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Tissue Engineering of 3D Organotypic Microtissues by Acoustic Assembly

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Abstract

There is a rapidly growing interest in generation of 3D organotypic microtissues with human physiologically relevant structure, function, and cell population in a wide range of applications including drug screening, in vitro physiological/pathological models, and regenerative medicine. Here, we provide a detailed procedure to generate structurally defined 3D organotypic microtissues from cells or cell spheroids using acoustic waves as a biocompatible and scaffold-free tissue engineering tool.

Keywords 3D organotypic microtissues, Acoustic assembly, Acoustic waves, Cardiac scaffold, Cardiomyocyte, Cell assembly, Cell spheroids, In vitro tissue model, Regenerative medicine, Spheroid assembly, Tissue engineering

1 Introduction

In vitro 3D human organotypic models are being increasingly investigated in drug screening and basic biomedical research applications as more physiologically relevant platforms in comparison to the conventional cell culture models [1–3]. The ability to generate 3D organotypic microtissues representing basic cellular structure, such as the hepatic acinus in the liver and the nephron in the kidney, will enable diverse applications in basic and transitional medical research. 3D bioprinting has recently drawn great attention in biofabrication of 3D human tissue/organ mimics due to its unsurpassed flexibility in defining the spatial organization of heterogeneous cell and biomaterial constructs [4, 5]. However, a number of challenges still constrain the clinical applications of bioprinting modalities including (1) long processing time in large-scale biofabrication technologies, (2) low cytocompatibility particularly during high-speed printing, and (3) difficulty to achieve physiologically relevant cell packing density and close cell proximity resembling those in native tissues, due to the limitations in the mixing ratio of cells in the bioink (biomaterial). Herein, we demonstrate a unique tissue engineering approach for generating 3D organotypic microtissues directly from

cells or cell spheroids without the need of scaffold/bioink during structure formation. This approach utilizes Faraday wave, a type of surface acoustic wave existing at the air-liquid interface, to drive cells in a fluidic environment and pack them into 3D architecture at the bottom of an assembly chamber [6–8]. The geometry of the generated 3D architecture can be pre-designed by a mathematical-physical model using numerical simulation and can be flexibly tuned by acoustic frequency and amplitude. This acoustic assembly process only takes a few seconds regardless of the number of cells in the chamber, thus representing a more efficient method to generate 3D tissue constructs with high cell packing density, in comparison to 3D bioprinting and other bioengineering approaches [9]. In this chapter, we describe two experimental procedures including (1) assembly of fibroblast spheroids into organotypic microtissues [6] and (2) assembly of human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) into organotypic microtissues with physiologically relevant structure and function (Fig. 1) [8]. We envision that this tissue engineering tool will find broad applications in designing and developing 3D tissue models for drug screening and personalized medicine.

2 Materials

Prepare solutions with Milli-Q or equivalently purified water. Diligently follow all waste disposal regulations when disposing waste materials. All reagents, unless specified, are purely analytical chemicals from Sigma.

2.1 Devices for Acoustic Assembly

1. Arbitrary function generator (33510B, Agilent, CA, USA) (Fig. 2).
2. Audio power amplifier (Dayton Audio DTA-120 Class T Mini Amplifier 60 WPC, USA) (*see* **Note 1**).
3. Vibration exciter (U56001, 3B Scientific, Tucker, GA, USA).
4. Assembly chamber (20 mm × 20 mm × 1.55 mm) (*see* **Note 2**).
5. Metric tilt platform (Edmund Optics, NJ, USA) (*see* **Note 3**).
6. Vibration isolation pad (Grainger, CA, USA) (*see* **Note 4**).
7. Accelerometer (MMA7341L, Freescale Semiconductor, TX, USA).
8. Bubble level (Spirit Level, Hoefer, MA).

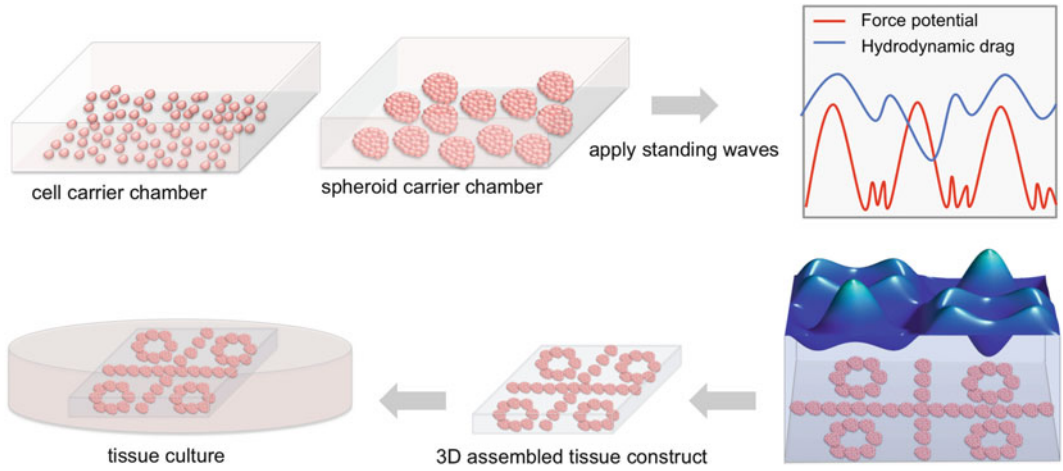

Fig. 1 Illustration of acoustic node assembly of organotypic microtissues. Cells/spheroids are first loaded in the assembly chamber. Then standing waves are applied to assemble cells/spheroids into pre-defined patterns based on node and antinode zones on standing waves in a fluidic environment. Further assembled cells/spheroids are immobilized in fibrin hydrogel and maturated in culture media

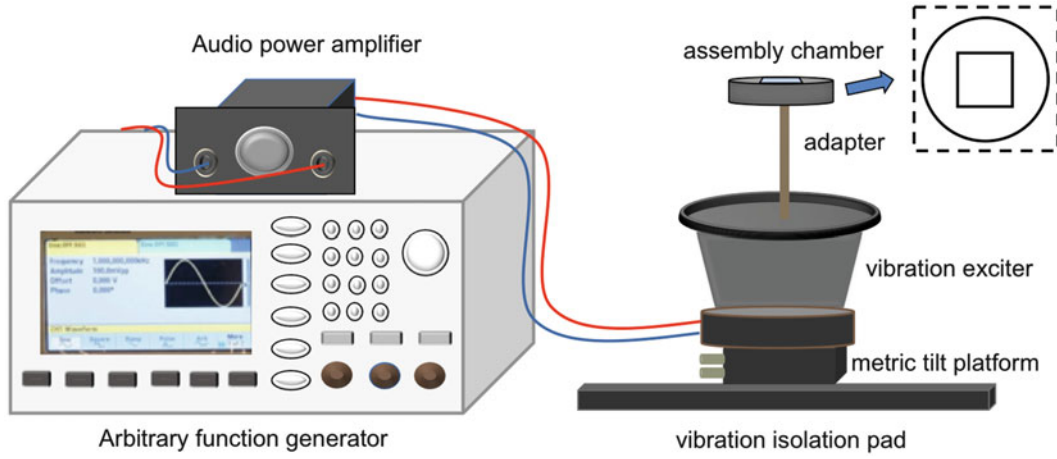

Fig. 2 Schematic of experimental setup for acoustic assembly

2.2 Culture Medium

2.2.1 Mouse Fibroblast Cell Culture Medium

Mouse fibroblast cells culture medium: Dulbecco's Modified Eagle Medium (DMEM)–High Glucose (HG) (Cat. No. 11965092, ThermoFisher Scientific) supplemented with 10% Fetal Bovine Serum (FBS) (Cat. No. F2442, Sigma-Aldrich), and 1% penicillin-streptomycin (Cat. No. 15140122, ThermoFisher Scientific).

2.2.2 hiPSC Culture and Differentiation Media

1. hiPSC culture medium (E8): E8 medium consists of DMEM-F12 (50:50 mixture of DMEM and Ham's F12 medium; Cat.

No. 10-092-CM, Corning), 20 μg/mL *Escherichia coli*-derived recombinant human insulin (Dance Pharmaceuticals/CS Bio), 64 μg/mL L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Cat. No. A8960, Sigma-Aldrich), 10.7 μg/mL *O. sativa*-derived recombinant human transferrin (Cat. No. T3705, Optiferrin, Invitria/Sigma-Aldrich), 14 ng/mL sodium selenite (Cat. No. 214485, Sigma-Aldrich), 100 ng/mL recombinant human FGF2 (Cat. No. 100-18B, 154 amino acid, *E. coli*-derived, Peprotech), and 2 ng/mL recombinant human TGFβ1 (Cat. No. 100-21, 112 amino acid, HEK293-derived, Peprotech) [10].

2. Cardiomyocyte differentiation medium (CDM3): CDM3 medium consists of RPMI 1640 medium (Cat. No. 11875, ThermoFisher Scientific), 500 μg/mL *O. sativa*–derived recombinant human albumin (Cat. No. A0237, Sigma-Aldrich, 75 mg/mL stock solution in water for injection (WFI) quality H_2O, stored at −20 °C), and 213 μg/mL L-ascorbic acid 2-phosphate (A8960, Sigma-Aldrich, 64 mg/mL stock solution in WFI H_2O, stored at −20 °C) [10] (Fig. 3).

2.3 Assembly Media

2.3.1 Assembly Media for Mouse Fibroblast Spheroids

1. Fibrinogen solution: Dissolve 20 mg fibrinogen (Cat. No. F8630, Sigma) in 2 mL PBS without Mg^{2+} and Ca^{2+} (Cat. No. 10010023, ThermoFisher Scientific) on ice.
2. Thrombin solution: Dissolve 5 mg thrombin (Cat. No. T4648-1KU, Sigma) with 35 mL dH_2O to storage solution (125 UN/mL); dilute the storage solution with PBS without Mg^{2+} and Ca^{2+} in a 1–50 ratio on ice.
3. Fibrin hydrogel: Mix 500 μL of 10 mg/mL fibrinogen with 150 μL 2.5 UN/mL thrombin on ice (*see* **Note 5**).

2.3.2 Assembly Media for hiPSC-Derived Cardiomyocytes

1. Hank's Balanced Salt Solution with calcium and magnesium HBSS (Cat. No. 55037C, Sigma-Aldrich).

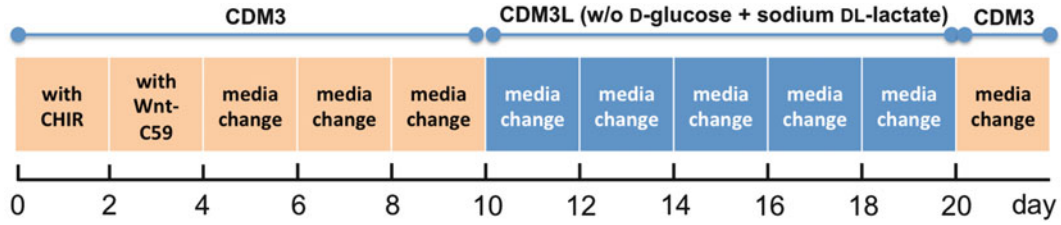

Fig. 3 Schematic of optimized chemically defined cardiac differentiation protocol [10]

2. Fibrinogen solution: Dissolve 10 mg fibrinogen in 2 mL HBSS with calcium and magnesium on ice.
3. Thrombin solution: Dilute the storage solution (125 UN/mL) with HBSS with calcium and magnesium in a 1–50 ratio on ice.
4. Fibrin hydrogel: Mix 550 μL of 5 mg/mL fibrinogen with 150 μL 2.5 UN/mL thrombin on ice.

2.4 Reagents for the Immunohisto chemistry

2.4.1 Solutions

1. Phosphate-buffered saline (PBS), Cat. No. 10010023 Gibco, ThermoFisher Scientific.
2. Live/Dead viability/cytotoxicity kit (L-3224, Molecular Probes, ThermoFisher Scientific).
3. The Live/Dead staining solution: Using Live/Dead viability/cytotoxicity kit mix 20 μL ethidium homodimer and 5 μL Calcein AM in 10 mL PBS (*see* **Note 6**).
4. 4% Paraformaldehyde (Cat. No. 15710, Electron Microscopy Sciences): Dilute 16% paraformaldehyde to 4% with H_2O.
5. Blocking Buffer (PBS/5% normal goat serum (Cat. No. 5425, Cell Signaling Technology)/0.1% Saponin, Cat. No. 47036, Sigma-Aldrich): Add 2.5 mL serum from the same species as the secondary antibody (normal goat serum) to 47.5 mL 1× PBS and mix well. While stirring, add Saponin to final concentration of 0.1% (*see* **Note 7**).
6. Antibody Dilution Buffer (1% BSA/0.1% Saponin): Add 0.4 g BSA to 40 mL 1× PBS and mix well. While stirring, add Saponin to final concentration of 0.1% (*see* **Note 8**).

2.4.2 Antibodies

1. Primary antibodies for mouse fibroblast cells: Mouse Anti-collagen type 1 (Cat. No. ab6308, Abcam), Rabbit Anti-Ki67 (Cat. No. ab15580, Abcam).
2. Secondary antibodies for mouse fibroblast cells: Donkey anti-mouse Alexa Fluor 488 (Cat. No. 715-545-150), and Rhodamine Red™-X (RRX) AffiniPure Donkey Anti-Rabbit IgG (H + L) (Cat. No. 711-295-152, Jackson Immuno Research).
3. Primary antibodies for hiPSC-CMs: mouse anti-sarcomeric α-actinin (Cat. No. A7811, Sigma–Aldrich), mouse anti--α-smooth muscle actin (Cat. No. A2547, Sigma-Aldrich), rabbit anti-vimentin (Cat. No. ab92547, Abcam), rabbit anti-nanog (Cat. No. ab21624, Abcam), rabbit anti-CD31 (Cat. No. ab28364, Abcam), rabbit anti-Connexin 43 (Cat. No. ab11370, Abcam), goat anti-AFP (Cat. No. sc-8108,

Santa Cruz Biotechnology), and mouse anti-NeuN (Cat. No. MAB377, Sigma-Aldrich).

4. Secondary antibodies for iPSC-CMs: Alexa Fluor 488 goat anti-rabbit and anti-mouse, Alexa Fluor 568 goat anti-mouse, Alexa Fluor 546 goat anti-mouse, Alexa Fluor 647 goat anti-rabbit, and Alexa Fluor 594 donkey anti-goat (all ThermoFisher Scientific) (*see* **Note 9**).

3 Methods

3.1 Mouse Fibroblast Cells

3.1.1 Spheroid Formation and Assembly

1. Culture NIH 3T3 mouse fibroblast cells in DMEM supplemented with 10% (v/v) FBS, 1% penicillin, and streptomycin.
2. Harvest and plate cells in 60 mm Petri dishes (pure virgin polystyrene) (ES3510, Medsupply Partners, USA) at 1×10^6 cells/dish for 2 days (*see* **Note 10**).
3. Rinse the dish with PBS and collect formed cell spheroids.
4. Centrifuge for 3 min at a speed of 800 rpm.
5. Discard the solution. Resuspend spheroids in 500 μL 10 mg/mL human fibrinogen (Cat. No. F8630, Sigma) prepared in PBS.
6. Add 150 μL of thrombin (Cat. No. T6884, Sigma) at 0.5 IU/mL final concentration and mix homogenously.
7. Transfer the solution to the assembly chamber (20 mm × 20 mm × 1.5 mm) with a 1 mL pipette (*see* **Note 11**).
8. Apply acoustic surface standing waves at 20–200 Hz frequency and 1.2–1.8 g amplitude ranges, resulting in formation of various assembly patterns (*see* **Note 12**).
9. Terminate acoustic surface standing waves at $t = 15$ s.
10. Immobilize (cross-linking of the fibrin) the assembled structures in formed fibrin hydrogel for 20 min at room temperature or 10 min at 37 °C. Room temperature control is critical to sustain consistent cross-linking rate.
11. Recover constructs on cover slip and culture in corresponding cell culture media [DMEM supplemented with 10% FBS, 1% penicillin-streptomycin (Cat. No. 15140-122, ThermoFisher Scientific), and 0.1 TIU/mL aprotinin (Cat. No. A1153, Sigma-Aldrich)] at 5% CO_2 and 37 °C at a humidified atmosphere.

3.1.2 Cell Viability and Proliferation Assays

1. Cut the hydrogel into nine aliquots with a blade and place each hydrogel construct in a 24-well plate (*see* **Note 13**).
2. Add 0.5 mL the Live/Dead staining solution; make sure the construct is submerged by the staining solution.
3. Incubate constructs at 37 °C in humidified incubator for 15 min in Live/Dead staining solution (*see* **Note 14**).
4. Rinse once with PBS.
5. Image with fluorescent microscope immediately.

3.1.3 Immunocytochemistry

1. Fix fibrin hydrogels containing microtissues with 4% paraformaldehyde (*see* **Note 15**).
2. Allow cells to fix for 20 min at room temperature.
3. Aspirate fixative; wash construct with excessive PBS three times for 5 min each.
4. Add 0.3% Triton-X 100 (Cat. No. T9284, Sigma-Aldrich) and block with 1% BSA (Cat. No. A2153, Sigma-Aldrich).
5. Place the plate in the shaker for 60 min.
6. Aspirate blocking solution; apply diluted primary antibody.
7. Stain spheroids overnight at 4 °C for primary antibodies.
8. Rinse three times with PBS for 5 min each in the shaker.
9. Stain 2 h for secondary antibodies at room temperature in the shaker in the dark.
10. Rinse in PBS as in step 8.
11. Stain cytoskeleton with phalloidin Alexa Fluor 647 (Cat. No. A22287, ThermoFisher Scientific) and use DAPI (Cat. No. 62247, ThermoFisher Scientific) as nuclear staining.
12. Keep immunostained constructs at dark and analyze with confocal microscopy as soon as possible (*see* **Note 16**).

3.2 hiPSCs

3.2.1 Culture and Assembly of Cells

1. Isolate the obtained hiPSC clones and culture (using E8 medium) on six-well tissue culture plates (Greiner) coated with 1:200 growth factor-reduced Matrigel (9 μg/cm^2) (Cat. No. 354230, Corning).
2. Medium is changed every other day (48 h). Cells are passaged every 4 days (split at 1:10 or 1:12 ratios) at ~85% confluence using 0.5 mM EDTA. 2 μM Thiazovivin (Cat. No. S1459, Selleck Chemicals) is added for the first 24 h after each passage.
3. Treat hiPSCs with a small molecule inhibitor of GSK3B signaling, 6 μM CHIR99021 (Cat. No. C6556, LC Laboratories), in CDM3 media for 2 days (*see* Fig. 3).

4. Treat cells subsequently with CDM3 media supplemented with a Wnt signaling inhibitor, 2 μM Wnt-C59 (Cat. No. S7037, Selleck Chemicals), for another 2 days.
5. At days 4–8 of differentiation, use CDM3 media without any factors. Change the media every other day (*see* **Note 17**).
6. Culture (starve) cardiomyocytes (CMs) in CDM3 media without glucose, supplemented with 5 mM sodium DL-lactate (Cat. No. 71720, Sigma-Aldrich), for 4 days. This step is necessary to purify the culture from the non-CM cells.
7. Use TrypLE Express (Cat. No. 12605010, ThermoFisher Scientific) for 3–5 min to dissociate CMs into single cells.
8. Count CMs with cell count plate; ensure cells are at a density of 1.0×10^6 cells/mL (or desired cell density).
9. Once dissociated, transfer the hiPSC-CMs cells into a 15 mL centrifuge tube on ice and pipette a few times to break them into single cell suspension.
10. Centrifuge cell suspension at a speed of 1200 rpm for 4 min.
11. Discard the supernatant culture medium and resuspend hiPSC-CMs in fibrin prepolymer solution on ice at a density of 1.7×10^6 cells/mL.
12. Place an 18 mm × 18 mm coverslip at the bottom chamber in advance (*see* **Note 18**).
13. Transfer the solution to the assembly chamber (20 mm × 20 mm × 1.5 mm) with a 1 mL pipette (*see* **Note 19**).
14. Wait until the solution gravitationally sediments onto the glass coverslip at the bottom of the chamber (~1 min). Apply the Faraday waves (10 s for 5 times with intervals of 10 s). Randomly distributed cells at the substrate are driven by the hydrodynamic drag field and patterned into the pre-defined 3D pattern. For the selected circle-square hybrid patterning of hiPSC-CMs, the waveform generator should be excited at 127 Hz and 110 mV (*see* **Note 20**).
15. Terminate the acoustic assembly device.
16. Add an additional 25 μL of thrombin (2.5 UN/mL) to the cast solution to accelerate the cross-linking of fibrinogen and wait for ~10 min.
17. Transfer the assembly chamber to a tissue culture incubator and incubate at 37 °C for another 15 min.
18. Detach cell-encapsulating fibrin scaffold from the assembly chamber using a blade and transfer the scaffold into a well of six-well tissue culture plate.

19. Put the six-well plate into cell incubator and culture for further development and maturation of the 3D tissue (*see* **Note 21**).

3.2.2 Characterization of Developed Tissue

Immunohistochemical Analysis of Patterned Constructs

1. Rinse 3D fibrin scaffolds (encapsulating hiPSC-CMs) three times for 20 min each with DPBS (Cat. No. 14190144, ThermoFisher Scientific).
2. Fix with 4% paraformaldehyde for 60 min on rocker at room temperature.
3. Rinse with DPBS three times for 20 min each.
4. Permeabilize with 0.1% Saponin for 60 min at room temperature with gentle agitation.
5. Block in blocking solution (10% goat serum, 0.1% Saponin, and 1% BSA solution) for 3 h on rocker at room temperature.
6. Drain and stain samples with 1:200 dilution of primary antibodies.
7. Incubate overnight at 4 °C in the blocking solution.
8. Wash with DPBS, four times for 30 min each on the rocker.
9. Incubate for 3 h at room temperature in the dark with 1:200 secondary antibodies in the blocking solution.
10. Wash with DPBS, three times for 30 min each.
11. Stain cell nuclei with NucBlue Fixed Cell Stain (Cat. No. R37606, ThermoFisher Scientific) in DPBS.
12. Perform the confocal imaging (*see* **Note 22**).
13. Count (NucBlue stained) cell nuclei for total cell number and α-actinin + NucBlue stained cells for the CM number.

hiPSC-CM Metabolic Activity Assay

1. Add AlamarBlue reagent (Cat. No. DAL1025, ThermoFisher Scientific) to each well at 10% of the culture volume (CDM3) at different time points in culture.
2. Incubate cellular constructs at 37 °C for 4 h.
3. Use acellular fibrin gels as control, for background reference subtraction.
4. Read the absorbance of 100 mL of medium at 550 and 600 nm using a microplate reader (Cytation 5 Cell Imaging Multi-Mode Reader, BioTek Instruments) and calculate the percentage of reduced AlamarBlue according to the manufacturer's instructions.

Motion Analysis of Beating Cardiomyocytes

1. At (desired) serial time points during culture, use an optical microscope with 10× objective at a speed of 12.5 frames per second.

2. Transform the raw videos to 8 bit gray scale and then analyze using an image velocimetry script in MATLAB (MathWorks) [11] (*see* **Note 23**).

4 Notes

1. The audio power amplifier is used to amplify signals from an arbitrary function generator to electrically drive vibration exciter.
2. The assembly chamber is fabricated from poly (methyl methacrylate) (PMMA) plates, double-sided adhesive (DSA) (iTapestore, Scotch Plains, NJ, USA) using a laser cutter (VersaLaser, Scottsdale, AZ, USA) [6]. The size and shape of the chamber can be customized according to the experimental requirements.
3. The metric tilt platform is used to adjust the horizontal level of liquid surface in the chamber together with a bubble level, which is placed on the chamber.
4. The vibration isolation pad is installed at the bottom of metric tilt platform to isolate mechanical perturbation.
5. The formulation of the solution should be carried out on ice; if not, the solution would link floc, which would result in Flocculent precipitate appear.
6. Live cells stained with Calcein AM (green) and dead cells stained for EthD-1 (red).
7. Dispense the blocking buffer with 1 mL each centrifuge tube and preserve at 4 °C.
8. When using any primary or fluorochrome-conjugated secondary antibody for the first time, titrate the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.
9. Using secondary antibodies that have been pre-absorbed with the serum of the other species (the mouse specific IgG antibody pre-absorbed with rat serum and vice versa) to avoid cross-reaction between these highly related animal species.
10. Spheroids are formed after 2 days of incubation. Spheroids sediment and distribute randomly on the substrate.
11. Wait until spheroids have landed onto cover slip (18 × 18 mm) placed at the bottom of the assembly chamber.
12. The force potential, U, that affects CMs with a radius R at the substrate exposed to a hydrodynamic drag field can be described as follows:

$$U = \left[-\frac{4}{3}\left(\rho_{\text{cell}} - \rho_{\text{liq}}\right)\omega g R^3 - \frac{24\nu\rho_{\text{liq}} R\omega e^{-\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)} \sin\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)}{k \sin h(kH)}\zeta_{\text{sh}} \right.$$
$$+\frac{3\pi^2\nu\rho_{\text{liq}} Rh\omega}{kL(\sin h(kH))^2}\left\{-3 + e^{-2\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)}\right.$$
$$+8e^{-\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)} \sin\left(\frac{\frac{\lambda}{2}-H}{\delta}\right) + 2e^{-\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)} \cos\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)$$
$$\left.\left.-2\sqrt{2}\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)e^{-\left(\frac{\frac{\lambda}{2}-H}{\delta}\right)} \cos\left(\frac{\frac{\lambda}{2}-H}{\delta}+\frac{\pi}{4}\right)\right\}\zeta_{\text{h}}\right]$$

Here, ρ_{cell} represents the density of the cell, ρ_{liq} shows the density of liquid, ω is the Faraday wave frequency, g is the gravitational constant, ν is the kinematic viscosity of the liquid, H is the thickness of the liquid, k is the Faraday wavenumber, h is the height of Faraday wave, λ is the Faraday wavelength, δ is the Stokes characteristic length, and ζ_{sh} is the sub-harmonic component of deformation of liquid surface. ζ_{h} and ζ_{sh} show wave functions and are adjusted by ω [8].

13. Each construct can be cultured in a well of a 24-well culture plate and can be used for different staining assays.
14. The staining time is related to the volume of the hydrogel, generally no more than 20 min.
15. Paraformaldehyde is toxic; use only in fume hood.
16. For long-term storage, store slides flat at 4 °C, protected from light.
17. Contracting cells were noted from day 7.
18. Make sure there is no bubble between the chamber and the coverslip.
19. Add the solution along the edge of the chamber and ensure that the coverslip is completely submerged.
20. Adjust the amplitude from low to high until the pattern appears.
21. Add 1 TIU/mL aprotinin (Cat. No. A1153, Sigma-Aldrich) into the culture media to inhibit cell-induced fibrin degradation.
22. 3D reconstruction and panoramic videos of the z-stacks are generated by the confocal microscope software (ZEN 2012,

Carl Zeiss Microscopy GMBH, MA). Cell density measurements using optical images are conducted by counting the number of cells in each focal plane (in focus).

23. Calculate contractile stress values from video recordings, based on the stiffness and depth of material, and the cell displacement, which is relative to the reference frame at resting states between contractions.

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