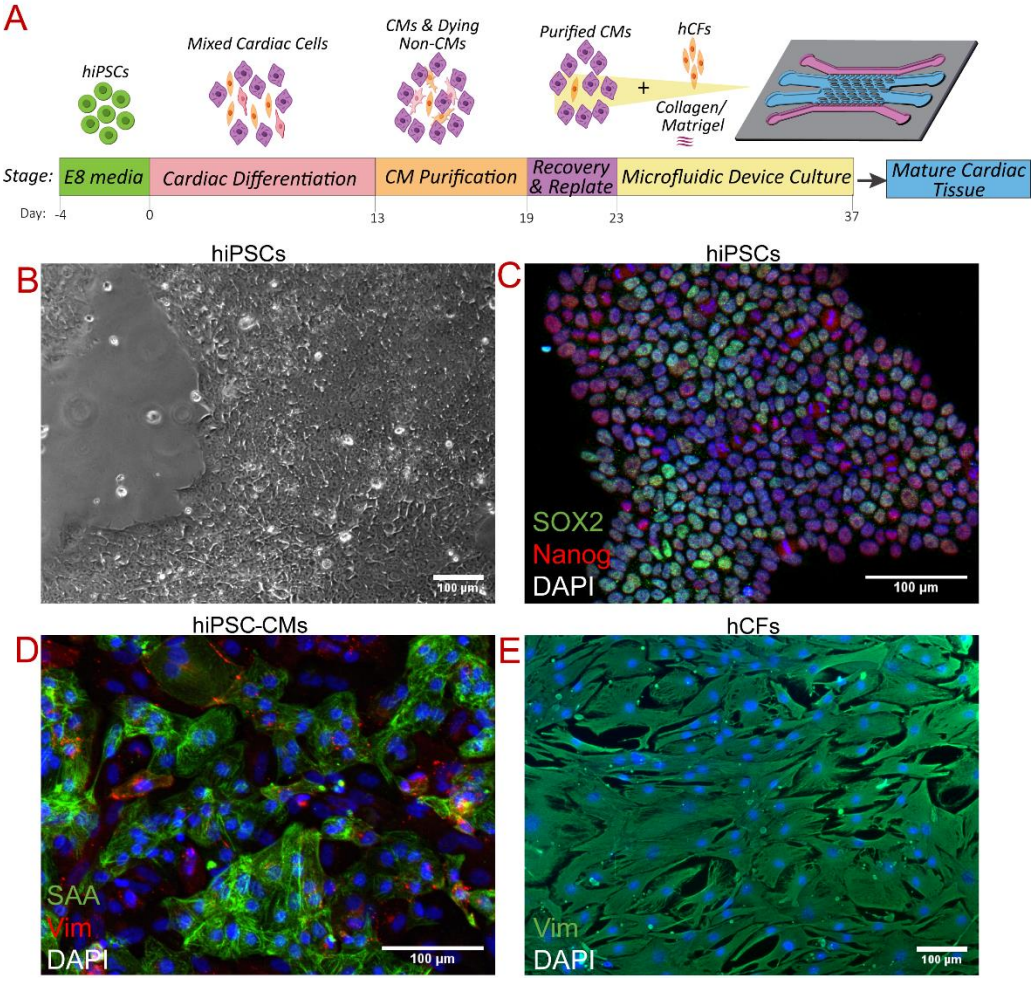
Response: We thank the reviewer for his/her helpful suggestions, and have included discussion about the chosen cellular composition, and possibility of adding other cell types. We have included the paragraph below for reference:

Discussion (pg. 11):

"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 a majority of the cell population within the heart[35]. 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 our 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[36], in addition to their well-documented role in immune response[37]. Therefore, macrophages could be incorporated in 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, that may lead to a population of inflammatory macrophages within the heart tissue."

Please add a schematic figure that outlines the timeline for cell culture (maintenance, differentiation, dissociation) and chip seeding to provide a clear picture of all the necessary steps for the reader.

Response: We thank the reviewer for his/her suggestion and have added a schematic to **Figure 1** that outlines the timeline from stem cell culture through cardiomyocyte differentiation and cardiac tissue formation within the chip. Please see the updated **Figure 1** below:



Updated Figure 1 Caption:

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**).

Minor Concerns:
Please provide the CAD technical drawings so the reader can fabricate these devices
Response: We thank the reviewer for his/her suggestion and have added the CAD file to the supplementary files, as **Supplementary File 2**.

Please comment on confirming the height of the SU8 mold
Response: We thank the reviewer for his/her suggestions, and we have added a comment about confirming both the height and the features of the SU8. Please refer to the passage below:

Protocol (pg. 3):

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

Please comment on daily media changes and possible evaporation given the small (~20uL) volume of media/chip. Any expected changes in concentration of cell culture nutrients overtime?

Response: We thank the reviewer for his/her concern, and we have added a comment to the manuscript to aid in culture of the chips to prevent evaporation and related changes in nutrients.

Protocol (pg. 8):

"*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 top of the channel inlets/outlets during routine media changes."

Please provide access to the custom MATLAB code to measure the beat rate and interbeat interval variability so that the reader can replicate these results

Response: We thank the reviewer for his/her suggestion. We have added our custom-written MATLAB programs to the supplementary files to extract to extract peaks to determine inter-beat interval variability from beating signals, as **Supplementary File 3**. With apologies, we unfortunately do not have the permission to disclose the code to extract the beating signals, as this was a gift from the former group of the PI. We have included the relevant passage from the manuscript here, where we refer to the supplemental file for inter-beat interval variability:

Protocol (pg. 8):

"*NOTE*: To assess tissue contractility, after extraction of beating signals, the supplementary custom-written MATLAB code be used to extract peaks (**Supplementary File 3**) to calculate inter-beat interval variability."

Please add a discussion point about how microfluidic devices may be fitted with electronics for real-time quantification of cellular processes, in particular measuring beat rate with a multi-electrode array.

Response: We thank the reviewer for his/her suggestion and have added a discussion point about adding electronics into the fabrication process, as provided below:

Discussion (pg. 12):

"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."

Are there any challenges with bonding PDMS to glass? Collapsed channels?

Response: We thank the reviewer for his/her inquiry and have included a note in the manuscript to aid in the bonding process to avoid channel collapse, as provided below:

Protocol (pg. 3):

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

Line 66: Please add a citation for the heart being complex and multicellular

Response: We thank the reviewer for his/her comment. We have added citations for the heart being complex (10.1007/0-387-22825-X_1) and multicellular (10.1161/CIRCRESAHA.115.307778).

Line 101: Please define hCFs

Line 119: Please define IPA

Line 140: Please define PDMS

Line 200: Please define the acronym pen/strep

Line 256: Please define DPBS (defined on line 270)

Response: We thank the reviewer for his/her suggestions, and we have added the appropriate definitions for the following abbreviations: hCFs (human cardiac fibroblasts), IPA (isopropyl alcohol), PDMS (polydimethylsiloxane), pen/strep (penicillin/streptomyocin), and DPBS (Dulbecco's phosphate buffered saline).

Line 167: What is the volume of one aliquot of Matrigel? Please also present this as a percentage

Response: We appreciate the reviewer's comment. The volume of one aliquot of Matrigel is lot-dependent, and provided by the vendor (Corning) for dilution into 25 mL of coating media. We have added more information in the manuscript, as also listed below:

Protocol (pg. 3):

"To coat plates with hESC-quality Matrigel, thaw one aliquot of Matrigel (lot-dependent volume, generally 200-300 µL; stored at -80 °C) into 25 mL of DMEM/F-12K on ice. Then aliquot 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 one hour."

Line 242: Please cite or provide some evidence that hCFs need to be used before passage 10

Response: We thank the reviewer for his/her insightful comment. We have added a relevant citation (10.1155/2020/9363809) for possible myofibroblast differentiation at high passages of hCFs.

Line 353: What frame rate?

Response: We thank the reviewer for his/her question. These videos were taken at a 25.23 frames/second framerate, however videos can be taken at the maximal framerate of the user's microscope/equipment can handle. We have added the appropriate information to the manuscript, and have included that passage below:

Protocol (pg. 8):

"4.1.1 Place devices in an environmentally-controlled stage incubator. Record 30-second videos of multiple spots within each device at the maximal frame rate."

Line 415: What is the CMs expected BPM?

Response: We thank the reviewer for his/her question. The recorded beating behavior of the CMs is spontaneous electrical activity, therefore the rate it is rather variable, and sensitive to many environmentally-determined factors, such as temperature. The more important factor that can be calculated based on the electrical activity of the cells is the inter-beat interval variability, as it defines the synchronicity of the tissue. The lower the variability, the more well-connected and synchronous the tissue.

Line 483: Why is adjustable stiffness of the microfluidic device important if the cardiac cells are encapsulated within a hydrogel?

Response: We thank the reviewer for his/her question. The easily adjustable stiffness quality of PDMS provides the protocol user with much flexibility during the fabrication process, as well as in experimentation. For example, if fluid flow is to be incorporated into the media channels, the stiffness of the PDMS channel plays a direct role in the amount of deformability of the channel[13], and thus on the amount of applied shear stress. Additionally, the stiffness of PDMS plays a role in amount of gas diffusion through the surface[14], thus affecting delivery of gas to encapsulated cardiac tissues, allowing the study of variable gas diffusion.

Line 500: Could this protocol be performed in a cold room to improve reliability? (also see major comment on the use of photocrosslinkable hydrogels)

Response: We thank the reviewer for his/her comment. We agree that performance of this protocol within a cold room may improve its reliability, due to the temperature sensitivity to materials. However, we are concerned that a majority of researchers may not have access to a cold room that is also equipped with a Biosafety Cabinet and cell culture incubator, which are pertinent for successful completion of the protocol. As per the reviewer's previous comment, we have added discussion about use of alternative hydrogels if temperature becomes a limiting factor in this protocol, which is included below:

Discussion (pg. 12):

"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)[47,49-51] or enzymatically crosslinked hydrogels (i.e., fibrin)[11,52,53], could be explored."

Response to Reviewer #3:

In their manuscript "Method to Develop 3D Organized Human Cardiac Tissue Within a Microfluidic Platform", Veldhuizen and Nikkhah present a protocol for development of 3D mature human pluripotent stem cell (hPSC) derived-human cardiac tissue within a microfluidic device. They describe microfluidic device creation through soft lithography; hPSC culture, differentiation, and purification to hPSC-cardiomyocytes (CMs); cardiac tissue formation by combining hPSC-CMs, human cardiac fibroblasts (hCFs), and a collagen-based hydrogel; and cardiac tissue structural and functional analyses. The authors envision that the proposed 3D anisotropic cardiac tissue on-chip model is suitable for fundamental biology studies, disease modeling, and for pharmaceutical testing.

Response: We thank the reviewer for his/her valuable comments on our manuscript.

Minor Concerns:

1. In Section 1.1 Photolithography, please provide a typical AutoCAD microfluidic device design as a figure.

Response: We thank the reviewer for his/her suggestion and have added the CAD file to the supplementary files, as **Supplementary File 2**.

2. From Section 4.2 Immunofluorescent Staining, add list of primary and secondary antibodies in a Supplementary table.

Response: We thank the reviewer for his/her suggestion and have added tables with the details for the primary and secondary antibodies in a new Supplementary File, as **Supplementary File 4**. We have also included the tables here for your reference:

Table of primary antibodies used in Immunofluorescent Staining:

Target	Species	Vendor	Dilution
Nanog	Mouse	Abcam	1:200
SOX2	Rabbit	Cell Signaling	1:100
Sarcomeric α-actinin (SAA)	Mouse	Sigma	1:200
Connexin43	Rabbit	Abcam	1:200
Vimentin	Rabbit	Cell Signaling	1:350

Table of secondary antibodies used in Immunofluorescent Staining:

Type	Wavelength	Vendor	Dilution
AlexaFlour; Anti-mouse	488 nm	ThermoFisher	1:500
AlexaFlour; Anti-rabbit	594 nm	ThermoFisher	1:500

3. In Figure 4A, is the staining with actin or sarcomeric alpha-actinin (as in Panel B)? Please clarify.

Response: We thank the reviewer for his/her suggestion and have added the clarification in the figure caption, as follows:

Updated Figure 4 Caption:

"Figure 4: **Representative characteristics of human cardiac tissue after culture for 14 days in 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 localization of gap junctions as evidenced through staining for sarcomeric α-actinin (SAA) and connexin 43 (CX43), respectively (**B**), and the spontaneous contraction is synchronous (**C**)."

4. For each Major section (1, 2, 3, 4), please provide an approximate duration needed to complete each section.

Response: We thank the reviewer for his/her suggestion, and we have added the approximate durations to each Major section heading.

This reviewer looks forward to the authors' responses.

Response: We thank the reviewer for his/her valuable comments on our manuscript.

References:

1 Ban, K., Bae, S. & Yoon, Y. S. Current Strategies and Challenges for Purification of Cardiomyocytes Derived from Human Pluripotent Stem Cells. *Theranostics* **7**, 2067-2077, doi:10.7150/thno.19427 (2017).

2 Uosaki, H. *et al.* Efficient and scalable purification of cardiomyocytes from human embryonic and induced pluripotent stem cells by VCAM1 surface expression. *PLoS One* **6**, e23657, doi:10.1371/journal.pone.0023657 (2011).

3 Dubois, N. C. *et al.* SIRPA is a specific cell-surface marker for isolating cardiomyocytes derived from human pluripotent stem cells. *Nature Biotechnology* **29**, 1011-U1082, doi:10.1038/nbt.2005 (2011).

4 Yang, X., Pabon, L. & Murry, C. E. Engineering Adolescence Maturation of Human Pluripotent Stem Cell–Derived Cardiomyocytes. *Circulation Research* **114**, 511-523, doi:10.1161/CIRCRESAHA.114.300558 (2014).

5 Veerman, C. C. *et al.* Immaturity of human stem-cell-derived cardiomyocytes in culture: fatal flaw or soluble problem? *Stem Cells Dev* **24**, 1035-1052, doi:10.1089/scd.2014.0533 (2015).

6 Jiang, Y., Park, P., Hong, S. M. & Ban, K. Maturation of Cardiomyocytes Derived from Human Pluripotent Stem Cells: Current Strategies and Limitations. *Molecules and Cells* **41**, 613-621, doi:10.14348/molcells.2018.0143 (2018).

7 Veldhuizen, J., Cutts, J., Brafman, D. A., Migrino, R. Q. & Nikkhah, M. Engineering anisotropic human stem cell-derived three-dimensional cardiac tissue on-a-chip. *Biomaterials* **256**, 120195, doi:10.1016/j.biomaterials.2020.120195 (2020).

8 Berthier, E., Young, E. W. & Beebe, D. Engineers are from PDMS-land, Biologists are from Polystyrenia. *Lab Chip* **12**, 1224-1237, doi:10.1039/c2lc20982a (2012).

9 Matellan, C. & Del Rio Hernandez, A. E. Cost-effective rapid prototyping and assembly of poly(methyl methacrylate) microfluidic devices. *Scientific Reports* **8**, 6971, doi:10.1038/s41598-018-25202-4 (2018).

10 Zhang, D. *et al.* Tissue-engineered cardiac patch for advanced functional maturation of human ESC-derived cardiomyocytes. *Biomaterials* **34**, 5813-5820, doi:10.1016/j.biomaterials.2013.04.026 (2013).

11 Shadrin, I. Y. *et al.* Cardiopatch platform enables maturation and scale-up of human pluripotent stem cell-derived engineered heart tissues. *Nat Commun* **8**, 1825, doi:10.1038/s41467-017-01946-x (2017).

12 Choi, J. R., Yong, K. W., Choi, J. Y. & Cowie, A. C. Recent advances in photo-crosslinkable hydrogels for biomedical applications. *Biotechniques* **66**, 40-53, doi:10.2144/btn-2018-0083 (2019).

13 Hardy, B. S., Uechi, K., Zhen, J. & Pirouz Kavehpour, H. The deformation of flexible PDMS microchannels under a pressure driven flow. *Lab Chip* **9**, 935-938, doi:10.1039/b813061b (2009).

14 Markov, D., Lillie, E., Garbett, S. & McCawley, L. Variation in diffusion of gases through PDMS due to plasma surface treatment and storage conditions. *Biomedical Microdevices*, doi:10.1007/s10544-013-9808-2 (2013).

Ira A. Fulton Schools of Engineering
Arizona State University

Mehdi Nikkhah, Ph.D.
Associate Professor of Bioengineering
Phone: (480)-965-0339
Email: mnikkhah@asu.edu

May 8, 2021

Professor Moshe Pritsker
Editor-in-Chief
Journal of Visual Experiments

Re Manuscript: **"Method to Develop 3D Organized Human Cardiac Tissue Within a Microfluidic Platform**" (JoVE62539)

Dear Prof. Pritsker:

We would like to thank you for handling our recent manuscript submission and giving us the opportunity to address the comments to our manuscript submitted to the Journal of Visual Experiments (JoVE). We would also like to thank the editor and reviewers for their valuable and constructive comments, which in our opinion have raised the quality of our manuscript.

In the attached response document, we have addressed in detail the reviewers' comments and suggestions, created a new schematic, added supplemental files, and edited the corresponding video, and have incorporated the requested details/clarifications to our revised manuscript. Please note that the response portions to specific points in the response document are marked in "Blue". The modified sections/Figures and newly added materials in the revised manuscript have been highlighted in green. The sections that are covered in the video remain highlighted in yellow. Please kindly note that we also updated the text within the manuscript to increase clarity, along with the added revisions.

Sincerely yours,

Mehdi Nikkhah, Ph.D.

School of Biological and Health Systems Engineering
P.O. Box 879709 Tempe, AZ 85287-9709
p: 480-965-3028 f: 480-727-7624 web: sbhse.engineering.asu.edu

Table of primary antibodies used in Immunofluorescent Staining:

Target	**Species**	**Vendor**	**Dilution**
Nanog	Mouse	Abcam	1:200
SOX2	Rabbit	Cell Signaling	1:100
Sarcomeric α-actinin (SAA)	Mouse	Sigma	1:200
Connexin43	Rabbit	Abcam	1:200
Vimentin	Rabbit	Cell Signaling	1:350

Table of secondary antibodies used in Immunofluorescent Staining:

Type	**Wavelength**	**Vendor**	**Dilution**
AlexaFlour; Anti-mouse	488 nm	ThermoFisher	1:500
AlexaFlour; Anti-rabbit	594 nm	ThermoFisher	1:500

Supplemental File 1

Supplemental File 2