Protocol for human Engineered Cardiac Tissue (hECT) Fabrication

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1. Introduction

We provide step-by-step instructions on how to create hECTs with our multi-hECT bioreactor platform using human induced pluripotent stem cell derived cardiomyocytes (hiPSC-CMs), and subsequently measure their contractile function. This protocol was published in: Methods Mol Biol 2018;1816:145-159. doi: 10.1007/978-1-4939-8597-5_11.

2. Materials

2.1. Cell Collection

- hiPSCs.
- mTeSR™ 1.
- 6 well tissue culture treated plate.
- +I media: RPMI 1640 + B-27 Supplement (50X) + 1% penicillin streptomycin.
- -I media: RPMI 1640 + B-27 Supplement Minus Insulin (50X) + 1% penicillin streptomycin.
- 0.025% trypsin.
- DMEM/F-12 Media, 1:1 Nutrient Mixture.
- CHIR99021 (30 mM stock solution).
- IWR-1 (10 mM stock solution).
- 1X phosphate-buffered saline (PBS, pH 7.4), sterile-filtered.
- 15 mL conical tubes.
- 1.5 mL Eppendorf tubes.

2.2. Tissue Formation

- 5 mg/ml type-I collagen.
- 1 M NaOH.
- 10X PBS.
- Sterile ultrapure deionized water.

- 10X Minimum Essential Medium (MEM).
- 0.2 N HEPES pH 9.
- Stem cell-qualified Matrigel.
- Petri dishes 60mm (60x15mm style) and 100mm (100mmx20mm style).

2.3. Bioreactor Construction

- CAD files with bioreactor design schematics are available upon specific request.
- Polysulfone.
- Polydimethylsiloxane.
- Black Teflon.
- Alcohol resistant black marker.
- 2% Bovine Serum Albumin (BSA).
- Vacuum grease.
- Tweezers.

2.4. Data Acquisition

- Laptop.
- GRASS S88× stimulator (Astro-Med, West Warwick, RI).
- High-speed camera.
- Dissecting microscope.
- Carbon plates (for multi-tissue data acquisition only).
- Tungsten wire.
- Plate heater.
- Boom microscope stand.

- Goose neck lamp.
- Vibration isolation table.
- Laminar flow hood.
- Mirror (20mm enhanced aluminum coated, right angle mirror, #45-594Edmund Optics).
- Laboratory Jack x2.
- LabVIEW (National Instruments, Austin, TX) and MATLAB (Natick, Massachusetts) software. LabVIEW program used for data acquisition, and MATLAB script used for data analysis, are available upon specific request.

3. Methods

3.1. Collecting Cells for hECTs

3.1.1Cardiomyocyte differentiation of hiPSCs

a. To start the cardiomyocyte differentiation at 80-90% confluency (confluency greatly affects differentiation efficiency) of hiPSCs in a 6-well plate, replace mTeSR™ 1 maintenance media (E8 media can also be used for maintenance) with 2 mL +I media containing CHIR99021 (10 μM final concentration) per well.
b. After 24 hours, wash with DMEM/F12 and replace with 2 mL -I media per well.
c. After 48 hours, wash with DMEM/F12 and replace with 2 mL -I media containing IWR-1 (5 μM final concentration) per well.
d. After 24 hours, wash with DMEM/F12 and replace with 2 mL -I media containing IWR-1 (5 μM final concentration) per well.

e. After 24 hours, wash with DMEM/F12 and replace with 2 mL -I media per well.
f. Repeat previous step.
g. After 24 hours, wash with DMEM/F12 and replace with 2 mL +I media per well.
h. Repeat previous step up to days 20-30 of differentiation. Beyond day 7 of differentiation, changing media every 48 hours (with 4 mL per well), rather than every 24 hours (with 2 mL per well), is also a viable option. After day 10 of differentiation, it is not necessary to wash with DMEM:F12.

3.1.2 Collecting hiPSC-CMs from the monolayer

i. On days 20-30 of differentiation, wash with 1X PBS. In our experience an effective time window to harvest the cells for hECT fabrication is 20-30 days of differentiation.
j. Add 1 mL 0.025% trypsin per well. TrypLE Express can also be used for dissociation.
k. Incubate for 5 minutes at 37^{o}C and 5% CO_2.
l. Remove cells from each well mechanically using the 0.025% trypsin from each well.
m. Place trypsin/cell mix into 15 mL conical tube, and neutralize with equal amount of cold +I media.
n. Centrifuge at 300xg for 5 min at 4^{o}C.

o. Aspirate and re-suspend with 10 mL +I media. Amount of +I used to re-suspend should be adjusted based on cell density.

p. Count cells using hemocytometer.

q. Centrifuge at 300xg for 5 min.

3.2. Preparing a Collagen/Matrigel Mix

1. Store all solutions on ice. Store cells at room temperature. All volumes listed below are per hECT for the single-tissue bioreactor. For the multi-tissue bioreactor, multiply each volume by 0.4-0.6. Using 0.4-0.6 scaling factors have proven success in our hands. Scaling factor is dependent on desired amount of matrigel/collagen mix to be used per tissue.
2. Dilute 27.56 µl of 5 mg/ml collagen stock to 3.125 mg/ml with 14.5 µl of sterile ultrapure deionized water, 0.68 µl of 1 M NaOH, and 4.75 µl of 10x PBS per hECT. Avoid bubbles.
3. Add 5.94 µl of 10x MEM and also 5.94 µl 0.2 N HEPES pH 9 to the dilute collagen mixture (previous step) to create the collagen mix.
4. Add 4.75 µl of stem cell-qualified Matrigel to the collagen mix (0.9 mg/ml final concentration)
5. Store the collagen/Matrigel mix on ice.

3.3. Preparing the Multi-Tissue Bioreactor

1. Mark the top of master mold posts with alcohol resistant black marker.
2. Sterilize. The PDMS molds can withstand sterilization cycles in steam autoclave up to 121^{o}C.
3. Insert master molds into polysulfone frame.

3.4. Forming hECTs Using the Multi-Tissue Bioreactor

1. Resume from step 3.1.2 (q).
2. Aspirate supernatant from hiPSC-CM pellet.
3. Add (40 μL of collagen/Matrigel mix per 1 million cells) to hiPSC-CM pellet.
4. Place baseplate in 60 mm tissue-culture treated dish.
5. Add 40 μL hiPSC-CMs + collagen/Matrigel mix into each well of the baseplate. To help avoid bubbles, add 10% more of the cell/extracellular mix into the pipette.
6. Insert bioreactor into baseplate. Confirm that bioreactor posts are submerged into the wells, but are not bent from touching the bottom of the well.
7. Discard top lid of 60 mm dish; place 60 mm dish into 100 mm tissue culture treated dish. Be sure that if you have relevant information on the lid, transcribe that information to the lid of the 100mm dish. It also helps to label the bottom of the dish to keep record of the bioreactor inside the dish.
8. Add 1 mL of +I media into edge of 60 mm dish.
9. Place into incubator for 2 hours at 37^{o}C and 5% CO_2.
10. Remove from incubator and bring to laminar flow hood.
11. Slowly add 14 mL +I media into 60 mm dish. To improve compaction, add DMEM containing 10% Neonatal Bovine Serum, 1% penicillin-streptomycin. May need to add more +I media to confirm that all hECTs are fully submerged into media.
12. Place into incubator for 48 hours at 37^{o}C and 5% CO_2. Check on compaction; it may be necessary to remove bioreactor ± 24 hours based on hECT compaction within bioreactor.

13. Remove from incubator; while holding the baseplate at the bottom of the 60 mm dish, slowly lift bioreactor out of baseplate, and place bioreactor into new 60 mm dish with 14 mL +I media.

3.5. Data Acquisition for Multi-hECT Bioreactors

1. Set up laminar hood with vibration isolation table, goose neck lamp, boom stand, dissecting microscope and high speed camera. For multi-hECT include the laboratory jack, place bottle caps equally distant with mirror in the center, place the 100mm dish containing the 60mm dish with the multi-tissue bioreactor carefully on top of the bottle caps, and align the microscope to view the reflection of the multi-tissue bioreactor on the mirror. The multi-tissue bioreactor is maintained in culture and tested with the hECT facing the bottom of the dish; the reflecting image on the mirror allows visualizing the tissue without requiring any manipulation. Connect grass stimulator and camera to laptop.
2. Adjust microscope magnification and limit region of interest to have both posts of one hECT in view.
3. Using a custom LabVIEW program (available upon specific request), record the displacement of the posts without electrical stimulation to analyze the spontaneous contractile properties of the hECT.
4. Place carbon plates adjacent to the single-tissue or the multi-tissue bioreactor respectively and connect them to the grass stimulator electrodes. To aid in connecting the carbon plates to the grass stimulator electrodes, the electrodes can be fitted with alligator clips soldered to the end of the electrodes; while

tungsten wire can be looped tightly around the carbon rods/plates; lastly, clasp the tungsten wire with the alligator clips.

5. Using a custom LabVIEW program, record the displacement of the posts with electrical stimulation The protocol to follow for electrical stimulation should be determined per the experiment. We typically pace the hECT starting at low frequency (0.25Hz), and then record hECT contractions (post displacement) at different frequencies using 0.25Hz increments).
6. Measure the post heights and tissue height on the posts by acquiring a side view image of the hECT. The beam bending equation $F = \frac{3\pi E R^4}{2a^2(3L-a)}\delta$ is used to calculate the force (F). Post length (L) and tissue height along the posts (a) are measured using a side view image of the hECT; the post displacement (δ) is measured during data acquisition using LabVIEW. The radius (R) and Young's modulus (E) are only required to be measured once upon fabrication of a bioreactor, and thereafter the values remain constant. Developed Force (DF) is the difference between the maximum and minimum force. For the multi-tissue bioreactor acquire side view images with the microscope looking directly through the 60mm dish.
7. The data is then processed using a custom MATLAB script (available upon specific request) to produce the results for the different twitch parameters that are analyzed.