**PROTOCOL 1: Generation of matured human cardiac tissues using iPS cells**

**AUTHORS**

Kacey Ronaldson-Bouchard1, Diogo Teles1,2, LouJin Song3, Kumi Morikawa3, Masayuki Yazawa3 and Gordana Vunjak-Novakovic1,4\*

1Department of Biomedical Engineering, Columbia University, New York, NY.

2Life and Health Sciences Research Institute (ICVS), School of Medicine, University of Minho, Braga, Portugal.

3Departments of Rehabilitation and Regenerative Medicine, and Pharmacology, College of Physicians and Surgeons, Columbia University, New York, NY.

4Department of Medicine, Columbia University, New York, NY.

\*Please address correspondence to: Gordana Vunjak-Novakovic, Columbia University, 622 West 168th Street, VC12-234, New York NY 10032, Tel: 212 305 2304; [gv2131@columbia.edu](mailto:gv2131@columbia.edu)

**KEYWORDS**

Human induced pluripotent stem cells; Cardiomyocytes; 3D Tissue; Electrical Stimulation; Maturation; Tissue engineering.

**MATERIALS**

**REAGENTS**

* Aprotinin from bovine lung (Sigma-Aldrich, cat. no. A3428)
* B-27™ Supplement (50X), serum free (Gibco by Life Technologies, cat. no. 17504044)
* Bovine Serum Albumin (Milipore Sigma, cat. no. 820451)
* Collagenase, Type 2 (Worthington, cat. no. LS004176)
* DMEM, high glucose (Gibco by Life Technologies, cat. no. 11965092)
* Fetal Bovine Serum (Atlanta Biologicals, cat. no. S11150)
* Fibrinogen from human plasma (Sigma-Aldrich, cat. no. F3879)
* HBSS, no calcium, no magnesium (Gibco by Life Technologies, cat. no. 14170112)
* HEPES (Corning, cat. no. 25-060-Cl)
* L-Ascorbic acid (Sigma-Aldrich, cat. no. A4544)
* Matrigel® Growth Factor Reduced Basement Membrane Matric (Corning, cat. no. 354230)
* mTeSR™1 (StemCell Technologies, cat. no. 85850)
* PBS (Corning, cat. no. 21-040-CV)
* Penicillin-streptomycin (Gibco by Life Technologies, cat. no. 15070063)
* PLL(20 kDa) grafted with PEG(2 kDa) (PLL-g-PEG) (Surface Solutions, cat. No. PLL(20)-g[3.5]- PEG(2))
* Recombinant Human BMP-4 Protein (R&D Systems, cat. no. 314-BP)
* Recombinant Human VEGF 165 Protein (R&D Systems, cat. no. 293-VE)
* Recombinant Human/Mouse/Rat Activin A Protein (R&D Systems, cat. no. 338-AC)
* RPMI 1640 (Gibco by Life Technologies, cat. no. 11875093)
* Thrombin from human plasma (Sigma-Aldrich, cat. no. T6884)
* Trypsin-EDTA (Gibco by Life Technologies, cat. no. 25200072)
* Tyrodes solution (Sigma, Cat #T2145, 4°C)
* CaCl (Fluka, Cat #2114-1L, RT/20-25°C)

**EQUIPMENT**

* 6 Well clear flat bottom TC-treated cell culture plates (Corning, cat. no. 353046)
* 500 mL Vacuum filter/Storage Bottle System (Corning, cat. no. 431097)
* T75 Cell culture flask (Corning, cat. no. 353136)
* 15 mL Centrifuge tube (Corning, cat. no. 352097)
* 50 mL Centrifuge tube (Corning, cat. no. 352098)
* Cell Culture Dish (Corning, cat. no. 430599)
* Stimulator (Grass, cat. no. S88X)
* Sylgard® 184 Silicone Elastomer Kit (Krayden, cat. no. DC2065622)
* Platinum wire (Ladd Research, cat. no. PW3N5)
* 0.22 µm Steriflip filter (Milipore Sigma, cat. no. SCGP00525)
* Myograph/Organ Bath System (ADInstruments, DMT840MD)
* CS4 Stimulator (ADInstruments, DMT100273)
* Myograph chamber stimulation lids (ADInstruments, DMT100238)
* LabChart Software (ADInstruments, LabChart 7)
* PowerLab 4/35 (ADInstruments, PL3504)

**REAGENT SETUP**

**Activin A** Dissolve at 100-500 µg/mL in sterile 4 mM HCl. Mix the solution thoroughly. Make aliquots and store at -20ºC to -80ºC.

**Aprotinin** Dissolve at 33mg/mL in diH2O. Mix the solution thoroughly. Make 50uL amounts (stock) and store at -20ºC.

**BMP-4** Dissolve at 50-200 µg/mL in sterile 4 mM HCl containing at least 0.1% BSA. Mix the solution thoroughly. Make aliquots and store at -20ºC to -80ºC.

**Cardiac differentiation medium** Cardiac differentiation medium (CDM) contains RPMI 1640, B-27™ supplement, 1% penicillin-streptomycin, 50 µg/mL ascorbic acid. Filter-sterilize the medium by passing it through a 0.22µm filter. Store it at 4ºC for up to 1 week. Pre-warm before every medium change.

**Collagenase solution** Dissolve at 2mg/mL in HBSS. Mix the solution thoroughly. Filter-sterilize the medium by passing it through a 0.22 µm filter and store it at 4ºC. Pre-warm before use.

**Fibrinogen** Dissolve at 33mg/mL in 20mM HEPES buffer in 0.9% saline, over several hours at 37ºC. Filter-sterilize the medium by passing it through a 0.22 µm filter and store it at 4ºC. Make 1mL aliquots (stock), and store them at -20ºC.

**hiPSC medium** hiPSC medium contains mTeSR™1 basal medium, mTeSR™1 5X supplement and 1% penicillin-streptomycin. Filter-sterilize the medium by passing it through a 0.22 µm filter. Store it at 4ºC for up to 1 week. Pre-warm before use.

**L-Ascorbic acid** Dissolve at 106.4 mg/mL in dH2O. Filter-sterilize it by passing it through a 0.22µm filter. Make 1mL aliquots and store it at 4ºC.

**Matrigel® growth factor reduced basement membrane matrix** Thaw matrigel growth factor reduced basement membrane matrix at 4ºC. Make 600 µL aliquots and store them at -20ºC. Avoid thaw/freeze cycles.

**PLL-g-PEG** Dissolve at 1mg/mL in HEPES buffer. Mix the solution thoroughly. Filter-sterilize the medium by passing it through a 0.22 µm filter and store it at 4ºC.

**Thrombin** Dissolve at 25U/mL in 0.1% BSA in PBS. Mix the solution thoroughly. Make 100μL amounts (stock) and store at -80ºC.

**VEGF** Dissolve at 100 µg/mL in sterile PBS containing at least 0.1% BSA. Mix the solution thoroughly. Make aliquots and store at -20ºC to -80ºC.

**Modified Tyrode’s Solution** Mix Tyrode’s solution so that it has the following composition: 129mM NaCl, 5mM KCl, 2mM CaCl2, 1mM MgCl2, 30mM Glucose, 25mM HEPES. Check that the pH is 7.4 and adjust accordingly until achieved. Add B-27 to solution so that it constitutes 2% of the solution. Prepare fresh for each experiment.

**EQUIPMENT SETUP**

**Matrigel coating of cell culture plates** Thaw matrigel growth factor reduced basement membrane matrix at 4ºC. Dilute it 1:60 in cold RPMI 1640. Coat the growth surface of the plates with 1 mL per 9.5 cm2. Incubate the plates at 37ºC for 30 min.

**PLL-g-PEG coating of bioreactor platform** After being autoclaved, remove pillars of the platform andadd 200 µL of PLL-g-PEG to one well of the bioreactor platform, after being autoclaved. Incubate at room temperature for 1 hr. Wash the wells with 200 µL of PBS twice. Put the formed pillars back into the platform.

**PROCEDURE**

**Expansion of human induced pluripotent stem cells**

1. Culture human induced pluripotent stem cells (hiPSC) on matrigel coated wells of a 6-well cell culture plate. They should be on hiPSC medium and the medium should be changed daily (2mL of pre-warmed medium per 9.5 cm2 growth surface).
2. Culture hiPSC in an incubator set to 37ºC, 5% CO2, 21% O2 and 90% humidity.
3. Passage hiPSC at 80% confluent or start cardiac differentiation.

**Cardiac differentiation**

1. At 80% confluence, start cardiac differentiation by changing the medium to CDM freshly supplemented with 50 ng/mL activin A and 25 ng/mL BMP-4. This is D0 of differentiation.
2. On Day 1, change media to CDM freshly supplemented with 10 ng/mL VEGF.
3. On Day 3, change media to CDM with no additional supplements.
4. Change media every 2 days after this point.
5. Beating cells should begin to appear at Day 10.
6. Culture cells in an incubator set to 37ºC, 5% CO2, 21% O2 and 90% humidity through the entire differentiation.
7. On day 12 of differentiation, generate cardiac tissues with hiPSC-derived cardiomyocytes (hiPSC-CM).

**Fibroblast culture**

1. Culture normal human dermal fibroblasts (NHDF) on T75 cell culture flask, in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS).
2. Passage NHDF when 90% confluent.

**Pillars and tissue bioreactor platform formation**

1. The tissue bioreactor platforms are fabricated out of polycarbonate utilizing a computer controlled milling machine. Each platform contains 12 wells for tissue culture and electrical stimulation of the cardiac tissues is provided via carbon rods with platinum wires.
2. Clean the pillars’ support structures, made of polycarbonate utilizing a computer numerical control milling machine, in a 70% ethanol bath followed by a water bath.
3. Prepare polydimethylsiloxane (PDMS) by mixing silicone elastomer base with curing agent, at a 10:1 ratio. Leave in the vacuum chamber for 20 min.
4. Assemble the pillars’ molds made of delrin (polyoxyomethylene), with the pillar’s support structures inside and pour PDMS into them. Leave in the vacuum chamber for 20 min.
5. Centrifuge at 400 RCF for 5 min;
6. Cure in the oven for 1 hr at 60-70ºC;
7. Open the molds, trim and clean the pillars and its support structures and autoclave them with the bioreactor platform.

**Generation of cardiac tissues**

1. Remove fibroblast medium and wash carefully NHDF one time with PBS at room temperature.
2. Aspirate PBS and add 15 mL of 0.25% trypsin-EDTA per 75 cm2 of growth surface.
3. Incubate for 15min in a 37ºC, 5% CO2, 21% O2 and 90% humidity incubator. Monitor microscopically the progress of cell dissociation.
4. Pipette gently and harvest cell suspension into a 50 mL tube with 10 mL of DMEM supplemented with 10% FBS.
5. Flush the cell culture flask with fresh DMEM supplemented with 10% FBS and place it in the same Falcon tube.
6. Centrifuge at 130 RCF for 5 min at room temperature.
7. Discard the supernatant and resuspend the cells in 10 mL of fresh DMEM supplemented with 10% FBS.
8. Do step 25 and 26 one more time.
9. Put cells on ice while dissociating hiPSC-CM.
10. Remove CDM from the wells with hiPSC-CM.
11. Wash carefully hiPSC-CM one time with room temperature PBS.
12. Aspirate PBS and add 1mL of collagenase solution per 9.5 cm2 of growth surface.
13. Incubate for 15 min in a 37ºC, 5% CO2, 21% O2 and 90% humidity incubator. Monitor microscopically the progress of cell dissociation.
14. Gently pipette the collagenase solution and add another mL of fresh collagenase solution.
15. Incubate for another 10 min at 37ºC, 5% CO2, 21% O2 and 90% humidity incubator. Monitor microscopically the progress of cell dissociation.
16. Gently pipette and harvest the dissociated cells to a 50 mL Falcon tube with pre-warmed CDM supplemented with 10% FBS.
17. Flush the cell culture wells with CDM supplemented with 10% FBS and place it in the same Falcon tube.
18. Centrifuge at 130 RCF for 5 min at room temperature.
19. Discard the supernatant and resuspend the cells in 10 mL of CDM supplemented with 10% FBS.
20. Do step 36 and 37 one more time.
21. Put one PLL-g-PEG coated tissue bioreactor platform inside a cell culture dish.
22. Mix hiPSC-CM and NHDF, at a ratio of 75% hiPSC-CM and 25% NHDF, 1 million cells per 100 µL.
23. Centrifuge at 130 RCF for 5min at room temperature.
24. Resuspend the cells in 84uL of fibrinogen per tissue.
25. Add 16μL of thrombin to each well of the bioreactor platform.
26. Add 168μL of the hydrogel to each well.
27. Add 16μL of thrombin to each well.
28. Keep culture plates in a 37ºC, 5% CO2, 21% O2 and 90% humidity incubator and incubate for 30min.
29. Add CDM supplemented with 0.02 mg/mL of aprotinin to the wells.
30. Keep culture plates in a 37ºC, 5% CO2, 21% O2 and 90% humidity incubator and change CDM supplemented with 0.02 mg/mL aprotinin every two days for the first 7 days.
31. On day 7, change to cardiac medium without aprotinin supplementation.
32. If cardiac tissues are attached to the bioreactor wall anywhere, gently detach using a sterile needle.
33. Start electrical stimulation.

**Electrical stimulation of cardiac tissues**

1. Connect one alligator test lead to one platinum wire and another alligator test lead to the other platinum wire of the stimulation platform. Secure the test leads with tape outside the incubator so they do not move when the incubator door is open and closed. Connect both alligator test leads to a stimulator. Make sure the stimulator is turned off when performing this step.
2. Stimulate with trains of 2 ms duration monophasic square pulses, delivered at a frequency of 2 Hz and amplitude of 4.5 V/cm.
3. Increase the frequency daily 0.33 Hz from 2 Hz (Day 0 of stimulation) until 6 Hz on day 12.
4. From day 12 to day 15 of stimulation, keep the stimulation at 6 Hz.
5. At day 15, drop the stimulation frequency to 2 Hz and keep it at 2Hz until day 21 of stimulation.
6. Keep cardiac tissues in a 37ºC, 5% CO2, 21% O2 and 90% humidity incubator and replace 50% of the culture medium every 2 days.

**Organ bath measurements of cardiac tissues**

1. Set up the organ bath:
   1. Fill each chamber of the organ bath with 6 mL of modified Tyrodes solution (129mM NaCl, 5mM KCl, 2mM CaCl2, 1mM MgCl2, 30mM Glucose, 25mM HEPES, pH 7.4) supplemented with 2% B-27.
   2. Turn on computer, powerlab, organ bath and turn heat on (set temperature to 37°C).
   3. Calibrate force transducers (Note: ensure the chambers have stabilized at 37°C before moving to this step).
      1. Open LabChart 8.
      2. Start a new experiment.
      3. On the organ bath, follow the directions to calibrate each chamber using a 2g weight.
      4. On the computer, make sure to also calibrate the corresponding chambers force:
         1. Select the trace containing the force measurement.
         2. On the top right of each channel, select the drop-down menu and select unit calibration.
         3. Select the baseline part of the force trace and set this to “0”.
         4. Select the part of the trace after the 2g weight as added and set this to “9.81”.
         5. Set the unit to “mN”.
   4. Turn on gas supply (95% O2/5% CO2) and open the bubbler (silver knob behind each chamber) so that a slow bubbling of the gas can be seen within each chamber. Let equilibrate for 15 minutes.
2. Load engineered cardiac tissues into organ bath chambers:
   1. Move the holder closer to the force transducer by turning the dial attached to each organ bath chamber.
   2. Place your tissue in the organ bath so it is suspended in the modified Tyrodes solution.
   3. Using forceps, gently move the tissue towards the hooks until the tissue is fully mounted on both hooks (use caution to not touch the force transducer).
   4. Replace the lid and let the tissue equilibrate for 15 minutes.
3. Begin electrical stimulation:
   1. Turn the stimulator on to a frequency of 2 Hz, 5 ms pulse duration, and 80-100 mA and let the tissue equilibrate for 15 minutes.
4. Determine optimal length for maximal force generation (Frank-Starling Response)
   1. Adjust the length of the tissue at stepwise increments to determine the maximal length at which the tissue exerts the maximal force:
      1. Record the initial length of the tissue.
      2. Turn the dial 0.1mM (noting the new length in the experimental notes) and let the tissue force equilibrate for a minimum of 1 minute.
      3. When subsequent increases in length no longer increase the force generated, stop increasing the length and leave the tissue at this maximal length for the remainder of the experiment.
5. Obtain the calcium-induced calcium response (CICR)
   1. Continuously add 0.2 or 0.4 mM increments of CaCl into the organ bath (noting the new concentration in the experimental logbook) until a maximum of 2.8 mM is reached.
6. Obtain the force-frequency response (FFR)
   1. Increase the frequency of stimulation by 1 Hz every 30-60 seconds until 6 Hz is reached
7. Obtain the post-rest potentiation (PRP)
   1. After reaching 6 Hz following the FFR, turn the stimulation off and wait for an additional 10, 30, or 60 seconds.
   2. Turn the stimulation back on to a frequency of 1 Hz (this first beat will detail the post-rest potentiation)
8. Perform any additional measurements (i.e. using drugs such as Isoproterenol)
9. Upon conclusion of the experiment, measure tissue cross-sectional area:
   1. Gently remove tissue from holders.
   2. Using calipers, measure the width and thickness of the tissue in the middle section (length-wise) of the tissue.
   3. Record these measurements for use in determining the cross-sectional area when analyzing the force data.
   4. Calculate the twitch force as an average of the difference between cyclic peak maximum and minimum forces. Calculate stress as a ration of the force and cross-sectional area.

**PROTOCOL 2: Electrophysiological recordings of cardiomyocytes isolated from engineered human cardiac tissues derived from pluripotent stem cells**

Kumi Morikawa1, LouJin Song1, Kacey Ronaldson-Bouchard1, Gordana Vunjak-Novakovic2,3 and Masayuki Yazawa1\*

1 Department of Rehabilitation and Regenerative Medicine, Department of Pharmacology, Columbia Stem Cell Initiative, College of Physicians and Surgeons, Columbia University, New York, NY.

2 Laboratory for Stem Cells and Tissue Engineering, Department of Biomedical Engineering, Columbia University, New York, NY.

3 Department of Medicine, Columbia University, New York, NY.

\*Please address correspondence to:

Masayuki Yazawa, Columbia University, 630 West 168th Street, PS 7-431, New York NY 10032, Tel: 212 305 1052; [my2387@columbia.edu](mailto:my2387@columbia.edu)

**INTRODUCTION**  
Electrophysiological characterization is crucial for validating the function of human cardiomyocytes derived from pluripotent stem cells. To examine the maturity of human cardiomyocytes generated, whole-cell patch clamp recordings for action potentials and *I*K1 current are required. Here we report a summary of electrophysiological recording procedures using human mature cardiomyocytes isolated from engineered cardiac tissues derived from pluripotent stem cells. As observed in rodent primary myocytes, human mature engineered myocytes could be more difficult for whole-cell patch clamp recordings compared to conventional cardiomyocytes spontaneously contracting, which are differentiated from human pluripotent stem cells. Especially, we found that time window to record the electrical activities in such mature myocytes was narrower than ones in human conventional cardiomyocytes spontaneously beating. The whole workflow could be useful to facilitate electrophysiological characterization of human engineered cardiomyocytes in order to unveil the pathophysiological mechanisms underlying cardiac disease and test drug candidates.

**MATERIALS**

**REAGENTS**

1. Cardiac tissue dissociation solution

20 unit/ml papain (Sigma-Aldrich, #76220, from *Caripa papaya*)

1.1mM EDTA (Thermo Scientific, #15575038, 0.5 mM solution stock, pH8.0)

67uM 2-Mercaptoethanol (Sigma-Aldrich, #M3148)

5.5mM L-Cystein HCl (Sigma-Aldrich, #C7880)

1x EBSS (Thermo Scientific, #24010043)

2. Glass-bottom dish coating solution

Geltrex (100x stock, Thermo Scientific, #A1413302)

in DMEM/F12-GlutaMax I (Thermo Scientific, #10565042)

3. Cardiomyocyte culture media

DMEM/F12-GlutaMax I 500ml

Fetal bovine serum (Hyclone, #SH30070.03, final 5%) 27ml

Penicillin / streptomycin (Thermo Fisher, # 15140122) 5.3ml

MEM Non-essential amino acid (Thermo Fisher, # 11140050) 5.3ml

2-Mercaptoethanol 3.5μl

4. External action potential recording solution

140mM NaCl (Sigma-Aldrich, #S7653)

5.4mM KCl (Sigma-Aldrich, #P9333)

1mM MgCl2 (Sigma-Aldrich, #M2670)

10mM D-glucose (Sigma-Aldrich, #G7021)

1.8mM CaCl2 (Sigma-Aldrich, #C7902)

10mM HEPES (Sigma-Aldrich, #H3375)

pH7.4 with NaOH at 25 oC

5. Internal/pipette action potential recording solution

120mM K D-gluconate (Sigma-Aldrich, #G4500)

25mM KCl

4mM MgATP (Sigma-Aldrich, #A9187)

2mM NaGTP (Sigma-Aldrich, #G8877)

4mM Na2-phospho-creatin (Sigma-Aldrich, #P7936)

10mM EGTA (Sigma-Aldrich, #E3889)

1mM CaCl2

10mM HEPES

pH 7.4 with KOH at 25 oC

6. External *I*K1 recording solution

160mM NMDG (N-Methyl-D-glucamine, Sigma-Aldrich, #66930)

5.4mM KCl

2mM MgCl2

10mM D-glucose

10μM nisoldipine (Sigma-Aldrich, #N0165)

1μM E-4031 (TOCRIS, #1808)

10mM HEPES

pH7.2 with HCl at 25 oC

7. BaCl2 (0.5mM, Sigma-Aldrich, #342920) in the above external solution (reagent 6)

8. Internal *I*K1 recording solution

150mM K-gluconate

5mM EGTA

1mM MgATP

10mM HEPES

pH 7.2 with KOH at 25 oC

**EQUIPMENT**

* Patch-clamp amplifier (Molecular Devices, MultiClamp 700B and Digidata 1440)
* Inverted microscope equipped with differential interface optics (Nikon, Ti-U) on a standard vibration table and faraday cage
* Patch clamp and imaging software (Molecular Devices, pClamp10 and MetaMorph imaging software MM-40000)
* EMCCD camera (Photometrics Evolve Intelligent, 512X512 EMCCD Digital Monochrome, #EVO-512-M-FW-16-AC)
* Micropipette puller (Sutter Instrument, Model P-97)
* Borosilicate glass (Sutter Instrument, #BF150-110-10)
* Pipette manipulator (Sutter instrument, #MPC-325-2)
* Heating system (Warner instrument, #TC324C)
* Quick exchange platform for 35-40mm dish (Warner instrument, #QE-1)
* Standard Water bath or heat block (for 37 oC tube incubation for tissue dissociation)

**PROCEDURES**

1. Prepare the papain-based dissociation solution (reagent 1, 10 ml) freshly. Incubate the dissociation solution in 37 oC water bath for 30 min to activate papain. Add DNase solution (Worthington, #LS002145, 5 µl/mL) into the papain solution before use.

2. Incubate 35 mm glass-bottom dishes (MatTek, P35-1.5-7-c) with Geltrex coating solution (reagent 2) for one hour at 37 oC and one hour at room temperature. Aspirate the solution right before plating cardiomyocytes

3. Wash engineered cardiac tissues with 1x PBS (phosphate buffered saline, Thermo Fisher, #70011044, no calcium/magnesium addition) twice. Next, incubate the tissues in the papain-based dissociation solution containing DNase for 15 min at 37 oC (one cardiac tissue in 10 ml of the papain solution in 15 ml tube). Gently shake the tube every 2 minutes. After 15-min incubation, gently pipette the tissues with 10ml serological plastic pipette 10 times.

4. Terminate the papain activity using the serum-containing culture media (reagent 3).

5. Centrifuge the tube, aspirate all of the solution, wash with the myocyte culture media (reagent 3) using another centrifugation (200xg, 5 min) and add fresh culture media into the tube

6. (Critical step) Replate the dissociated single cardiomyocyte (~100 uL) on only glass part of the Geltrex-coated dishes (7mm diameter). Low cell confluency (~2-5%) is required to utilize dissociate myocyte efficiently using multiple electrophysiological recording conditions. A dish containing sterile water without cover can be placed next to two myocyte dishes in a 100mm dish in order to keep humidity and prevent the medium from being dried. Twenty four hours after myocyte plating, add 2ml cardiomyocyte culture media (reagent 3) into the dishes.

7. Two or three days after myocyte plating, wash the dishes using patch clamp external recording solution (reagent 4, at 37oC in case of action potential recording) once, and add the recording solution (~2ml/dish at 37oC). (Critical timing) at the following day, the dissociated myocytes are not fully recovered. More than four days after myocyte dissociation, the cells (without any spontaneous contraction) are no longer healthy for electrophysiological recordings. Therefore, the whole-cell patch clamp recordings should be completed in 2-3 days after the myocyte dissociation and plating. Particularly, this optimized protocol enables obtaining healthy patchable single cardiomyocytes without spontaneous beating from early/intensity cardiac tissues (see “associated publication”).

8. Find single cardiomyocytes (no spontaneous beating) on the dish using 20x objective lens (Nikon, #MRD30205, CFI Plan Apochromat Lambda DM 20x Objective Lens NA 0.75 WD 1.00MM).

9. Fill the internal solution (reagent 5 in case of action potential recording) into a glass pipette, and install the pipette into the electrode connected with the manipulator holder. Move the pipette in the external solution using the manipulator controller (speed 0 or 1).

10. Examine pipette resistance (smaller, ~8 MΩ is better, i.e. using a larger pipette with 2-6 MΩ resistance often induces failure of sealing and breaking through and/or results in leak trouble in cardiomyocytes). (Critical step) Move the pipette tip on the top of the target cell slowly (speed 4) using the 20x objective lens. Also, confirm that there is no bubble inside the glass pipette. If any bubbles or debris are observed, apply pressure using a 10ml syringe to get rid of bubbles and debris after keeping the pipette far from the target cell.

11. Move the pipette down slowly (speed 6, down to ~30μm above the cell). Switch the objective lens to 40x one (Nikon, #MRD00405, CFI Plan Apochromat Lambda 40x Dry Objective Lens NA 0.95 WD 0.21MM). Again confirm that pipette resistance and pressure are stable and there are no bubble and debris inside the pipette. Switch to intermediate magnification changer 1.5X (Nikon). Use PC monitor to see the target myocyte with MetaMorph software and ECDD camera instead of 10x eyepiece of the microscope.

12. Seal the cell using a glass pipette using mouth (gentle pressure). (Critical step) Seal thicker and elastic region of the target cell (but not close to the nucleus). Hold potential at -60 mV after sealing is successful (>3 GΩ). Next, apply a small amount of pressure multiple times using mouth suction (i.e. sharp pulses) to the electrode/pipette in order to break through the plasma membrane of the sealed cardiomyocyte. Immediately after breaking through, the cell capacitance should be measured using the whole cell clamp mode in pClamp 10 software. And then switch the mode to current-clamp to make sure that there is no spontaneous action potentials in the patched cardiomyocytes. Start action potential recordings.

13. In case of *I*K1 current recording in human pluripotent stem cell-derived cardiomyocyte1-2, reagent 6 and 8 are used as the external and internal solutions, respectively. Reagent 7 is used for measuring Ba2+-sensitive current to dissect *I*K1 current in the cardiomyocytes. Use 2-sec long voltage-clamp applied from -130 to +10 mV (holding at -40 mV, 0.1 Hz 2-sec voltage pulse). The *I*K1 reversal potential (Ba2+-sensitive current) had a negative slope conductance consistent with inward rectification, as previously described. The current-voltage plot can be analyzed before and after the addition of 0.5 mM BaCl2 for 2min.

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| **Figure 1. Electrophysiological recording setup** C:\Users\kacey\AppData\Local\Microsoft\Windows\INetCache\Content.Word\Morikawa_Nat_Protocol_Exchange_Fig_v1.tif |
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**ASSOCIATED PUBLICATION**

Electromechanical stimulation advances maturation of human cardiac tissues grown from pluripotent stem cells

Kacey Ronaldson-Bouchard, Stephen Ma, Keith Yeager, Timothy Chen, LouJin Song, Dario Sirabella, Kumi Morikawa, Diogo Teles, Masayuki Yazawa and Gordana Vunjak-Novakovic

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