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Generating Self-assembling Human Heart Organoids Derived from Pluripotent Stem Cells

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TITLE:

Generating Self-assembling Human Heart Organoids Derived from Pluripotent Stem Cells

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SUMMARY:

Here, we describe a protocol to create developmentally relevant human heart organoids (hHOs) efficiently using human pluripotent stem cells by self-organization. The protocol relies on the sequential activation of developmental cues and produces highly complex, functionally relevant human heart tissues.

ABSTRACT:

The ability to study human cardiac development in health and disease is highly limited by the capacity to model the complexity of the human heart *in vitro*. Developing more efficient organ-like platforms that can model complex *in vivo* phenotypes, such as organoids and organs-on-a-chip, will enhance the ability to study human heart development and disease. This paper describes a protocol to generate highly complex human heart organoids (hHOs) by self-organization using human pluripotent stem cells and stepwise developmental pathway activation using small molecule inhibitors. Embryoid bodies (EBs) are generated in a 96-well plate with round-bottom, ultra-low attachment wells, facilitating suspension culture of individualized constructs.

The EBs undergo differentiation into hHOs by a three-step Wnt signaling modulation strategy, which involves an initial Wnt pathway activation to induce cardiac mesoderm fate, a second step of Wnt inhibition to create definitive cardiac lineages, and a third Wnt activation step to induce proepicardial organ tissues. These steps, carried out in a 96-well format, are highly efficient, reproducible, and produce large amounts of organoids per run. Analysis by immunofluorescence imaging from day 3 to day 11 of differentiation reveals first and second heart field specifications

and highly complex tissues inside hHOs at day 15, including myocardial tissue with regions of atrial and ventricular cardiomyocytes, as well as internal chambers lined with endocardial tissue. The organoids also exhibit an intricate vascular network throughout the structure with an external lining of epicardial tissue. From a functional standpoint, hHOs beat robustly and present normal calcium activity as determined by Fluo-4 live imaging. Overall, this protocol constitutes a solid platform for *in vitro* studies in human organ-like cardiac tissues.

INTRODUCTION:

Congenital heart defects (CHDs) are the most common type of congenital defect in humans and affect approximately 1% of all live births[1–3]. Under most circumstances, the reasons for CHDs remain unknown. The ability to create human heart models in the lab that closely resemble the developing human heart constitutes a significant step forward to directly study the underlying causes of CHDs in humans rather than in surrogate animal models.

The epitome of laboratory-grown tissue models are organoids, 3D cell constructs that resemble an organ of interest in cell composition and physiological function. Organoids are often derived from stem cells or progenitor cells and have been successfully used to model many organs such as the brain[4,5], kidney[6,7], intestine[8,9], lung[10,11], liver[12,13], and pancreas[14,15], just to name a few. Recent studies have emerged demonstrating the feasibility of creating self-assembling heart organoids to study heart development *in vitro*. These models include using mouse embryonic stem cells (mESCs) to model early heart development[16,17] up to atrioventricular specification[18] and human pluripotent stem cells (hPSCs) to generate multi-germ layer cardiac-endoderm organoids[19] and chambered cardioids[20] with highly complex cellular composition.

This paper presents a novel 3-step WNT modulation protocol to generate highly complex hHOs in an efficient and cost-effective manner. Organoids are generated in 96-well plates, resulting in a scalable, high-throughput system that can be easily automated. This method relies on creating hPSC aggregates and triggering developmental steps of cardiogenesis, including mesoderm and cardiac mesoderm formation, first and second heart field specification, proepicardial organ formation, and atrioventricular specification. After 15 days of differentiation, hHOs contain all major cell lineages found in the heart, well-defined internal chambers, atrial and ventricular chambers, and a vascular network throughout the organoid. This highly sophisticated and reproducible heart organoid system is amenable to investigating structural, functional, molecular, and transcriptomic analyses in the study of heart development, and diseases, and pharmacological screening.

PROTOCOL:

1. hPSC culture and maintenance

NOTE: The hPSCs or human embryonic stem cells (hESCs) need to be cultured for at least 2 consecutive passages after thawing before being used to generate EBs for differentiation or further cryopreservation. hPSCs are cultured in PSC medium (see the **Table of Materials**) on basement-membrane-extracellular matrix (BM-ECM)-coated 6-well culture plates. When

performing medium changes on hPSCs in 6-well plates, add the medium directly to the inner side of the well rather than directly on top of the cells to prevent unwanted cell detachment or stress. Users should be wary of pre-warming PSC media that should not be warmed at 37 °C; all PSC media used in this protocol were thermostable.

1.1. To coat the well-plates with the BM-ECM, thaw one aliquot of the BM-ECM (stored at -20 °C according to the manufacturer's instructions) on ice and mix 0.5 mg of the BM-ECM with 12 mL of cold Dulbecco's modified Eagle's medium (DMEM)/F12 medium (stored at 4 °C). Distribute 2 mL of the DMEM/F12–BM-ECM mixture onto each well of a 6-well plate and incubate at 37 °C for at least 2 h.

1.2. To thaw the cells, first, thaw the hPSC cryovial in a 37 °C bead or water bath for 1–2 min until only a small amount of ice is visible. Transfer the thawed cells to a centrifuge tube and slowly add 8–9 mL of the PSC medium supplemented with 2 µM of the ROCK inhibitor, thiazovivin (Thiaz), and centrifuge at 300 × *g* for 5 min. Remove the supernatant and resuspend the cell pellet in the PSC medium supplemented with 2 µM of Thiaz. Distribute the cells in the culture medium into 1–2 wells depending on the cryovial cell concentration and culture at 37 °C, 5% CO_2 for 24 h before changing the PSC medium.

1.3. Change the medium on the cells at 48 h intervals. Perform washes and medium changes using DMEM/F12 (1 mL/well) and the PSC medium (2 mL/well), respectively.

NOTE: Washes help remove cell waste and debris while fresh media changes provide cells with a renewed source of nutrients.

1.4. Passage the cells upon subconfluency (60–80% confluent) by aspirating the medium, then washing each well with 1 mL of 1x Dulbecco's phosphate-buffered solution (no calcium, no magnesium; DPBS). Aspirate the DPBS and add 1 mL of the dissociation reagent for hPSCs (see the **Table of Materials**), followed by the aspiration of all but a thin film of the reagent after 10 s.

1.5. Incubate for 2–5 min with the thin film of the dissociation reagent for hPSCs until gaps form between cells.

NOTE: The time to stop the dissociation is cell-line-dependent.

1.6. Add 1 mL of the PSC medium supplemented with 2 µM of Thiaz (PSC medium+Thiaz) to the well and gently tap the plate to induce cell detachment. Pipette the detached cells in the medium 1–2 times to break up any large colonies, and resuspend the cells in PSC medium+Thiaz in a 1:6 well ratio (cells from 1 well resuspended in 12 mL of culture medium). Replate the cells on BM-ECM-coated wells.

2. Generation of 3D self-assembling human heart organoids

2.1. Embryoid body (EB) formation:

NOTE: It is imperative to limit observable differentiated cells prior to embryoid body formation. Two to three wells of a 6-well plate at a 60–80% confluency will yield enough cells for a single 96-well plate of organoids. All media should be aliquoted and warmed in a 37 °C bead or water bath before any medium changes to minimize temperature shock to the EBs or organoids (this does not include cell dissociation reagents). See **Figure 1A,B.**

2.1.1. Day -2

2.1.1.1. To create EBs, on day -2, wash sub-confluent hPSCs (60–80% confluent) with DPBS for at least 10 s to wash any cell debris and aspirate the DPBS.

2.1.1.2. To detach the cells and release them into a single-cell state, add 1 mL of room-temperature cell dissociation reagent (see the **Table of Materials**) to each well for 3–6 min. Gently tap the plate ~5 times every minute to induce detachment while checking under the microscope. Add 1 mL of PSC medium+Thiaz to stop the reaction.

2.1.1.3. To collect the cells and break up any remaining aggregates, pipette the media up and down in the well 2–3 times to generate a single-cell suspension. Transfer the single-cell suspension to a centrifuge tube and spin for 5 min at 300 × *g*.

2.1.1.4. To obtain the desired cell concentration, discard the supernatant and resuspend the cells in 1 mL of PSC medium+Thiaz. Count the cells using a cell counter or hemocytometer and dilute the cells in PSC medium+Thiaz to a concentration of 100,000 cells/mL.

2.1.1.5. To distribute the cells for EB formation, use a multichannel pipette, add 100 µL (10,000 cells) to each well of a round-bottom ultra-low attachment 96-well plate. Centrifuge the plate at 100 × *g* for 3 min and incubate for 24 h at 37 °C, 5% CO_2.

2.1.2. Day -1

2.1.2.1. Carefully remove 50 µL of medium from each well and add 200 µL of fresh PSC medium warmed to 37 °C to achieve a final volume of 250 µL per well. Incubate the cells for 24 h at 37 °C, 5% CO_2.

NOTE: Remove and add medium carefully on the side of the well to avoid disturbing the EBs at the bottom of the well. Due to the delicate nature of the EBs and the suspension culture, it is necessary to leave a small volume of liquid in each well when changing the medium to avoid disturbing the EBs.

2.2. Human Heart Organoid (hHO) Differentiation:

NOTE: All media should be warmed in a 37 °C bead or water bath prior to any media changes. Remove and add medium carefully on the side of the well to avoid disturbing the developing

organoids at the bottom of the well. Washes are not needed between media changes to minimize agitation and allow the gradual removal of inhibitors and growth factors. RPMI with 2% B-27 supplement (**Table of Materials**) was used throughout the differentiation protocol. B-27 supplement contains insulin unless specified (insulin-free in days 0–5). See **Figure 1C.**

2.2.1. Day 0

2.2.1.1. To initiate differentiation towards a mesoderm lineage, remove 166 µL of medium from each well (~2/3rd of total well volume) and add 166 µL of RPMI 1640 containing insulin-free B-27 supplement, 6 µM CHIR99021, 1.875 ng/mL bone morphogenetic protein 4 (BMP4), and 1.5 ng/mL Activin A for a final well concentration of 4 µM CHIR99021, 1.25 ng/mL BMP4, and 1 ng/mL Activin A. Incubate for 24 h at 37 °C, 5% CO_2.

2.2.2. Day 1

2.2.2.1. Remove 166 µL of medium from each well and add 166 µL of fresh RPMI 1640 with insulin-free B-27 supplement. Incubate for 24 h at 37 °C, 5% CO_2.

2.2.3. Day 2

2.2.3.1. To induce cardiac mesoderm specification, remove 166 µL of medium from each well and add 166 µL of RPMI 1640 containing insulin-free B-27 supplement and 3 µM Wnt-C59 for a final well concentration of 2 µM Wnt-C59. Incubate for 48 h at 37 °C, 5% CO_2.

2.2.4. Day 4

2.2.4.1. Remove 166 µL of medium from each well and add 166 µL of fresh RPMI 1640 with insulin-free B-27 supplement. Incubate for 48 h at 37 °C, 5% CO_2.

2.2.5. Day 6

2.2.5.1. Remove 166 µL of medium from each well and add 166 µL of RPMI 1640 with B-27 supplement. Incubate for 24 h at 37 °C, 5% CO_2.

2.2.6. Day 7

2.2.6.1. To induce proepicardial differentiation, remove 166 µL of medium from each well and add 166 µL of fresh RPMI 1640 containing B-27 supplement and 3 µM CHIR99021 for a final well concentration of 2 µM CHIR99021. Incubate for 1 h at 37 °C, 5% CO_2.

2.2.6.2. Remove 166 µL of medium from each well and add 166 µL of fresh RPMI 1640 containing B-27 supplement. Incubate for 48 h at 37 °C, 5% CO_2.

NOTE: Extra caution is advised at this second medium change on day 7 as the organoids are more prone to movement because of the media changes.

2.2.6.3. From day 7 onwards until collection or transfer for analyses or experimentation, perform medium changes every 48 h by removing 166 µL of medium from each well and add 166 µL of fresh RPMI 1640 containing B-27 supplement.

NOTE: Organoids are ready for analyses and experimentation at day 15 unless earlier developmental stages are of interest. They can be cultured past day 15 for long-term culture or maturation experiments.

3. Organoid analysis

3.1. Transferring whole organoids (live or fixed)

NOTE: For live organoid transfer, ensure that pipette tips used are sterile.

3.1.1. Cut the tip off a P200 pipette tip 5–10 mm from the tip opening, resulting in a wide opening of ~2–3 mm diameter.

3.1.2. Insert the tip straight into the round-bottom well containing the organoid so that the pipette is completely vertical (perpendicular to the plate). Ensure that the pipette plunger is already pressed all the way before inserting the tip into the medium.

3.1.3. Slowly release the pipette plunger, taking up enough medium (100–200 µL) to collect the organoid.

3.1.4. Transfer the organoid in medium to the target destination (e.g., for fixing, live imaging, electrophysiology recording, new plate culture).

3.2. Fixing organoids

NOTE: Fixing and staining organoids can be done either in the 96-well culture plate or microcentrifuge tubes. Paraformaldehyde (PFA) should be handled only in a fume hood.

3.2.1. For fixation in microcentrifuge tubes, transfer live organoids to separate tubes with 1–8 organoids per tube.

NOTE: Do not exceed 8 organoids per tube.

3.2.2. Carefully remove and discard as much medium from the tube as possible without touching the organoids.

3.2.3. Add 4% PFA to each tube or well (300–400 µL per microcentrifuge tube and 100–200 µL per well of a 96-well plate). Incubate at room temperature for 30–45 min.

NOTE: Incubation times over 1 h may require antigen retrieval steps and are not recommended.

3.2.4. Safely discard the PFA without disturbing the organoids. Perform 3 washes with DPBS supplemented with 1.5 g/L glycine (DPBS/Gly), using the same volume used for the 4% PFA, waiting 5 min between washes. Remove DPBS/Gly and proceed to immunostaining or other analyses or add DPBS and store at 4 °C for future use for up to 2 weeks.

NOTE: Storing fixed organoids for longer than 2 weeks may result in tissue degradation and contamination and is not recommended.

3.3. Whole-mount immunofluorescent staining

3.3.1. Add 100 µL of blocking/permeabilization solution (10% normal donkey serum + 0.5% bovine serum albumin (BSA) + 0.5% Triton X-100 in 1x DPBS) to each well or tube containing the fixed organoids. Incubate at room temperature overnight on a shaker.

NOTE: Do not exceed 8 organoids per tube.

3.3.2. Carefully remove and discard as much of the blocking solution as possible without touching the organoids. Perform 3 washes with DPBS, waiting 5 min between washes.

3.3.3. Prepare the primary antibody solution (1% normal donkey serum + 0.5% BSA + 0.5% Triton X-100 in 1x DPBS) with the desired primary antibodies at the recommended concentrations. Incubate at 4 °C for 24 h on a shaker.

3.3.4. Carefully remove and discard as much of the antibody solution as possible without touching the organoids. Perform 3 washes with DPBS, waiting 5 min between washes.

3.3.5. Prepare secondary antibody solution (1% normal donkey serum + 0.5% BSA + 0.5% Triton X-100 in 1x DPBS) with the desired secondary antibodies at the recommended concentrations. If the antibodies are fluorescently labeled, incubate at 4 °C in the dark (e.g., covered in aluminum foil) for 24 h on a shaker.

3.3.6. Carefully remove and discard as much of the antibody solution as possible without touching the organoids. Perform 3 washes with DPBS, waiting 5 min between washes.

3.3.7. Prepare slides with beads (90–300 µm in diameter) mounted in a mounting medium (see the **Table of Materials**) near the edges of the slide where the coverslip with the organoids will be placed.

NOTE: It is recommended to allow the mounting medium around the beads to dry before proceeding; this will prevent the beads from moving around. See **Figure 2**.

3.3.8. Transfer the stained organoids using a cut pipette tip onto the slide, between the beads, ensuring spacing to avoid contact between the organoids once on the slide. Use the corner of a rolled-up laboratory wipe to carefully remove excess liquid around the organoid.

3.3.9. Cover the organoids with mounting-clearing medium (fructose–glycerol clearing solution is 60% (vol/vol) glycerol and 2.5 M fructose)[37] using 120–150 µL of the mounting-clearing medium per slide.

NOTE: It is recommended to use a cut pipette tip when working with the mounting-clearing medium as it is very viscous.

3.3.10. Hover the coverslip over the slide with the organoids covered with mounting-clearing solution and slowly press the coverslip over the slide, ensuring the organoids are between the mounted beads.

3.3.11. Seal the perimeter of the coverslip on the slide using top coat nail varnish. Allow the slide to dry in the dark at room temperature for 1 h. Store at 4 °C in the dark for long-term storage.

3.4. Calcium transient imaging in live heart organoids

NOTE: According to the manufacturer's instructions, Fluo4-AM was reconstituted in dimethyl sulfoxide (DMSO) to a final stock solution concentration of 0.5 mM. Fluo4-AM was added directly to the organoid well in the 96-well plate.

3.4.1. Perform 2 washes on the organoids using RPMI 1640 medium.

3.4.1.1. Remove 166 µL of the spent medium from the well.

3.4.1.2. Add 166 µL of warmed RPMI 1640 medium, remove 166 µL of medium, and add 166 µL of fresh RPMI 1640 medium.

NOTE: The washes are done to remove waste material and cell debris. Two-thirds of the medium is removed from the wells during the washes to avoid disturbing the organoids at the bottom of the well before the functional assay.

3.4.2. Add Fluo4-AM medium to the organoids.

3.4.2.1. Add Fluo4-AM reconstituted in DMSO to RPMI 1640 containing B-27 supplement to prepare a 1.5 µM solution.

3.4.2.2. Remove 166 µL of medium from the well.

3.4.2.3. Add 166 µL of 1.5 µM Fluo4-AM in RPMI 1640 containing B-27 supplement for a final well concentration of 1 µM. Incubate at 37 °C, 5% CO_2 for 30 min.

3.4.3. Perform 2 washes as in step 3.4.1.

3.4.4. Add 166 µL of RPMI 1640 containing B-27 supplement to the well.

3.4.5. Using a cut tip of a P200 pipette tip, transfer the organoid to a glass-bottom Petri dish (e.g., 8-well chambered cover glass with #1.5 high-performance coverglass) with 100–200 µL of medium.

NOTE: See section 3.1 on transferring whole organoids.

3.4.6. Image the organoids live under a microscope with a temperature- and CO_2-controlled chamber at 37 °C, 5% CO_2.

3.4.7. Record several 10–20 s videos at various locations across the organoid, showing the increase and decrease in fluorescence intensity levels as the calcium enters and exits the cells.

NOTE: For high-resolution recordings, it is recommended to record at a speed of 10 fps or faster; 50 fps is recommended.

3.4.8. Analyze the videos using image analysis software (e.g., ImageJ) by selecting regions of interest and measuring intensity levels over time.

3.4.9. Normalize the intensity recordings using $\Delta F/F_0$ vs. time in milliseconds and plot.

REPRESENTATIVE RESULTS:

To achieve self-organizing hHO *in vitro*, we modified and combined differentiation protocols previously described for 2D monolayer differentiation of cardiomyocytes[21] and epicardial cells[22] using Wnt pathway modulators and for 3D precardiac organoids[16] using the growth factors BMP4 and Activin A. Using the 96-well plate EB and hHO differentiation protocol described here and shown in **Figure 1**, the concentrations and exposure durations of the Wnt pathway activator CHIR99021 were optimized to yield highly reproducible and complex hHOs derived from human PSCs. hPSCs or hESCs cultured in PSC medium to 60–80% confluency in colony-like formation with minimal to no visible differentiation are ideal for EB generation (**Figure 1B**).

EBs were allowed to incubate for 48 h with a medium change after 24 h before starting differentiation at day 0. On day 0, the EBs should appear as a dark spherical aggregate at the center of each well under a light microscope (**Figure 1B**). The differentiation protocol starts on day 0 with the Wnt pathway activation and growth factor addition for exactly 24 h. This mesoderm induction followed by a cardiac mesoderm induction on day 2 using the Wnt pathway inhibitor Wnt-C59 will result in a significant enlargement of the organoid from ~200 µm in

diameter to 500–800 μm in diameter at day 4 and to as much as 1 mm (organoids my experience a slight reduction in size by day 15 (**Figure 1C**). The hHO will begin beating as early as day 6 (**Video 1**), with 100% of the organoids showing visible beating by day 10 (**Video 2**) (unless undergoing drug treatment or if inadequate hPSCs were used to generate the EBs). This has been observed in 5 distinct hPSC cell lines[23].

To evaluate the capacity of the hHOs to represent various steps of the physiological development of the heart, we collected organoids at various time points throughout the differentiation protocol and looked for the presence and transcriptomic expression of heart field markers. Immunofluorescent staining for the first heart field (FHF) marker, HAND1, and the second heart field (SHF) marker, HAND2, revealed their nuclear presence in these cardiac progenitor cells arising at around day 3 and day 5, respectively (**Figure 3A**).

The expression of both markers happens at regions of the organoids that diminish in size after day 7 for the FHF and after day 9 for the SHF. Interestingly, high-magnification images of day 7 organoids revealed that most HAND1-expressing cells were cardiomyocyte in origin (as shown by the cardiomyocyte-specific marker TNNT2). In contrast, many of the HAND2-expressing cells did not express the cardiomyocyte marker (**Figure 3B**). This observation is in agreement with the precardiac organoids derived from mouse ESCs demonstrating the development of non-myocyte cells from SHF progenitor cells[16]. It is important to note that the RNA-Sequencing data show that the RNA transcripts for both HAND1 and HAND2 were expressed from day 3 onwards, with the FHF marker being more highly expressed between days 3 and 11 and the SHF marker being more highly expressed after day 13 (**Figure 3C**).

Immunofluorescence staining revealed the presence of markers of various cell-type lineages that make up the human heart. Myocardial tissue (identifiable using the cardiomyocyte-specific marker TNNT2) adjacent to epicardial tissue (marked by the nuclear transcription factor WT1 and the epithelial membrane marker TJP1) (**Figure 4A**). Endocardial cells expressing NFATC1 were detected lining the walls of internal chamber-like structures within the organoids (**Figure 4B**). Endothelial cells in a vessel-like network can be seen as early as day 13 of differentiation (**Figure 4C**). Lastly, we report the presence of cardiac fibroblasts intermixed throughout the organoid (**Figure 4D**). These cell-type markers were also observed in the RNA-Seq gene expression profiles (**Figure 4E**). The composition of cell types in the organoids, as measured by area of the organoid they occupy, were found to be ~58% cardiomyocytes, with the rest comprising of non-myocyte cardiac cells, including epicardial cells (~15%), endocardial cells (~13%), cardiac fibroblasts (~12%), and endothelial cells (~1%) (**Figure 4F**).

The electrophysiological function of the organoids was measured by live calcium imaging of individual cells in whole organoids. Fluo-4 fluorescence intensity varies over time due to calcium entry and exit from the cell, revealing regular action potentials (**Figure 5A**). Heatmaps showing calcium intensities over a high-magnification region of the organoid show the increased intensity because of calcium transients in individual cells (**Figure 5B** and **Video 3**).

FIGURE AND TABLE LEGENDS:

Figure 1: Embryoid body generation and heart organoid differentiation steps. (**A**) (**1–2**) Dissociated cells are seeded into wells of a 96-well ultra-low attachment plate via a multichannel pipette. (**3**) The 96-well plate is then centrifuged, which allows the cells to aggregate in the center. (**4**) Over time, following the addition of growth factors and pathway modulators, the embryoid body begins to differentiate into several cardiac lineages and form spatially and physiologically relevant distinct cell populations surrounding internal microchambers. (**B**) Representative images of the progression of embryoid body generation, beginning with 2-dimensional iPSC culture (left) and ending with a Day 0 embryoid body (right); scale bar = 500 µm. (**C**) Summary of human heart organoid differentiation protocol, including chemical pathway modulators and inhibitors with respective time points, durations, and developing organoid images under light microscopy from day 1 to day 15; scale bar = 500 µm.

Figure 2: Whole organoid mounting on slides for imaging. Steps for preparing slides and mounting organoids for imaging. (**A**) Placement of microbeads onto the periphery of a glass slide. (**B**) Covering the microbeads with mounting medium. (**C**) Transferring organoids onto the slide between the beads and removing excess liquid surrounding the organoids. (**D**) Covering the organoids with clearing/mounting medium. (**E**) Placing the coverslip on top of the slide with organoids and beads.

Figure 3: First heart field and second heart field specification in hHOs recapitulates physiological human heart development. (**A**) Confocal immunofluorescence images of day 3 to day 11 hHOs showing formation of FHF (HAND1, top) and SHF (HAND2, bottom), and cardiomyocytes (TNNT2) and nuclear dye DAPI; scale bars = 500 µm. (**B**) High-magnification images of day 7 organoids showing co-localization of HAND1 and HAND2 with the cardiomyocyte marker TNNT2; scale bars = 50 µm. (**C**) RNA-Seq gene expression profiles of FHF marker HAND1 (red) and SHF marker HAND2 (blue) from day 0 to day 19. Abbreviations: hHOs = human heart organoids; FHF = first heart field; SHF = second heart field; HAND = heart and neural crest derivatives expressed; TNNT2 = cardiac troponin T2; DAPI = 4',6-diamidino-2-phenylindole.

Figure 4: hHOs develop multiple cardiac lineages. (**A–D**) Confocal immunofluorescence images of day 15 hHOs showing formation of cardiomyocytes (TNNT2) and staining with nuclear dye DAPI in non-myocyte cardiac cells. (**A**) Whole organoid and high magnification of epicardial marker WT1 (green) and epithelial membrane marker TJP1 (white) showing epicardial cells of epithelial origin on top and adjacent to myocardial tissue; scale bar = 500 µm, inset = 50 µm. (**B**) Endocardial marker NFATC1 (green) expression on the lining of chambers; scale bar = 500 µm. (**C**) Endothelial vessel network in hHOs shown by PECAM1 (green). Scale bar = 500 µm. (**D**) Cardiac fibroblasts markers THY1 and VIM shown in green and white, respectively, distributed throughout the organoid. Scale bar = 500 µm. (**E**) RNA-Seq gene expression profiles of the major cell types present in the hHOs from days 0 to 19 of differentiation. Abbreviations: hHOs = human heart organoids; TNNT2 = cardiac troponin T2; DAPI = 4',6-diamidino-2-phenylindole; WT1 = Wilm's tumor-1 transcription factor; NFATC1 = cytoplasmic nuclear factor of activated T cell; PECAM1 = platelet endothelial cell adhesion molecule-1; VIM = vimentin. (**F**) Pie chart of average tissue type

composition in hHOs, calculated as the percentage area with the respective cell marker over an entire organoid by nuclear staining across three z-planes throughout the organoid using ImageJ.

Figure 5: Fluo-4 live calcium transient recordings in live human heart organoids. (**A**) Representative calcium transient recordings of individual cardiomyocytes within whole organoids. (**B**) Heatmap showing low and peak calcium levels between action potentials as determined by Fluo-4 intensity; scale bars = 10 µm.

Video 1: Live imaging of representative organoid derived from hPSCs at day 6 of differentiation under light microscopy at room temperature. Abbreviation: hPSC = human pluripotent stem cell.

Video 2: Live imaging of representative organoid derived from hPSCs at day 15 of differentiation under light microscopy at room temperature. Abbreviation: hPSC = human pluripotent stem cell.

Video 3: Live recording of day 10 organoid showing heatmap of calcium transients under a fluorescence microscope.

DISCUSSION:

Recent advances in human stem cell-derived cardiomyocytes and other cells of cardiac origin have been used to model human heart development[22,24,25] and disease[26–28] and as tools to screen therapeutics[29,30] and toxic agents[31,32]. Here, we report an easy-to-implement, highly reproducible protocol to generate and differentiate EBs into highly complex hHOs. This protocol has been successful in multiple cell lines, including hPSCs and hESCs[23], showing consistent beating frequencies and cell type organization. This protocol draws aspects from previously described protocols for cardiomyocyte differentiation[24], epicardial cell differentiation[22], and precardiac organoids derived from mouse ESCs[16] and optimizes the stepwise modulation of canonical WNT signaling using chemical inhibitors and growth factors in a fully defined medium. Several optimization methodologies were employed in the generation of this protocol.

First, the chemical inhibitor concentrations and exposure durations, as well as the addition of growth factors, have been optimized for the 3D environment and are discussed in previous work[23]. These were optimized to elucidate structures with physiological complexity and representation of the *in vivo* human heart, with physiological composition and ratios of cardiomyocytes to non-myocyte cardiac cell types (epicardial cells, cardiac fibroblasts). Second, the two-thirds medium change strategy allows minimal agitation of the EBs/organoids, as they sit in suspension near the bottom of the well, while also facilitating a gradient exposure to chemical inhibitors and growth factors when the medium is refreshed. The combination of cardiac mesoderm differentiation through Wnt pathway activation, followed by inhibition[24], and the subsequent induction of proepicardial specification via a second Wnt pathway activation[22], allows for a single protocol to yield highly complex hHOs. The organoids grow up to 1 mm after 15 days of differentiation and can be easily transferred for live or fixed analyses and assays. Third, given the large size of the organoids, the use of microbeads or other similar structures to maintain

space between the slide and coverslip was found to better preserve the 3D structure of the organoids and improve the imaging process.

This developing human heart model allows access to otherwise inaccessible stages of heart development, such as early first and second heart field specification—observed between days 3 and 9 of differentiation—and organization into cardiac progenitor cells that give rise to heart tissues, including the myocardium, endocardium, epicardium, endothelial vasculature, and supporting cardiac fibroblasts, which were observed on day 15 of differentiation. The tissue types present in the heart organoids derived from this protocol are highly representative of the human fetal heart in both composition[33] and transcriptomic profile[23,34]. They can therefore facilitate tissue-tissue and cell-cell higher-order interactions resembling that of the *in vivo* heart. This protocol was highly efficient and reproducible across experiments and cell lines, yielding organoids that comprise mostly of cardiomyocytes and include non-myocyte cardiac cells, such as epicardial cells, endocardial cells, cardiac fibroblasts, and endothelial cells, representing the physiological composition[23,33,35].

Analyses of the ultrastructure of the forming cardiomyocytes via transmission electron microscopy and the development of chambers and a vascular network via optical coherence tomography and confocal imaging are discussed in detail in previous work[23]. A great advantage of this heart organoid protocol over other existing protocols recently published[17–20,36] is the robust formation of an endothelial network throughout the organoid, allowing the ability to investigate vascular development and disease in the early human heart, without the need for further external inductions to the protocol. Lastly, functional analysis of the heart organoids is achievable through various approaches, including the use of a calcium-sensitive dye to track the calcium transients in the cardiomyocytes across the organoid. Using high-resolution microscopy, we recorded the fluorescence intensity of calcium entering and exiting cells and observed highly representative action potentials. Other possible functional analyses methods include the use of a transgenic line with a calcium-sensitive indicator or direct recording using a microelectrode array[23].

The heart organoids described here are recapitulative of the developing human fetal heart, yet are limited in demonstrating more mature, adult-like features. Future protocols may build on the protocol described here to induce maturation in these organoids and yield constructs that better model the adult heart. Moreover, this protocol is designed to create miniature models of the human heart and is limited to research studies of heart development and disease or for pharmaceuticals screening and may not be suitable as a means of clinical intervention such as replacement of heart tissue via transplantation. Overall, we describe here an easy-to-follow and cost-effective protocol to generate highly reproducible and sophisticated human heart organoids that can facilitate research studies in human heart development, disease etiology, and pharmacological screening.

ACKNOWLEDGMENTS:
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DISCLOSURES:

The authors have no conflicts of interest to declare.

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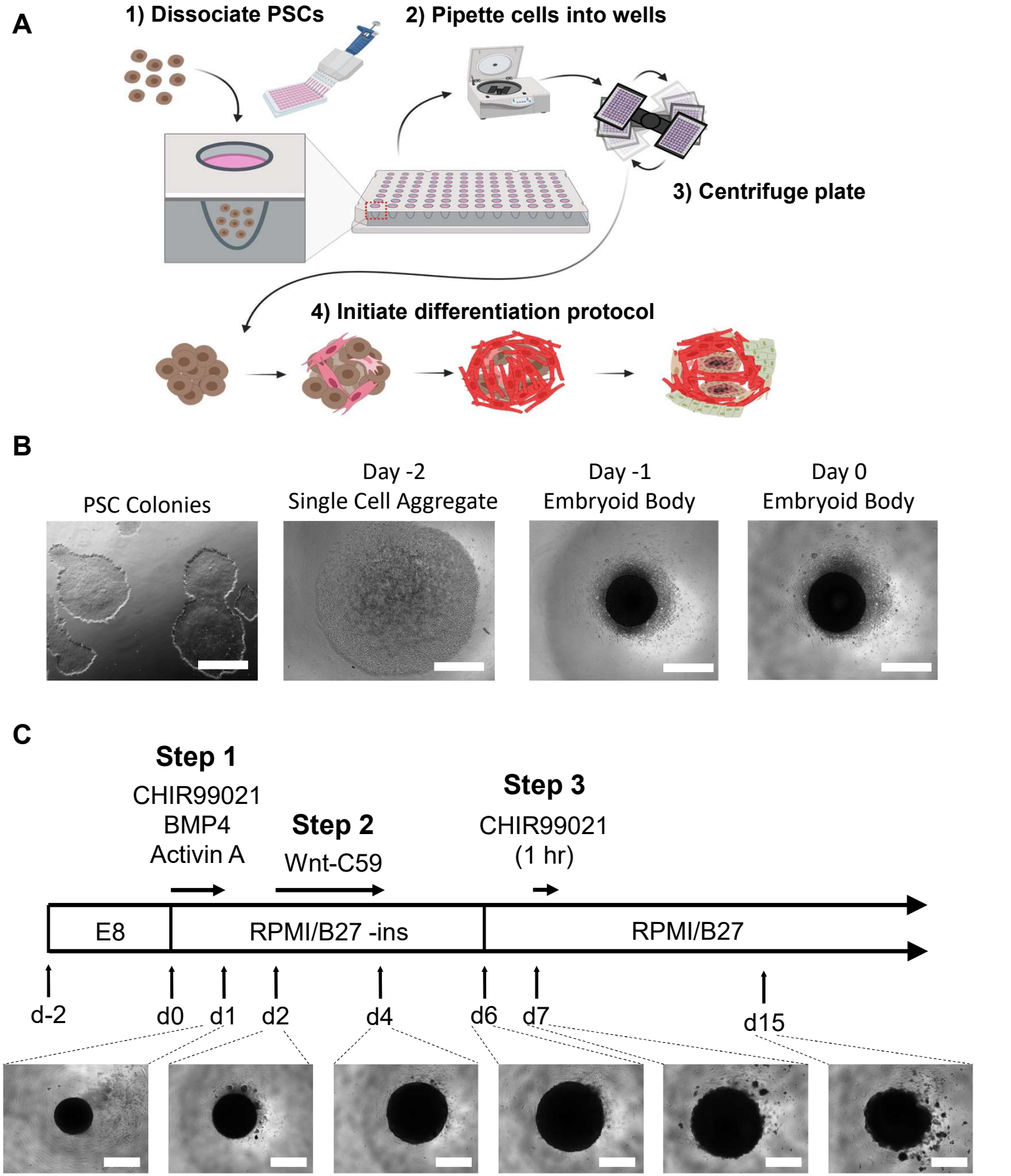
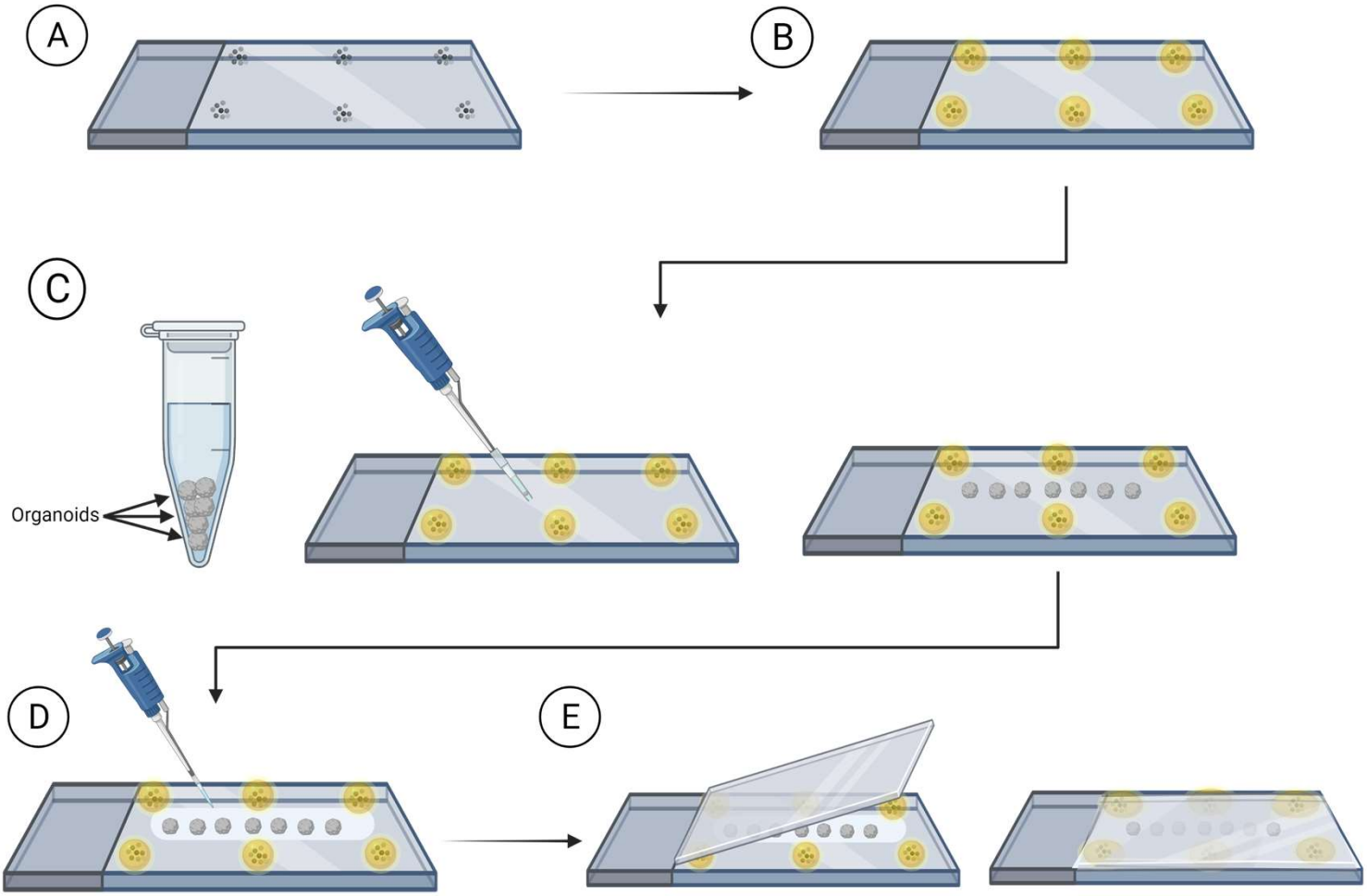


Figure 2



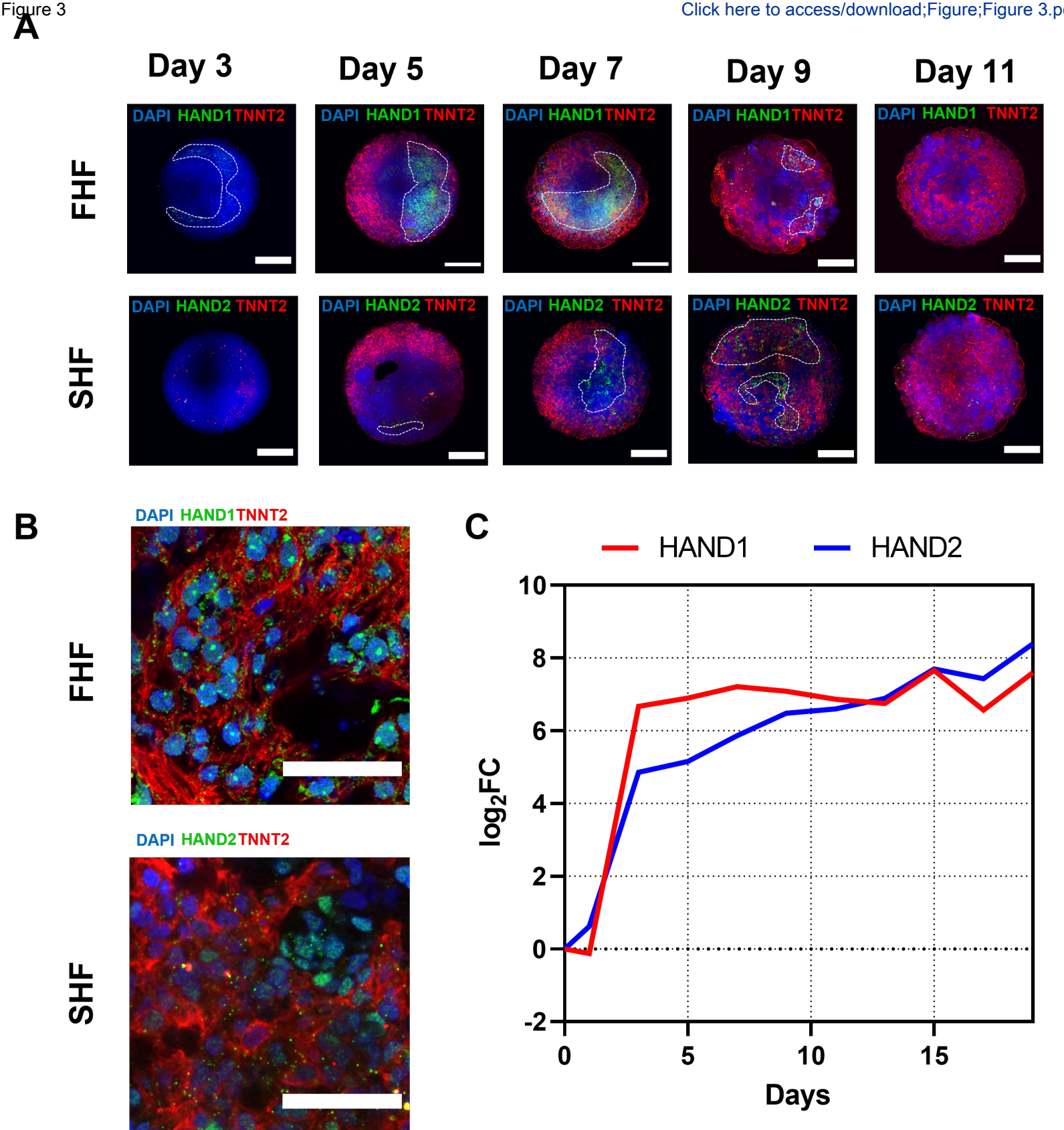


Figure 4

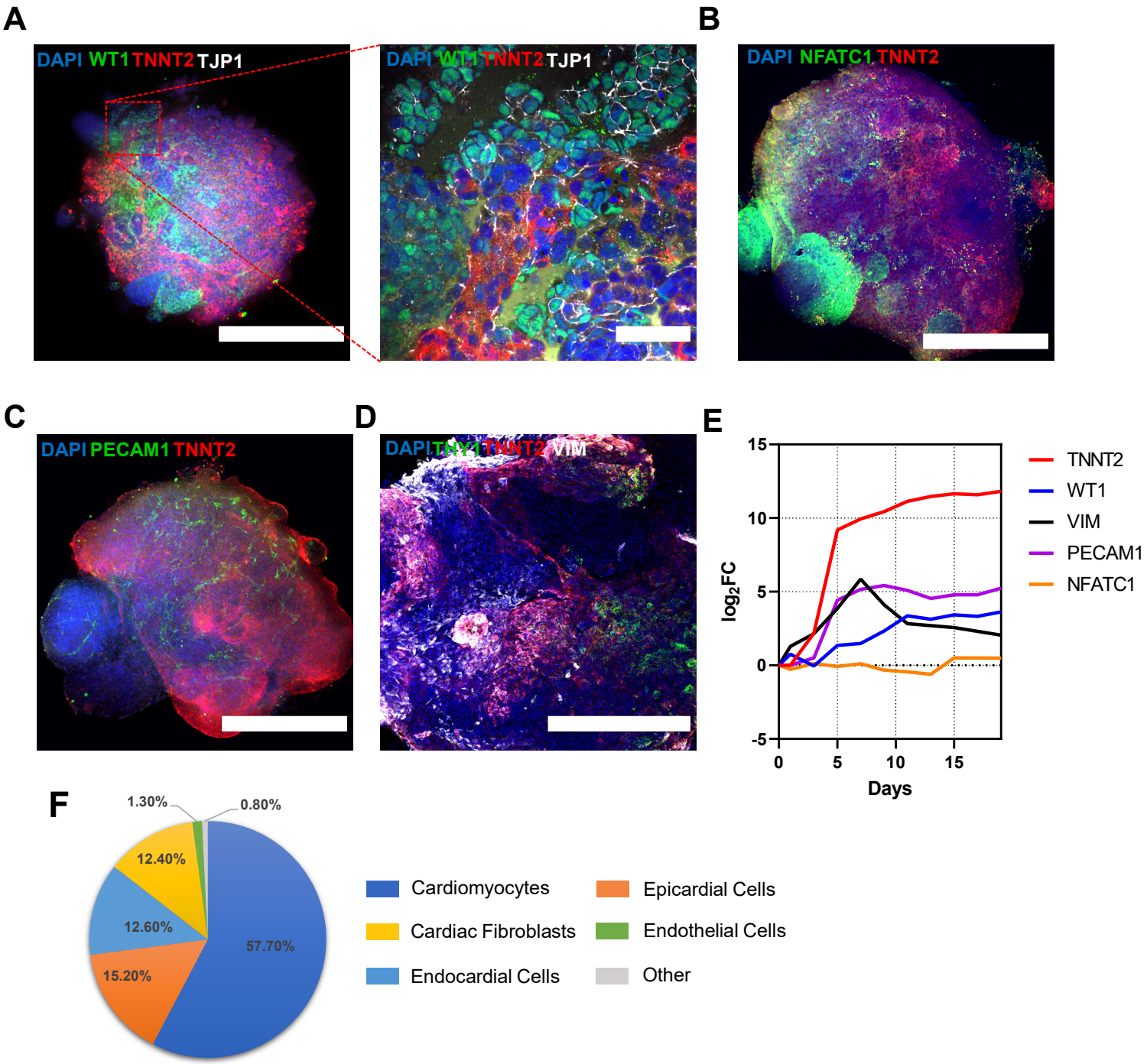
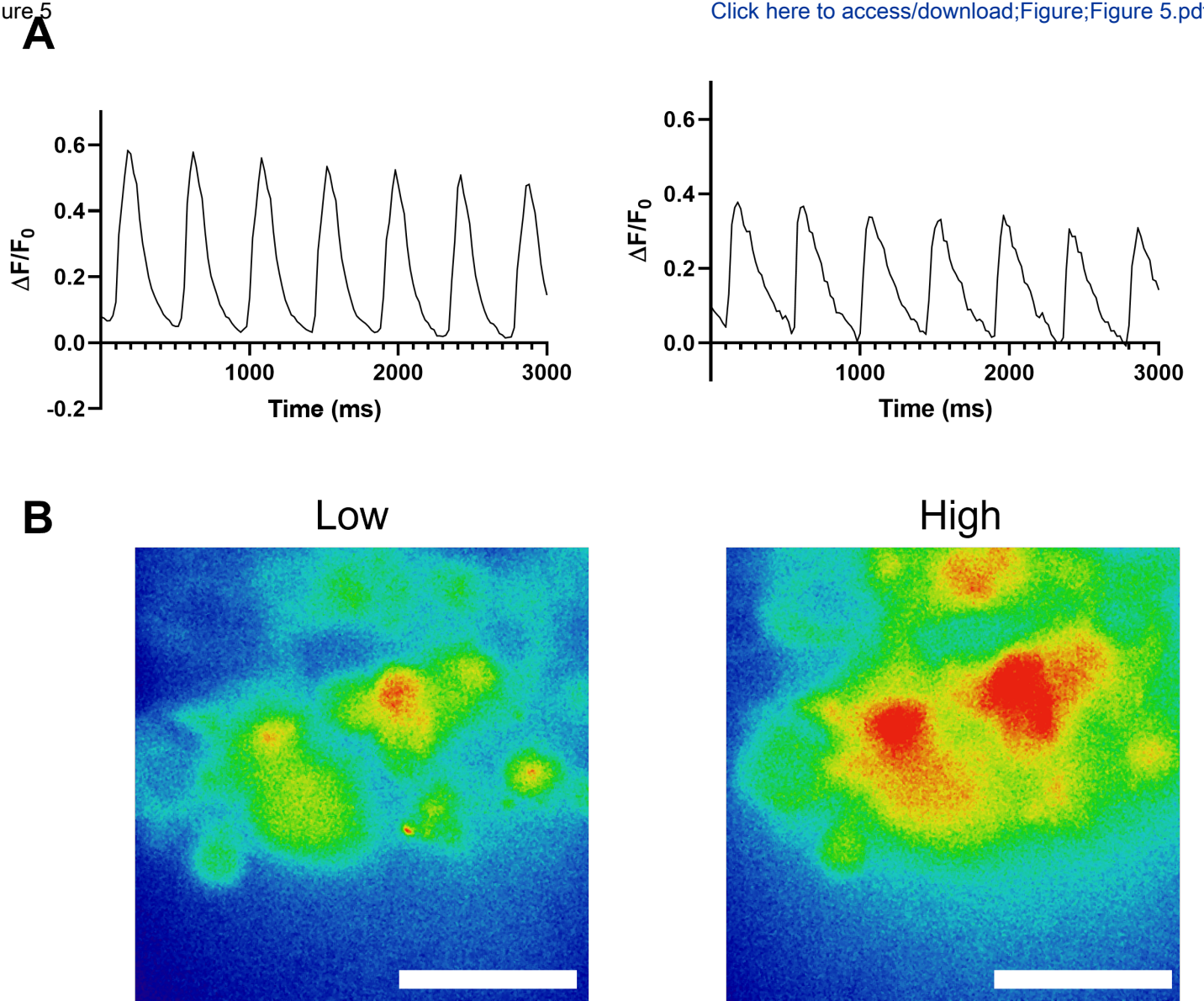
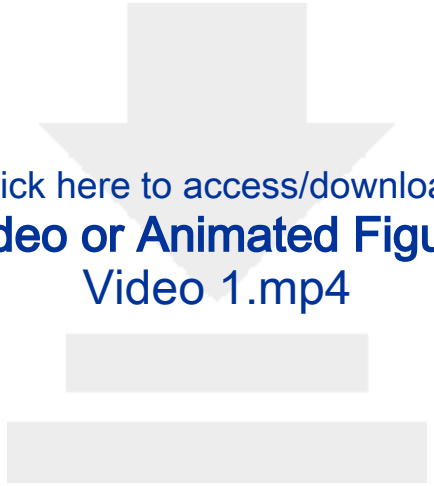


Figure 5



video 1



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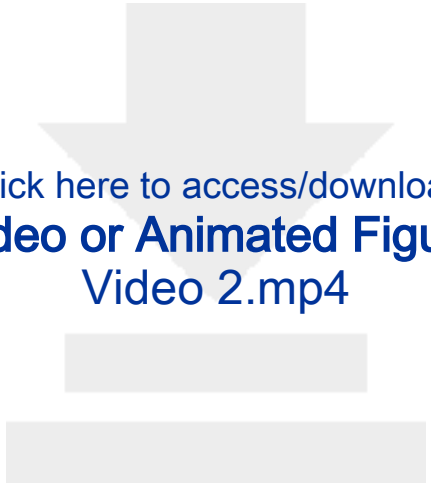
Video or Animated Figure

Video 1.mp4

video 2



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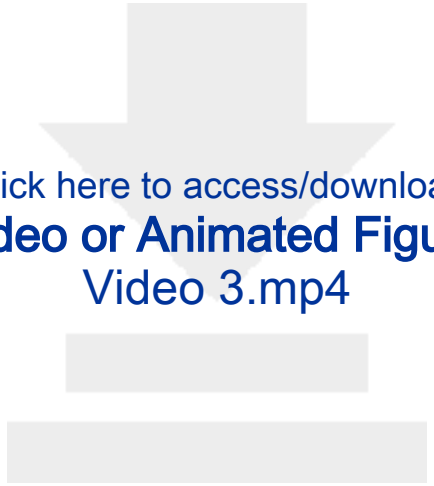
Video 2.mp4

video 3



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Video or Animated Figure

Video 3.mp4

Rebuttal Letter

We want to thank the editors and reviewers for their time and constructive criticism. A detailed point by point rebuttal follows.
Note: Original reviewers' comments have been highlighted in blue for clarity.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have proofread the manuscript and ensured all abbreviations are defined at first use.

2. Please provide an email address for each author.

Here are the email addresses for all authors:

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3. Please add a Summary (before the abstract) to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

We included a summary before the abstract.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript (this includes the figures and the legends) and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Matrigel; ReLeSR; Accutase; Eppendorf; Vectashield Vibrance etc

We have revised the text to comply with this comment.

5. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed all personal pronouns from the protocol.

6. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. 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. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

We have optimized the text to cover all the desired points for the video while also explaining the “how” questions.

7. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points and one-inch margins on all the side. Please include a ONE LINE SPACE between each protocol step and then HIGHLIGHT up to 3 pages of protocol text for inclusion in the protocol section of the video.

The text has been formatted according to the comment and up to 3 pages of the protocol have been highlighted.

8. 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 modifications and troubleshooting of the technique

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

The Discussion section has been revised to cover and include the aforementioned points.

9. Please use “h” for hour(s) (text and images).

We have modified the text so only “h” is used for hours.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ITALICS). Volume (BOLD) (Issue), FirstPage–LastPage (YEAR).] For 6 and more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate the journal names. Make sure all references have page numbers or if early online publication, include doi.

All the references now comply with this format.

11. Please add all items (tubes, solvents, equipment, software etc) in the Table of Materials so that it serves as a handy reference for users to get everything ready for the protocol. Please use only English names of the materials used in the table and elsewhere. Please sort the Materials Table alphabetically by the name of the material.

The materials table has been updated as requested.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This study describe a novel protocol to generate highly complex human heart organoids (hHOs) by self-organization using human pluripotent stem cells, which constitutes a solid platform for in vitro studies in human organ-like cardiac tissues. Although this research is of certain value, the article consequently fails to reach the qualification for publication and further modification is needed.

We thank the reviewer for their comment and constructive criticism. We have addressed all the comments below in detail to improve the manuscript.

Major Concerns:

1. The expression of the whole article needs to be revised and improved in detail, and the writing of the full text is not rigorous enough. For example, many units of numerical values and operation details are not written completely.

We thank the reviewer for this comment. We have revised the manuscript in detail and in accordance to all comments by the reviewers and editors to improve detail and rigor (please see track changes in the document).

2.There are many debatable points in the experimental method. For example, in this paper, PBS was used to wash the cells, but for stem cell differentiation, PBS should not be used, but DPBS should be used to wash the cells. Besides, E8 medium should not be preheated at 37℃ routinely. And why is there no washing link in the process of EB induced differentiation before changing the differentiation solution every day?

We thank the reviewer for this comment and have clarified our meaning to these points in the text. DPBS without calcium or magnesium is used for routine passaging as described many times before, as the lack of these cadherin cofactors softens cell-cell adhesions. For other types of wash, where cell dissociation is not sought, we use the corresponding basal medium (DMEM, RPMI, etc or DPBS with calcium and magnesium). We have addressed this in the manuscript, so it is better clarified. Regarding E8, it is true that it should not be pre-warm due to the labile nature of FGF present in its composition at 37 C. However, the formulation of E8 we used is more advanced (E8 Flex), and uses thermostable FGF in its composition. Thus, warming cycles are not particularly deleterious in this case. Regardless of that, we always prepare an aliquot of media (E8 or RPMI depending on the culture) with the amount needed and warm it in a 37℃ bath to approximately room temp, not 37 C. The stock media bottles are not warmed to 37℃ and therefore the media is not routinely warmed, just when it is about to be used. We have updated the text to better reflect all of these procedures. Regarding no washing of the EBs during differentiation, this is a key step of our new protocol (Lewis-Israeli et al, 2021, Nat Comms; Israeli et al, bioRXiv, 2020; Lewis-Israeli et al, 2021, Research Square). The EBs and subsequent organoids are in suspension in a low attachment round bottom well, and are quite delicate in nature, for this reason we minimize the required media changes, and found that washes between media changes are unnecessary to yield successful and reproducible heart organoids. We have updated the text to better clarify this.

3.What is the principle of adding CHIR99021 on the 7th day of induction? According to our experience, after the formation of cardiac progenitor cells, CHIR99021 will cause the massive cell death and affect the differentiation and maturation of normal cardiomyocytes. I wonder if you have observed the same phenomenon in the operation of your induction scheme?

We thank the reviewer for this comment and have better explained the reasoning in the text. Briefly, the purpose of adding CHIR99021 on the 7th day of differentiation is to induce proepicardial organ formation (more details on the protocol can be found at Lewis-Israeli et al., Nat Comms 2021). This step is based on a 2017 Nature Protocols paper (Bao et. al., 2017) based on a 2016 study (Bao 2016), found that a second CHIR99021 exposure around days 7-9 directs differentiation towards epicardial cells in 2D monolayer culture. We have adapted this protocol and optimized it to induce partial epicardial differentiation in our organoids.

4.Why is the beat frequency of hHOs in the video 2 on the 10th day of induction significantly lower than that on the 6th day of induction in the video 1? In the calcium transient detection, the hHOs with strong pulsation and fast frequency are detected by the organoids induced for how many days? If it is the calcium transient result of organoids on the 10th day of differentiation, why is it so different from the pulsation in Video 2? Please explain the above phenomena in detail.

We thank the reviewer for this question. Videos of the organoids were taken at room temperature and are therefore not an accurate indication of the beating frequency, which can vary due to the room conditions (temperature and lack of an incubator chamer), but are presented to provide a brightfield microscopy indication of the robustness of the beating. The calcium transients recordings were taken at optimal temperature and CO2 levels using an incubation chamber, and are therefore accurate representation of the beating frequencies. We have clarified this in the text.

5. The priority is not prominent in discussion part. The analysis of discussion section should be closely integrated with the results of present experiment, not only focus on describing the function of each detection index. Deeply analysis and discussion combined with the actual research data are absent to support author's point.

We have updated and revised the discussion to better reflect the presented results.

6.It is suggested that the ultrastructural development of hHOs should be detected by transmission electron microscope. Meanwhile, the induction method in this paper should be compared with the recently published hHOs to reflect the advanced differentiation method in this paper. For example, compare and discuss with the article entitled "Cardioids Reveal Self-organizing Principles of Human Cardiogenesis" published in the journal of Cell.

We have referenced our previous publication for TEM data on ultrastructure of organoids (Lewis-Israeli et al, 2021, Nat Comms; Israeli et al, bioRXiv, 2020; Lewis-Israeli et al, 2021, Research Square) and have discussed the advantages of this protocol over previously describe heart organoid protocols including Hofbauer 2021;Drakhlis 2021;Rossi 2020;Lee 2020;Richards 2020.

Reviewer #2:

Manuscript summary:

The authors describe a detailed protocol to generate human heart organoids (hHOs) by self-organization using human pluripotent stem cells. They also use small molecule inhibitors to activate developmental pathway along the process.

1. This protocol is highly efficient as the authors say, but they didn't discuss the efficiency of the induction.

We thank the reviewer for raising this point, and have addressed this in the text to clarify the efficiency of the induction.

2. WNT pathway activator is CHIR99021, but the authors mistakenly wrote as CHIR99201 in some places (Line 311), please check it. The authors modified the concentrations and exposure durations of the CHIR99021. What evidences support for the author to choose the current concentration and exposure durations of CHIR99021? Is it more efficient than using the conventional concentration?

We thank the reviewer for spotting this mistake and have corrected it. The concentrations and exposure durations have been optimized and are discussed in our previous work (Lewis-Israeli et al, 2021, Nat Comms; Israeli et al, bioRXiv, 2020; Lewis-Israeli et al, 2021, Research Square). We have referenced to this in the text.

3. Positive control and negative control are missing in RNA-Seq, and I want to know why there is no obvious change in the expression level of NFATC1 in Figure 4.

We thank the reviewer for this comment. The RNA-seq data presented corresponds to a time course experiment on the heart organoid gene expression, as such there are no negative and positive controls (d0 could be considered a negative control in the sense that iPSCs do not express cardiac genes, for example). A much more detailed analysis of our RNA-seq data can be found in our previous work in this respect (Lewis-Israeli et al, 2021, Nat Comms; Israeli et al, bioRXiv, 2020; Lewis-Israeli et al, 2021, Research Square), with a positive control on age-matched fetal tissues. Since the purpose of this manuscript is to detail and clarify the protocol and methodology, and the data presented is just representative of the results expected, we have stayed out of discussing experimental results found in our other work in greater detail (if the editors think more data in this respect is necessary, we will be happy to add it).

The presence of the endocardial marker NFATC1 is clear from the confocal image provided here and in our previous work (Lewis-Israeli et. al., 2020), and is demonstrated in a similar manner in other heart organoid publications (Drakhalis et al., 2021). Since direct detection of the protein overrides the more indirect RNA measures, we believe this claim is sufficiently justified.