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# Preparation of mesh-shaped engineered cardiac tissues derived from human iPS cells for in vivo myocardial repair.

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Dr. Ronald Myers,
Science editor
*JoVE*

April 14th, 2020

JoVE61246

Dear Dr. Myers,

We appreciate the preferable considerations on our manuscript entitled "**Preparation of mesh-shaped engineered cardiac tissues derived from human iPS cells for in vivo myocardial repair**" for publication in *JoVE*. According to valuable comments from the reviewers, we revised the manuscript.

We believe that we could sufficiently revised the manuscript for the publication in *JoVE* and we look forward to receiving your positive reply.

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Sincerely,

Hidetoshi Masumoto, MD, PhD

**TITLE:**

# Preparation of Mesh-Shaped Engineered Cardiac Tissues Derived from Human iPS Cells for In Vivo Myocardial Repair

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**KEYWORDS:**

Engineered cardiac tissue, tissue engineering, cardiac regeneration, cell therapy, heart failure, induced pluripotent stem cell, cardiac differentiation, cell culture

**SUMMARY:**

The present protocol generates mesh-shaped engineered cardiac tissues containing cardiovascular cells derived from human induced pluripotent stem cells to allow the investigation of cell implantation therapy for heart diseases.

**ABSTRACT:**

The current protocol describes methods to generate scalable, mesh-shaped engineered cardiac tissues (ECTs) composed of cardiovascular cells derived from human induced pluripotent stem cells (hiPSCs), which are developed towards the goal of clinical use. HiPSC-derived

cardiomyocytes, endothelial cells, and vascular mural cells are mixed with gel matrix and then poured into a polydimethylsiloxane (PDMS) tissue mold with rectangular internal staggered posts. By culture day 14 ECTs mature into a 1.5 cm x 1.5 cm mesh structure with 0.5 mm diameter myofiber bundles. Cardiomyocytes align to the long-axis of each bundle and spontaneously beat synchronously. This approach can be scaled up to a larger (3.0 cm x 3.0 cm) mesh ECT while preserving construct maturation and function. Thus, mesh-shaped ECTs generated from hiPSC-derived cardiac cells may be feasible for cardiac regeneration paradigms.

**INTRODUCTION:**

Numerous preclinical studies and clinical trials have confirmed the efficiency of cell-based cardiac regenerative therapies for failing hearts[1–3]. Among various cell types, human induced pluripotent stem cells (hiPSCs) are promising cell sources by virtue of their proliferative ability, potential to generate various cardiovascular lineages[4,5], and allogenicity. In addition, tissue engineering technologies have made it possible to transfer millions of cells onto a damaged heart[5–8].

Previously, we reported the generation of three-dimensional (3D) linear engineered cardiac tissues (ECTs) from hiPSC-derived cardiovascular lineages using a commercially available culture system for 3D bioartificial tissues[5,7]. We found that the coexistence of vascular endothelial cells and mural cells with cardiomyocytes within the ECT facilitated structural and electrophysiological tissue maturation. Furthermore, we validated the therapeutic potential of implanted hiPSC-ECTs in an immune tolerant rat myocardial infarction model to improve cardiac function, regenerate myocardium, and enhance angiogenesis[5]. However, the linear ECTs constructed by this method were 1 mm by 10 mm cylinders and therefore not suitable for the implantation in preclinical studies with larger animals or clinical use.

Based on the successful use of tissue molds to generate porous engineered tissue formation using rat skeletal myoblasts and cardiomyocytes[9], human ESC-derived cardiomyocytes[10] and mouse iPSCs[11], we developed a protocol to generate scalable hiPSC-derived larger implantable tissue using polydimethylsiloxane (PDMS) molds. We evaluated a range of mold geometries to determine the most effective mold characteristics. Mesh-shaped ECTs with multiple bundles and junctions exhibited excellent characteristics in cell viability, tissue function and scalability compared to plain-sheet or linear formats that lacked pores or junctions. We implanted the mesh-shaped ECT in a rat myocardial infarction model and confirmed its therapeutic effects similar to implanted cylindrical ECTs[12]. Here we describe the protocol to generate a hiPSC-derived mesh-shaped ECT.

**PROTOCOL:**

**1. Maintenance of hiPSCs and cardiovascular differentiation**

1.1. Expand and maintain hiPSCs on thin-coat basement membrane matrix (growth factor reduced, 1:60 dilution) in conditioned medium extracted from mouse embryonic fibroblasts (MEF-CM) with human basic fibroblast growth factor (hbFGF)[4].

NOTE: We used a hiPSCs (4-factor (Oct3/4, Sox2, Klf4 and c-Myc) line: 201B6). Add hbFGF at the appropriate concentration for each cell line. Laminin-511 fragment can also be used for the coating of culture dish instead of basement membrane matrix. Commercially available medium designed for feeder-free culture of hiPSCs can be used as a substitute for MEF-CM.

1.2. Use Versene (0.48 mM ethylenediaminetetraacetic acid (EDTA) solution) to detach and dissociate cells when cell confluency reaches 90–100%.

NOTE: Other commercially available products for cell dissociation can also be used.

1.3. Plate cells at a density of 10,000 cells/$mm^2$ on matrix-coated cell culture plates in MEF-CM with hbFGF and culture for two to three days.

1.4. When the culture becomes fully confluent, cover the cells with matrix (1:60 dilution with MEF-CM) for one day.

1.5. Replace the MEF-CM with RPMI 1640 + B27 medium. Add 100 ng/mL of Activin A and 100 ng/mL of Wnt3A to the medium for one day.

NOTE: This is day 0 of differentiation. To upregulate canonical Wnt signaling, GSK3β inhibitors can also be used instead of Wnt3A.

1.6. On day 1 of differentiation, change the medium to new RPMI 1640 + B27 with 10 ng/mL of BMP4 and 10 ng/mL of hbFGF. Culture cells for two (MC protocol) or four (CM+EC protocol) days without medium change[5].

NOTE: CM+EC protocol is optimized to simultaneously induce cardiomyocytes (CMs) and vascular endothelial cells (ECs). MC protocol is optimized to preferentially induce vascular mural cells (MCs).

1.7. CM+EC protocol for the induction of CMs and ECs (**Figure 1A**).

1.7.1. Replace the medium on day 5 of differentiation with RPMI 1640 + B27 supplemented with 50 ng/mL of $VEGF_{165}$.

1.7.2. Change the culture medium every 48 h until day 13–15 of differentiation.

1.8. MC protocol for the induction of vascular mural cells (**Figure 1B**)

1.8.1. Replace the medium with RPMI1640 + 10% fetal bovine serum (FBS) at day 3 of differentiation.

1.8.2. Change the culture medium every 48 h until day 13–15 of differentiation.

**2. Cell harvest and lineage analysis on differentiation day 13–15**

2.1. Wash the cells with $Ca^{2+}$ and $Mg^{2+}$ free phosphate-buffered saline (PBS).

2.2. Add cell dissociation solution (containing proteases, collagenases and DNAses) in the cell culture dish to cover the plate. Incubate the plate for 5 min at 37 °C.

2.3. Collect and dissociate the cells with culture medium using a pipette after incubation.

2.4. Allocate 1 x $10^6$ cells for lineage analysis with flow cytometry. To eliminate dead cells, stain the cells with fixable viability dye.

2.5. Stain the cells with membrane surface markers in PBS with 5% FBS. Use the following dilutions of antibody in fluorescence activated cell sorting (FACS) staining buffer: anti-PDGFRβ (1:100), anti-VE cadherin (1:100), anti-TRA-1-60 (1:20).

2.6. For intracellular proteins, resuspend and fix the cells with 4% paraformaldehyde (PFA) in PBS.

2.7. Stain the cells with anti-cardiac isoform of Troponin T (cTnT) in PBS with 5% FBS and 0.75% Saponin, then label the cTnT antibody with 488 mouse IgG1 (dilution 1:50).

2.8. Resuspend the stained cells in PBS with 5% FBS and put them in FACS tubes with cell strainer.

2.9. Analyze the cell composition of the stained cells from each differentiation protocol with flow cytometry to facilitate the generation of cell suspensions with defined lineage distributions.

NOTE: While performing this procedure, the remaining cell suspension for ECTs is preserved in a 4 °C refrigerator.

**3. Fabrication of PDMS tissue mold**

3.1. Cast a 0.5 mm thick and over 30 mm x 30 mm layer of polydimethylsiloxane (PDMS) by mixing the prepolymer and cross-linking solution at a ratio of 10:1 and then cure at 80 °C for 3 h.

3.2. Cut the PDMS sheet and bond it with silicone adhesive to fabricate a 21 mm x 20.5 mm rectangular tray with 7 mm long, 0.5 mm wide, and 2.5 mm high rectangular posts at a staggered position. Horizontal post spacing between two lines of posts is 2.5 mm (**Figure 2B**).

3.3. Autoclave the tray at 120 °C for 20 min.

3.4. Coat the tray with 1% poloxamer 407 in PBS for 1 h. Rinse the poloxamer 407 and rinse the mold with PBS sufficiently prior to use.

**4. ECT construction**

4.1. After the cell lineage analysis, combine the cells from CM+EC and MC protocols so that the final concentration of MCs is 10 to 20% in a total cell number of six million cells per construct[5].

4.2. Suspend mixed six million cells in ECT culture medium (alpha minimum essential medium supplemented with 10% FBS, 50 µM 2-mercaptoethanol and 100 U/mL Penicillin-Streptomycin).

4.3. Preparation of matrix solution

4.3.1. Mix 133 µL of acid-soluble rat tail collagen type I solution (2 mg/mL, pH 3) with 17 µL of 10x minimum essential medium (MEM). Then mix the solution with 17 µL of alkali buffer (0.2 M $NaHCO_3$, 0.2 M HEPES, and 0.1 M NaOH).

NOTE: Collagen must be kept on ice (4 °C). Check buffer color and if the medium does not become pinkish when all solutions are mixed, add additional alkali buffer to the medium. The mixing steps must be mix collagen I + 10x MEM, and then add alkali buffer. DO NOT change this order. Do NOT generate bubbles in the mix.

4.3.2. Add 67 µL of basement membrane matrix to the neutralized collagen solution.

NOTE: The mixed solution must be kept on ice (4 °C).

4.4. Centrifuge the prepared cell suspension containing six million cells at 1,100 rpm (240 x *g*) for 5 min and resuspend the cells with 167 µL of high-glucose DMEM + 20% FBS + 1% penicillin-streptomycin (100x).

4.5. Mix the cell suspension and the matrix solution. The total volume of cell/matrix mixture for one construct is 400 µL.

NOTE: The cell/matrix mixture should be non-viscous and a pinkish color at this step. Keep it on ice as the gel will solidify at room temperature. The final concentration of collagen type I is 0.67 mg/mL.

4.6. Pour the cell/matrix mixture evenly over the poloxamer 407 coated PDMS tissue mold, which is placed in a six-well culture plate[12].

NOTE: Pour the mixture carefully to avoid generating bubbles in order to prevent filling defects in the poured gel.

4.7. Incubate the cell/matrix mixture in a standard $CO_2$ incubator (37 °C, 5 % $CO_2$) for 60 min.

4.8. After the tissue is formed, soak the tissue mold with 4 mL of ECT culture medium.

NOTE: Although the cell/matrix is crosslinked in 60 min, the construct is still very fragile. Add medium gently to avoid damaging it.

4.9. Culture the tissue for 14 days with medium change every day.

4.10. Prior to ECT implantation, remove the ECT from the loading posts gently using sterilized fine forceps.

NOTE: The final ECT dimensions after removal from the mold are less than the original mold. A 2.1 cm x 2.05 cm mold generates a released ECT of approximately 1.5 cm x 1.5 cm. A larger 3.9 cm x 4.05 cm mold generates an ECT of approximately 3 cm x 3 cm. Unloaded ECT shows intrinsic spontaneous beating in warm culture medium. Although it initially maintains a mesh structure, an unloaded ECT shrinks and condenses over time. It is possible to hold the tissue softly with fine forceps and then use 7-0 silk suture to attach the ECT to the epicardium.

**REPRESENTATIVE RESULTS:**

**Figure 1A,B** shows the schematics of CM+EC and MC protocol. After inducing CMs and ECs from CM+EC protocol and MCs from MC protocol, the cells are mixed adjusting final MC concentrations to represent 10 to 20% of total cells. The 2 $cm^2$ wide tissue mold is fabricated according to the design drawing from 0.5 mm thick PDMS sheet (**Figure 2A,B**). Six million of CM+EC+MC cells are combined with collagen I, and matrix and poured onto the tissue mold precoated with poloxamer 407 (**Figure 2C**). During preliminary experiments, we fabricated tissue molds with various patterns characterized by various post lengths and spacing and confirmed final geometries of ECTs (**Figure 3**). We selected the mold with 7 mm long posts with 2.5 mm wide intervals for this study.

Poloxamer 407 prevents cell adhesion to the mold and enabled the formation of characteristic mesh structure following rapid gel compaction in 14 days (**Figure 4**). This structure is maintained even after the ECT is released from its mold. The whole tissue is approximately 1.5 cm wide and 0.5 mm thick, and the width of each bundle in the mesh is approximately 0.5 mm in average.

It is possible to generate a 3 cm final width mesh ECT containing twenty-four million cells from a four times larger mold with the same staggered post design (**Figure 5A,B**). This larger mesh ECT can be removed from the mold easily and generates local active force equivalent to the smaller mesh ECT (**Figure 5C**).

**FIGURE LEGENDS:**

**Figure 1: Protocols to differentiate cardiovascular cells from human induced pluripotent stem cells.** Schematic diagrams of protocols used to induce cardiomyocytes and vascular endothelial cells (A, CM+EC protocol) and to induce vascular mural cells (B, MC protocol).CM = cardiomyocyte; EC = endothelial cell; MC = vascular mural cell; iPSC = induced pluripotent stem cell; MG = basement membrane matrix; ActA = Activin A; Wnt3a, BMP4, Bone morphogenetic

protein 4; bFGF = basic fibroblast growth factor; VEGF = vascular endothelial cell growth factor; FBS = fetal bovine serum. This figure is adapted from reference Masumoto et al.[5].

**Figure 2: Fabrication of PDMS tissue mold and ECT construction.** (**A**) Representative image of an ECT mold fabricated from 0.5 mm thick PDMS sheets. (**B**) Design of the PDMS tissue mold with 7 mm long internal posts arranged in staggered position; front view (upper panel) and side view (lower panel). (**C**) The schematic protocol of ECT construction from hiPSC-derived cardiovascular cells and matrix gel.

**Figure 3: Impact of tissue mold designs on final ECT geometries.** (**A**) Definitions of post length (PL) and horizontal post spacing (HPS). (**B**) A tissue mold without rectangular posts (PL0) and formed a sheet ECT. (**C**–**E**) Tissue molds with different PL/HPS and mesh ECTs. (**F**) A tissue mold with long parallel posts and formed a multiple linear ECT.

**Figure 4: Formation of mesh ECT following gel compaction.** Representative series of images of a mesh ECT maturation from day 0 to 14 are shown. Cell/matrix is poured in the PDMS tissue mold (Day 0_0 h), then culture medium is added one hour later (Day 0_1 h). On day 1, elliptical pores are observed around loading posts. The construct showed rapid gel compaction and matured into a mesh tissue thereafter. On day 14, the tissue is released from the mold. The unloaded ECT maintains the mesh geometry.

**Figure 5: Representative images of a larger mesh ECT intended for large animal preclinical trials and clinical trials.** (**A**) Design of the 4 cm x 4 cm PDMS tissue mold with 7 mm long posts and (**B**) an image of the mold. (**C**) Representative image of a 3 cm x 3 cm larger mesh ECT.

**Supplemental Figure 1: Pathological and electrophysiological evaluation of mesh ECTs.** (**A**) Representative image for a bundle in a mesh ECT stained with Hoechst 33342 (blue) for live cells and Ethidium Homodimer III (red) for dead cells. Scale bar: 250 μm. (**B**) Representative image of a three-dimensional confocal image of a bundle in a mesh ECT stained with cardiac troponin T (green). Local cardiomyocyte orientations within the bundle are visualized as small lines, where line color indicates magnitude in the circumferential (green), radial (red), and axial (blue) directions. (**C**) The spherical histogram displays local cardiomyocyte orientations. The volume of each ray represents the relative count for each direction and the thick red line shows the mean CM orientation. (B) and (C) are adapted from reference[13] with revision. (**D**) Representative image of contractile force measurement. A segment was cut off at the red dotted line in a 1.5 cm x 1.5 cm mesh ECT and attached to the muscle testing system using 10-0 nylon suture. The white arrowhead indicates force transducer and the yellow arrowhead indicates high-speed length controller. (**E**) Representative waveforms of active stress at different pacing frequencies from 1.5 Hz to 4 Hz.

**Supplemental Video 1: Intrinsic beating of a junction in a mesh ECT on day 14.**

**Supplemental Video 2: Intrinsic beating of a bundle in a mesh ECT on day 14.**

**Supplemental Video 3: Intrinsic beating of a mesh ECT on day 14 after released from the tissue mold.**

**DISCUSSION:**

Following the completion of our investigation of a linear format, hiPSC derived ECT[5], we adapted the protocol to mix hiPSC-derived CMs, ECs, and MCs to facilitate the in vitro expansion of vascular cells within ECTs and subsequent in vivo vascular coupling between ECTs and recipient myocardium.

To facilitate the generation of larger, implantable mesh ECT geometries we used thin PDMS sheets to design the 3D molds with loading posts arrayed at staggered positions. During preliminary experiments, we noted that ECTs adhered to the PDMS posts during gel compaction and so we modified our method to coat each mold with the surfactant poloxamer to prevent cell adhesion which is a critical step of the present protocol[9]. ECTs remain detached from the internal loading posts and the mold bottom during in vitro maturation, which facilitates gel compaction and ECT removal from the mold. Another critical step of the protocol is to evenly pour the cell-gel matrix mixture into the PDMS mold before the matrix solidifies. It is critical to accomplish the procedure within a short time. Bubbles in the cell-gel matrix mixture should also be avoided because it may cause structural vacuolation and functional disruption of the ECTs.

According to viability assays, approximately 97% of cells were alive within the day 14 constructs (**Supplemental Figure 1A**). **Supplemental Figure 1B** shows the whole mount confocal image stained with cTnT representing myofiber alignment parallel to the local bundle long axis (**Supplemental Figure 1C**)[13]. All constructs start intrinsic spontaneous beating in vitro within 72 h and continued beating throughout the duration of culture (**Supplemental Videos 1–3**). We analyzed electromechanical properties of 1.5 cm x 1.5 cm ECTs using a custom isolated muscle testing system (**Supplemental Figure 1D**). ECTs displayed maximal pacing capture rates of 4 Hz and generated an average active stress of 0.55 $mN/mm^2$ at 2 Hz, 5 V pacing protocol (n = 11, standard error of means = 0.063; **Supplemental Figure 1E**).

Although we selected 7 mm long internal loading posts with 5 mm intervals, it is possible to vary the final ECT geometry by arranging the length of loading posts and the interval between adjacent posts (**Figure 3**). Moreover, it is possible to expand the scale of 1.5 cm x 1.5 cm ECTs to larger formats such as 3 cm x 3 cm large mesh ECT. According to force measurement, 3 cm x 3 cm ECTs showed electromechanical properties similar to 1.5 cm x 1.5 cm ECTs[12]. The flexibility of the tissue shapes and the scalability with preserved tissue function is significances of the present protocol with respect to already existing methods.

One limitation of the present protocol is that the PDMS molds are hand-assembled from PDMS sheets. Although the construction of millimeter-unit molds would be feasible by hand-assembly, molds from photolithography or casting from master molds would be more suitable for expanded and stable ECT generation.

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**DISCLOSURES:**

The authors have no financial or scientific conflicts to disclose.

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Figure 1



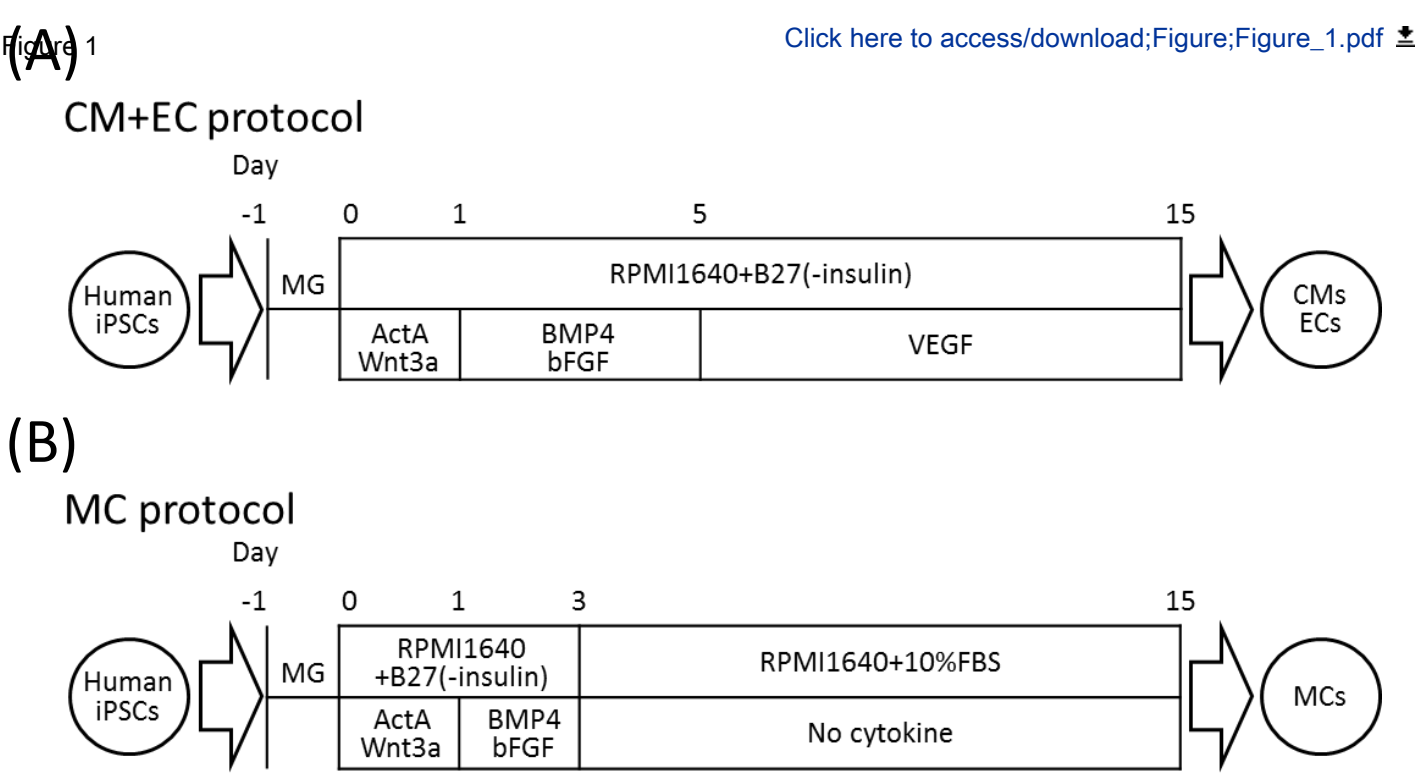
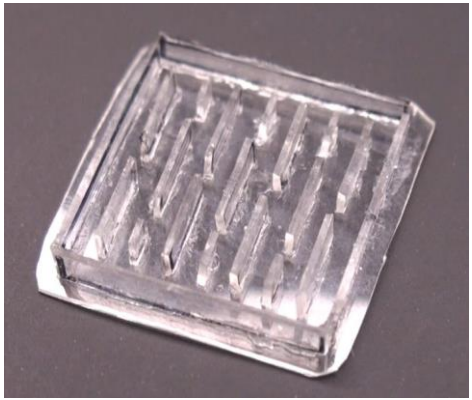


Figure 2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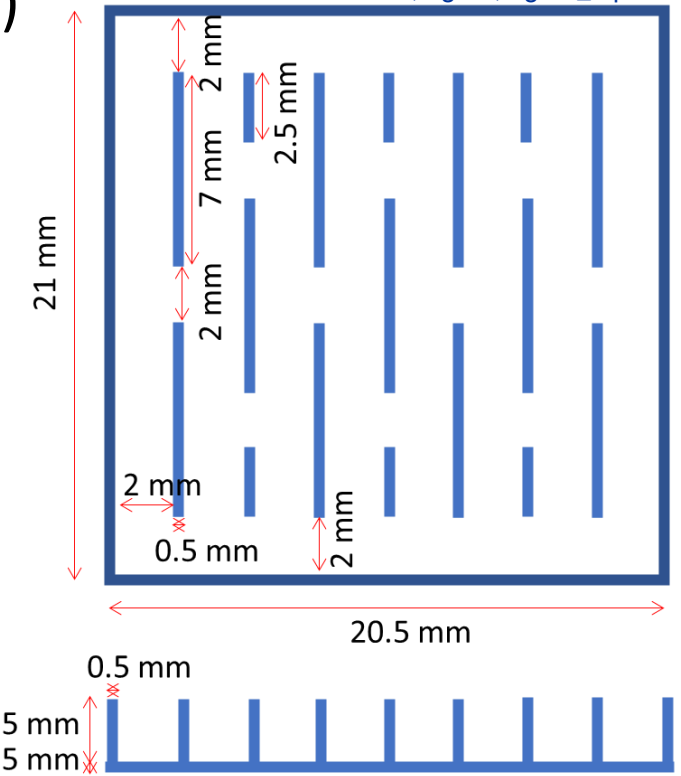
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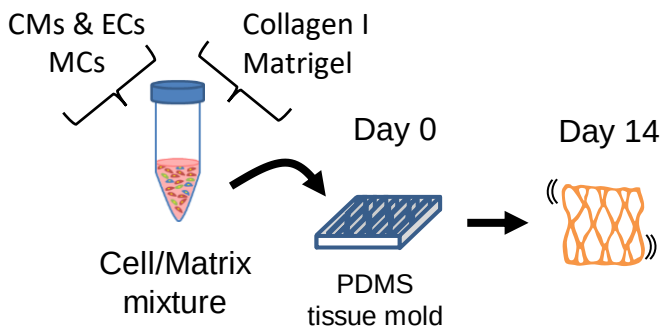

(C)
CMs & ECs
MCs
Collagen I
Matrigel
Day 0
Day 14
Cell/Matrix
mixture
PDMS
tissue mold

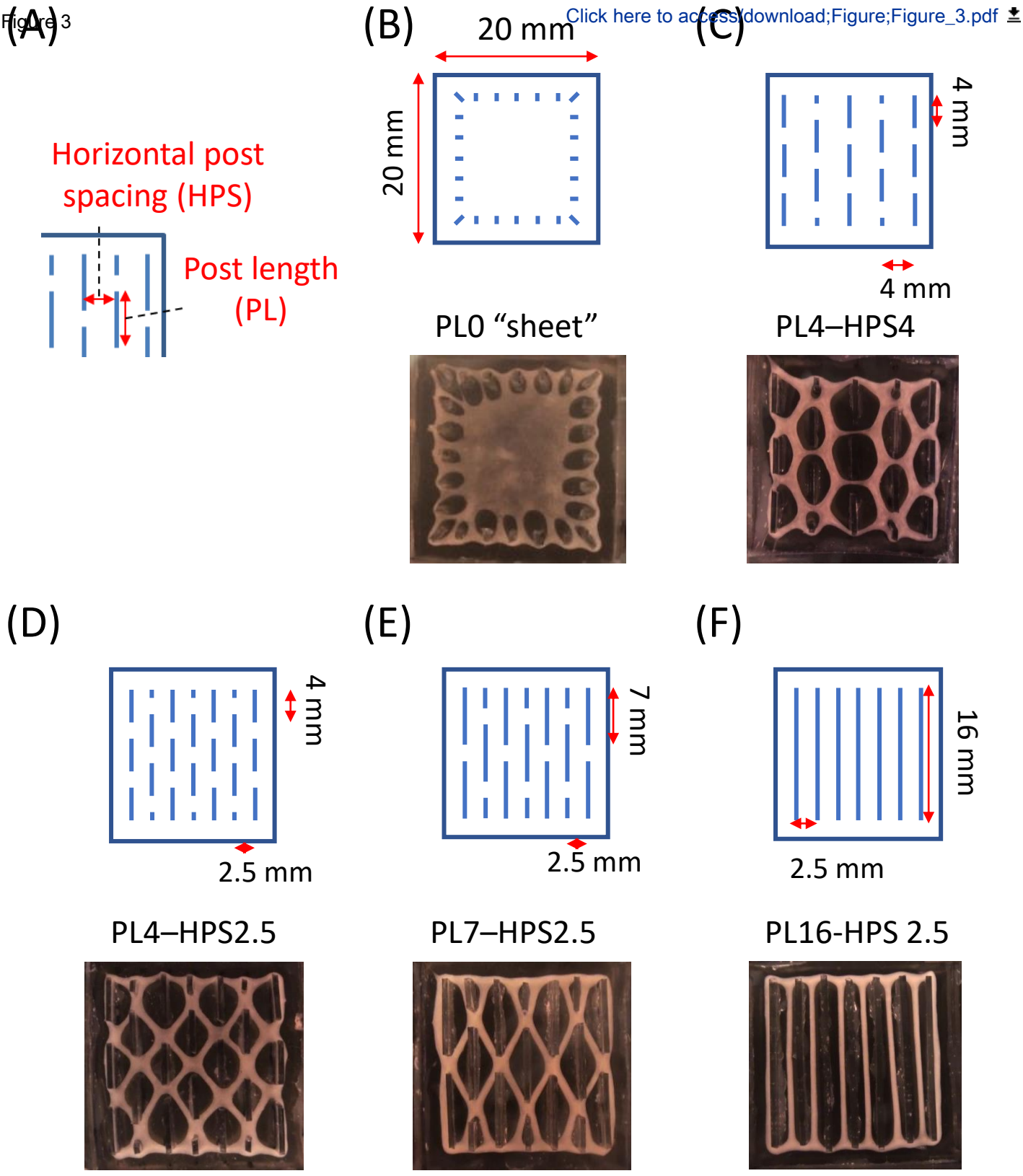


Figure 4



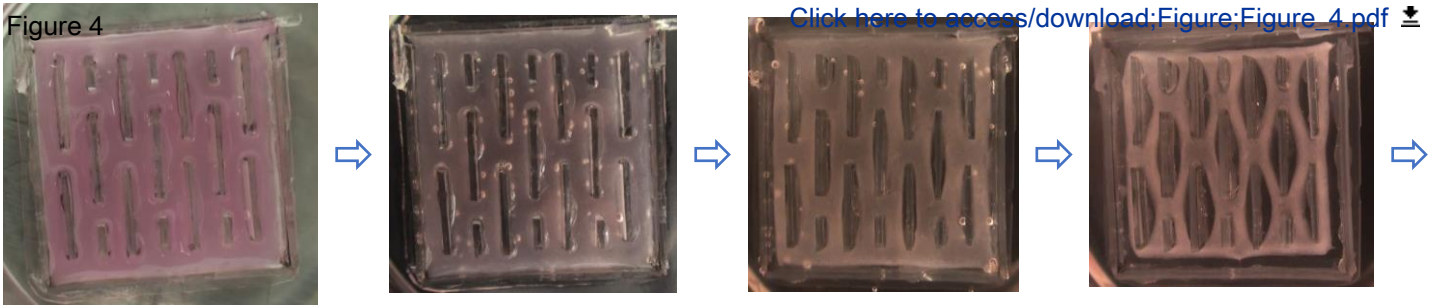
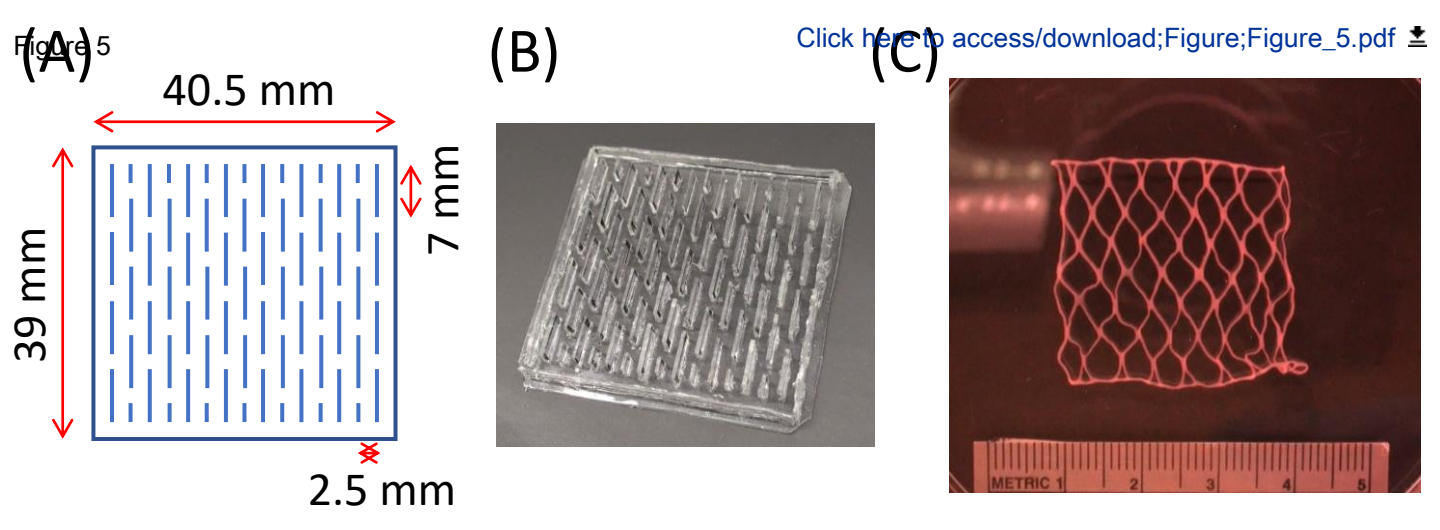


Figure 5





Supplementary Video 1

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**Video or Animated Figure**

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Supplementary Video 2

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Supplementary Video 3

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Video_3.wmv

Table of Materials



| Name of Material/ Equipment | Company |
|---|---|
| **Materials** | |
| Cell Culture Dishes 100x20 mm style | Falcon/ Thomas scientific |
| Multiwell Plates For Cell Culture 6well 50/CS | Falcon / Thomas scientific |
| Sylgard 184 Silicone Elastomer Kit | Dow Corning |
| | |
| **Reagents** | |
| Accumax | Innovative Cell Technologies |
| BMP4, recombinant (10µg) | R&D |
| Collagen, Type I solution from rat tail | Sigma |
| Growth factor-reduced Matrigel | Corning |
| Human VEGF (165) IS, premium grade | Miltenyi |
| Pluronic F-127, 0.2 µm filtered (10% Solution in Water) | Molecular Probes |
| Recombinant human bFGF | WAKO |
| Recombinant Human/Mouse/Rat ActivinA (50µg) | R&D |
| rh Wnt-3a (10µg) | R&D |
| Versene solution | Gibco |
| | |
| **Culture medium and supplements** | |
| 10x MEM | Invitrogen |
| 2 Mercaptro Ethanol | SIGMA |
| B27 supplement minus insulin | Gibco |
| DMEM, high glucose | Gibco |
| Fetal Bovine Serum (500ml) | Any |
| Fetal Bovine Serum (500ml) | Any |
| L-Glutamine | Gibco |
| NaHCO3 | Any |
| PBS 1x | Gibco |
| Penicillin-Streptomycin (5000 U/mL) | Gibco |
| RPMI1640 medium | Gibco |
| αMEM | Invitrogen |
| | |
| **Flowcytometry** | |

| | |
|---|---|
| anti-TRA-1-60, FITC, Clone: TRA-1-60, BD Biosciences | BD / Fisher |
| anti-Troponin T, Cardiac Isoform Ab-1, Clone: 13-11, Thermo Scientific Lab Vision | Fisher |
| BD FACS Clean Solution | BD |
| BD FACSFlow Sheath Fluid | BD |
| BD FACSRinse Solution | BD |
| EDTA | Any |
| Falcon Tube with Cell Strainer Cap (Case of 500) | Corning |
| Fetal Bovine Serum (500ml) | Any |
| LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation | Molecular Probes |
| PDGFRb; anti-CD140b, R-PE, Clone: 28D4, BD Biosciences | BD / Fisher |
| Saponin | Sigma-Aldrich |
| VEcad-FITC; anti-CD144, FITC, Clone: 55-7H1, BD Biosciences | BD / Fisher |
| Zenon Alexa Fluor 488 Mouse IgG1 Labeling Kit | Molecular Probes |

| Catalog Number | Comments/Description |
| --- | --- |
| 9380C51 | |
| 6902A01 | |
| 761036 | |
| | |
| AM-105 | |
| RSD-314-BP-010 | |
| C3867 | |
| 356231 | |
| 130-109-385 | |
| P-6866 | |
| 060-04543 | |
| 338-AC-050 | |
| 5036-WN | |
| 15040066 | |
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| 11430 | |
| M6250 | |
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| 15070-063 | |
| 21870092 | |
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47306-50G-F
560411
Z25002

Thank you for your detailed comments and useful suggestions that have helped us to considerably improve our manuscript. Taking your comments into careful consideration, we revised our manuscript. As indicated in the responses that follow, we have taken each of these comments and suggestions into account in the revised version of our manuscript. Modified or added sentences are highlighted in yellow.

**For editor:**

General:

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

Thank you for the comment. We checked the sentences again for grammar issues.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Flexcell FX-5000 Tissue Train, Matrigel, Versene, Accumax, LIVE/DEAD fixable Aqua dead cell stain, Zenon, Sylgard, Pluronic F127

We removed symbols such as "®" and limited the use of commercial language.

Protocol:

1. For each protocol step/substep, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Thank you for the suggestion. We added references to adequately indicate how to perform the protocol actions (Protocols 1.1., 1.6., 4.1. and 4.6.).

Specific Protocol steps:

1. 4.4: How long and how fast (in x g) to centrifuge?

Thank you for the comment. We added the setting of centrifuge we applied as follows:

"4.4. Centrifuge the prepared cell suspension containing six million cells at 1100 rpm, 240 G for 5 minutes" (lines 206-207).

Figures:

1. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account.

We confirmed the permission of reusing the figures in our former publications we referred in this report. Please refer marked part of PDF files: "Permissions requests_Nature Research" and "Permissions FAQs_Cambridge University Press".

2. Figures 2-4: Please include a space between numbers and their corresponding units ('Day 0', '21 mm', etc). Please use 'h' instead of 'hr' in Figure 3).

We are sorry for the mistake. We modified the way of writing numbers and units in the figures.

3. Discussion of Supplemental Figure 2 would better fit in the results section.

Thank you for the suggestion. We described the contents in the former Supplemental figure 2 in the Result section and change it to Figure 3. (lines 252-254).

Discussion:

1. Discussion: As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3–6 paragraphs with citations:

a) Critical steps within the protocol

b) Any limitations of the technique

c) The significance with respect to existing methods

Thank you for the suggestion. According to the comment, we revised the Discussion section to clarify critical steps of the protocol (lines 327, 329-333), the significance of the protocol (lines 347-349) and limitations (lines 350-353).

References:

1. Please do not abbreviate journal titles.

Thank you for the comment. We spelled out the journal titles.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

We confirmed that all materials and equipment used are listed in the Table of Material.

2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

We modified the table of materials as suggested.

**For Reviewer #1:**

Major Concerns:

This report shows that the authors can create ECTs composed of hiPSC-derived cardiomyocytes, endothelial cells and vascular mural cells. This is interesting work, acknowledging that other have reported the use of engineered constructs like this one. The authors need to cite references that are more current (see review Lancaster et al., Vessel Plus 2019;3:34. http://doi.org/10.20517/2574-1209.2019.16).

We appreciate introducing informative references and cited Lancaster's one (new #8).

8. Lancaster, J. J. et al. Surgical treatment for heart failure: cell-based therapy with engineered tissue. *Vessel Plus* 2019, (2019).

The reference list favors the work of their laboratory. The authors might want to consider dropping Ref # 1 because of issues raised regarding the validity of that work.

We agree the comment and removed old Reference #1 (Bolli et al., *Lancet*).

The authors ought to cite this recent report using hiPSC-derived cardiomyocytes on a cell

sheet implanted in a patient.
https://www.japantimes.co.jp/news/2020/01/28/national/science-health/osaka-university-transplants-ips-cell-basedheart-cells-worlds-first-clinical-trial/

Although we agree that the article in Japan Times is important in the field of iPS cell based regenerative therapy, we may not cite it in this occasion because the results of this study has not been reported yet and it seems to be too early to refer it in our present report. Instead, we will refer the initial result report of the clinical study in our future related works.

The authors make the statement is that they can scale this approach up to 3.0X3.0 cm but they will probably need larger ECTs for human hearts. There are potential issues as you scale up these constructs. The authors need to acknowledge these issues. Do they have any data on the scaled up versions to 3x3cms? Other labs (cited above) have created these constructs and implanted them in animal model of myocardial infarction and heart failure. That is what is required to show that these ECTs are functional tissues and can improve cardiac function.

Thank you for the comment. We agree that a 3x3 cm ECT is still small for human hearts to cover the injured area. Although we did not generate >3-cm ECTs in our work because it requires huge number of cells and costs, it would be possible to scale them up to >3-cm using larger tissue molds considering that the length and width of each bundle, basic structure of the mesh ECTs which secure the tissue function, do not differ regardless of the whole size of the ECTs. As an evidence for this point, we have performed electromechanical and histological evaluations of 3x3 cm ECTs and validated that the tissue function and histological findings were not different from those in 1.5x1.5 cm ECTs (Nakane, *Scientific Reports* 2017).

We added descriptions in Discussion section regarding this point: “According to force measurement, 3x3 cm ECTs showed electromechanical properties compatible with 1.5x1.5 cm ECTs.” (lines 346-347).

Lines325: "ECTs displayed maximal pacing capture rates of 4Hz and generated an average active stress of 0.55 mN/mm2 326 (2Hz, 5V pacing protocol, Supplemental Figure 1E). The authors need to show mean±SE with numbers for these data.

We added the number and SE in the sentence. (lines 341-342)

Line 322:" All constructs start intrinsic spontaneous beating in vitro within 72 hours and continued beating throughout the duration of culture." I suggest the authors show a video of this spontaneous beating ECT and show that the beating aligns with the long-axis of the bundles.

Thank you for the suggestion. We added three video files of beating ECTs: Supplementary Videos 1-3. (lines 312-316, 338)

Minor Concerns: Why are lines in the fabrication section Lines 165-246 yellowed?

According to the instructions from the journal, important steps for video shoot must be marked with yellow.

**For Reviewer #2:**

Major Concerns:
The authors do not discuss the manufacturing of the PDMS tissue mold sufficiently. The language to describe the creation is confusing and I would assume needs to have a master mold to cast onto. If this device was completely hand made it is hard to believe that the dimensions can easily be reproduced - it is not clear how important these dimensions are to the overall viability of the sample.

Thank you for the comment. We agree that the reproducibility is guaranteed by making tissue molds from a master mold produced by photolithography. However, in this experiment, it was enough to confirm the accuracy of tissue mold design by a millimeter unit, and it is not technically demanding to fabricate molds by hand. We fabricated ten tissue molds for the 15x15 mm mesh ECT by hand and confirmed uniform structure of constructs from all molds by measuring the width of each bundle. However, the stability of hand-made molds would depend on each individual manipulation skill, and molds from photolithography or casting from master molds would be more suitable for universal use.

We added this point as a limitation in the Discussion section (lines 350-353).

General comment: What is the thickness of these tissues? Although you include a viability (live/dead stain) test - I am still curious about the whether you are within the diffusion

limit.

Thank you for the comment. Tissues are about 0.5 mm thick and each bundle in a construct is about 0.5 mm wide. We confirmed high cell viability in the construct by live/dead staining and consider that the diffusion is satisfactory.

We added the thickness of a tissue in the Result section: “The whole tissue is approximately 1.5 cm wide and 0.5 mm thick, and the width of each bundle in the mesh is approximately 0.5 mm in average.”. (lines 258-260)

Include what iPSC lines you are differentiating.

We used 201B6 line and added a NOTE.
“NOTE: We used a hiPSCs [4-factor (Oct3/4, Sox2, Klf4 and c-Myc) line: 201B6.” (line 85)

Minor Concerns:
Line 227: do you incubate a test sample within a tube for reference to determine when you have reached a desirable gel texture? Additionally, I would state that the incubation step it to crosslink/gel the collagen mixture.

After some preliminary experiments, we determined that the cell/matrix mixture crosslinked in 60 minutes and we could add medium to it. However, the construct is still fragile, and we may break the structure if we vigorously splashes medium on it.

We modified the sentence as follows: “NOTE: Although the cell/matrix is crosslinked in 60 minutes, the construct is still very fragile. Add medium gently to avoid damaging it.”. (lines 228-229)

Lines 249-251 and 182-183: You make 2 different statements here. What is the correct protocol? Are you targeting 10-20% MCs or 10-20% MC + EC or 10-20% MCs and 10-20% ECs? Please clarify.

We are sorry for the confusion. We added MCs to adjust final MC ratios as 10 to 20% of total cells resulting in that the final ratio of CM: EC: MC was 3: 1: 1. To clarify that we target 10-20% MCs, we removed the sentence including 3: 1: 1.

Line 197: Do you need to adjust the entire alkali buffer or just the NaOH, if you are trying

to adjust the pH?

We need to adjust the pH of collagen solution with alkali buffer containing NaOH.

Line 207: I think you should add an additional line right before using the collagen stating that collagen needs to remain on ice for the duration of the experimental preparation.

Thank you for the comment. We added “NOTE: Collagen must be kept on ice (4 °C).”. (line 192)

Line 218: Trying to read this 5 way ratio is tough. I believe the only important part you are really concerned about is the final collagen concentration and the pH of the collagen. I would suggest making these sentences more clear.

We agree that the final collagen concentration is important and removed the ratio. “NOTE: The final concentration of collagen type I is 0.67 mg/mL.”. (line 216)

Line 172: Replace square with rectangular (technically you aren't making a square).

Thank you for your careful reading. We modified the sentences as you pointed out. (line 170)

Line 298: extra "("

Thanks!

Supplemental figures scale bars - you create 2 different sized sets of tissues and it is difficult to determine what is what.
Supplemental figure 1D: the left image it a top view is the one text to it a side view? Not sure what I am seeing here(caption does not specify - maybe on the figure including some arrows to describe what the other components are would be helpful, such as force transducer, etc)

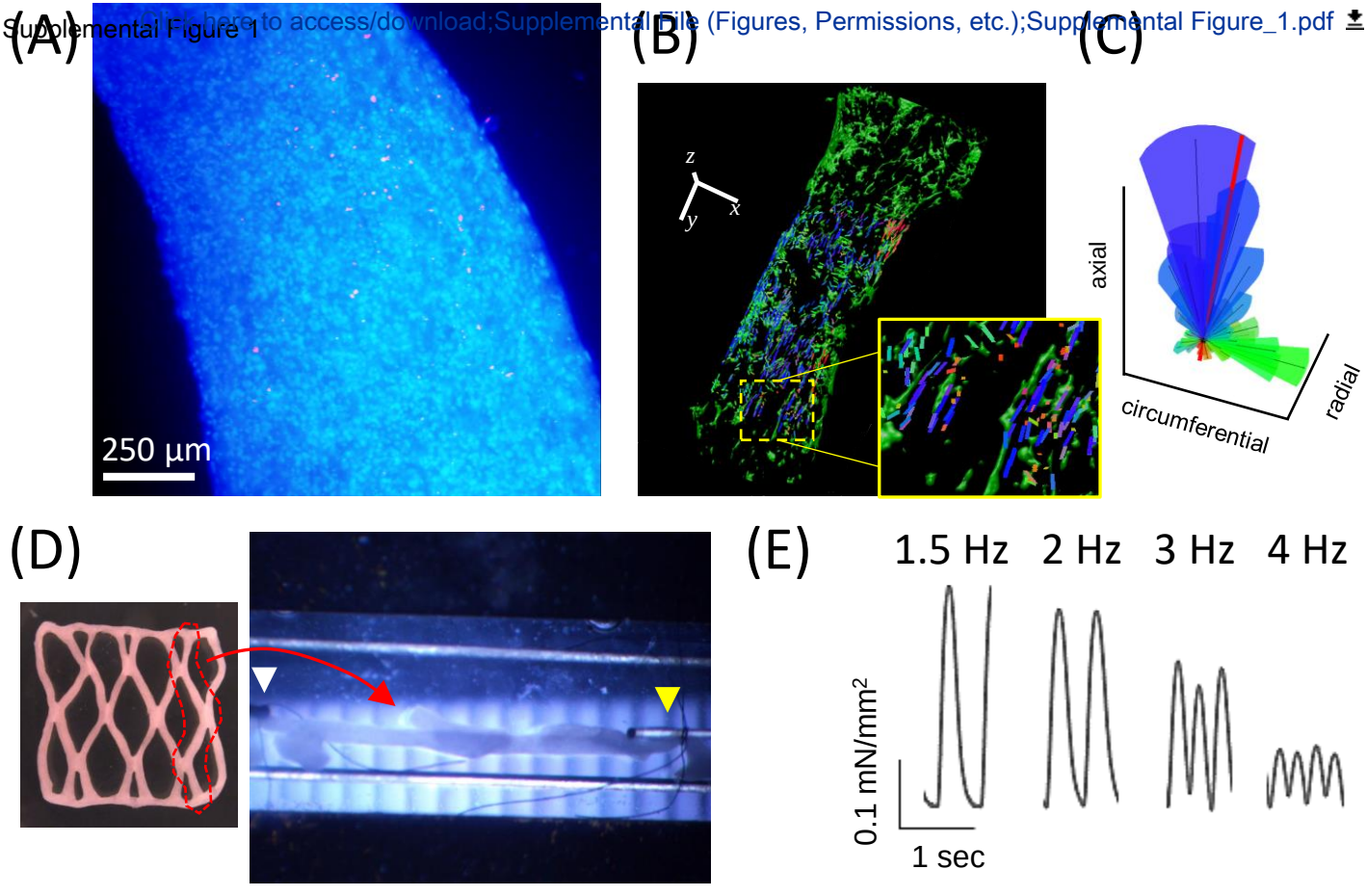
We added some explanations in the figure and the figure legend.
“The white arrowhead indicates force transducer and the yellow arrowhead indicates high-speed length controller.”. (lines 307-309)

Line 60-62: specific reference for your findings.

Thank you for the comment. We added the reference (#5) for the findings. (line 63)

We believe that we have adequately addressed all the concerns. We look forward to receiving the next review comments.

Best regards,
Hidetoshi Masumoto, MD, PhD



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