Chapter 2

Scalable Cardiac Differentiation of Human Pluripotent Stem Cells as Microwell-Generated, Size Controlled Three-Dimensional Aggregates

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Abstract

The formation of cells into more physiologically relevant three-dimensional multicellular aggregates is an important technique for the differentiation and manipulation of stem cells and their progeny. As industrial and clinical applications for these cells increase, it will be necessary to execute this procedure in a readily scalable format. We present here a method employing microwells to generate large numbers of human pluripotent stem cell aggregates and control their subsequent differentiation towards a cardiac fate.

Key words Cardiac differentiation, Microwells, Human pluripotent stem cells, Size controlled cell aggregates, Forced cell aggregation

1 Introduction

It has been known for a long time that cellular behavior in two-dimensional adherent culture does not fully recapitulate three-dimensional in vivo systems, and consequently there is a growing trend in many fields of research to employ three-dimensional tissue constructs. These may be divided into scaffolded constructs, where cells are cultured on or in an exogenous supporting matrix [1], and unscaffolded systems that consist primarily or exclusively of cells (which may then proceed to generate their own endogenous matrices) [2, 3]. Here, we will discuss a process for generating large numbers of uniform, unscaffolded aggregates of human pluripotent stem cells for differentiation towards a cardiac fate.

Conventionally, size controlled cellular aggregates have been generated in microcentrifuge tubes [4], as hanging drops [5], by micropatterning human embryonic stem cells (hESCs) colonies [6] or by centrifugation into U- or V-bottom plates [7, 8]; however throughput is limited using these approaches. Microwell-based systems are similar in concept to V-bottom plates; however

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the smaller size of the microwells permits very large numbers of uniform aggregates to be generated from a single culture-plate well in a mechanically simple system [9]. This approach has been used for a variety of applications including differentiation of pluripotent stem cells to ectodermal [10], endodermal [11], mesodermal [12], and extraembryonic [13] fates; chondrogenesis from mesenchymal stem cells [14]; and generation of uniform substrates for toxicological screening [15] and investigations of mechanobiology [16].

Using this approach to control aggregate size, we consequently determine surface area to volume ratio, which in turn modulates extraembryonic endoderm (ExE) commitment. Cardiogenic signals from this cell lineage then specify subsequent cardiomyogenesis [12].

2 Materials

All reagent preparation and cell culture handling should be carried out in a biological safety cabinet under sterile conditions.

2.1 General Equipment and Supplies

1. Biological safety cabinet.
2. Pipette aid.
3. Serological pipettes (5–25 mL).
4. Aspirator.
5. Aspirator or Pasteur pipettes.
6. 15 and 50 mL conical tubes.
7. Fume hood.
8. 0.22 μm syringe filter.
9. 5 % CO_2, *5 % O_2*, and humidity controlled cell culture incubator (Subheadings 3.1 and 3.4).
10. 5 % CO_2, *20 % O_2*, and humidity controlled cell culture incubator (Subheading 3.5).
11. Low speed centrifuge with a swinging bucket rotor fitted with a plate holder.
12. P2, P20, P200, and P1000 micropipettors with appropriate tips.
13. Inverted microscope with 4×, 10×, and 20× phase objectives.
14. Ultra-Low Attachment (ULA) 24 well plates.
15. 1.5 mL microcentrifuge tubes.
16. Bench-top microcentrifuge.

2.2 Basal Medium

1. L-Ascorbic Acid: Prepare a stock solution of 5 mg/mL in 4 °C, sterile ultrapure distilled water. Leave on ice and vortex

Table 1
Cytokines and buffers used for media preparation

Cytokine	Buffer	Stock Conc. (μg/mL)
Human Bone morphogenetic protein 4 (hBMP-4)	4 mM Hydrochloric acid, 0.1 % BSA	10
Human Fibroblast Growth Factor 2 (basic) (hbFGF)	Phosphate buffered saline (PBS), 0.1 % BSA	10
Human Vascular Endothelial Growth Factor (hVEGF)	Phosphate buffered saline (PBS), 0.1 % BSA	5
Activin A	Phosphate buffered saline (PBS), 0.1 % BSA	10
Human Dickkopf 1 homolog (hDkk-1)	Phosphate buffered saline (PBS), 0.1 % BSA	50

periodically until completely dissolved. Filter-sterilize using a 0.22 μm syringe filter, aliquot at 1 mL volume, and store the Ascorbic Acid aliquots at −20 °C. Use a freshly thawed aliquot each time medium is prepared.

2. Monothioglycerol: MTG should be aliquoted (1 mL) and stored frozen (−20 °C). When aliquots are thawed, they can be used for 3 months and then discarded. Aliquoting of MTG is strongly recommended as it minimizes the amount of oxidation due to repeated opening. On the day of media preparation, dilute 13 μL MTG in 1 mL StemPro-34. Discard unused diluted MTG.
3. Transferrin: Transferrin (stock concentration: 30 mg/mL) should be aliquoted (1 mL aliquots) and stored at −20 °C.
4. All cytokines are stored lyophilized at −20 °C until ready to be aliquoted in the following buffers and at the following concentrations, Table 1. Continue to store unused aliquots at −20 °C.
5. 4 mM Hydrochloric acid (HCl), 0.1 % BSA Buffer: In a fume hood, transfer 30 μL of 6.0 N HCl solution to 50 mL of ultrapure distilled water. Filter-sterilize the solution using a 0.22 μm syringe filter. Add 2 mL of 25 % BSA solution to the 50 mL HCl solution.
6. PBS, 0.1 % BSA Buffer: Add 20 μL of 25 % BSA solution for every mL PBS.
7. Supplemented DMEM/F-12: 1 % Penicillin/Streptomycin (Pen/Strep) and 1 % L-glutamine in DMEM/F-12.
8. hESC Wash Medium: 5 % Knockout Serum Replacement in Supplemented DMEM/F12.

9. StemPro-34: StemPro-34 is sold as a kit with two components. The supplement is kept at −20 °C and the liquid media at 4 °C. When combined, the media is stable for 30 days (*see* **Note 1**). StemPro-34 is always used with the supplement added in this protocol.
10. Modified StemPro-34: StemPro-34 modified with the addition of 1 % Pen/Strep, 1 % L-glutamine, 0.5 % Transferrin, 1 % Ascorbic Acid, 0.3 % MTG. This medium should be prepared on the day of use with freshly thawed Ascorbic Acid.

2.3 hESC Aggregate Formation Components

1. One 6 well plate culture of feeder-free or feeder-depleted (*see* **Note 2**) hESCs adapted to single cell passaging (e.g., enzymatic dissociation with TrypLE).
2. TrypLE Select.
3. hESC Wash Medium (preparation previously described in Subheading 2.2, **item 8**).
4. Hemocytometer.
5. Trypan Blue.
6. Aggrewell™ 400 plates (StemCell Technologies 27845).
7. Aggrewell™ Rinsing Solution (StemCell Technologies 07010).
8. Aggregation Medium: Modified StemPro-34, 0.5 ng/mL BMP4, 10 μM Y-27632 ROCK Inhibitor. 1 mL of medium is required per well of a 24 well Aggrewell™ 400 plate.
9. OPTIONAL: 37 μm strainer.

2.4 Cardiac Induction Components

1. Stage 1 Induction Medium: Modified StemPro-34, 10 ng/mL BMP4, 5 ng/mL bFGF, 6 ng/mL Activin A. 1 mL of medium is required per well of a 24 well Aggrewell™ 400 plate (*see* **Note 3**).
2. Stage 2 Induction Medium: Modified StemPro-34, 10 ng/mL VEGF, 150 ng/mL DKK1. 1 mL of medium is required per well of a 24 well ULA plate (*see* **Note 3**).
3. Stage 3 Induction Medium: Modified StemPro-34, 10 ng/mL VEGF, 150 ng/mL DKK1, 5 ng/mL bFGF. 1 mL of medium is required per well of a 24 well ULA plate (*see* **Note 3**).
4. hESC Wash Medium (prepared as previously described in Subheading 2.2, **item 8**).

2.5 Flow Cytometry

1. 1 mg/mL Collagenase Type II in Hank's Balanced Salt Solution.
2. TrypLE Select.
3. hESC Wash Medium (preparation previously described in Subheading 2.2, **item 8**).

4. DNase: DNase should be diluted in ultrapure distilled water to a concentration of 1 mg/mL, aliquoted, and stored at −20 °C.
5. 96 well assay plate (or 1.5 mL microcentrifuge tubes).
6. IntraPrep fixation and permeabilization kit.
7. Cardiac Troponin T antibody.
8. Allophycocyanin (APC)-conjugated goat-anti-mouse IgG antibody.
9. HF buffer solution: 2 % Fetal Bovine Serum (FBS) in Hank's Balanced Salt Solution.
10. 5 mL round-bottom flow cytometry analysis tubes.
11. Flow cytometer with the appropriate laser and filter to excite (i.e., 633–640 nm) and detect (i.e., 660/20 nm) APC.

3 Methods

A schematic of the process is shown in Fig. 1.

3.1 hESC Aggregate Formation

1. Add 0.5 mL Aggrewell™ Rinsing Solution to each well of the Aggrewell™ plate used. Ensure that the solution contacts the entire surface inside each microwell by centrifuging the plate at 840 × *g* for 2 min. Incubate the Aggrewell™ plate containing the Rinsing Solution for 30–60 min at room temperature. Aspirate the solution and wash twice with 1 mL PBS per well, centrifuging at 840 × *g* for 2 min after each PBS addition.
2. Completely dissociate the hESCs to single cells: Aspirate the culture medium from each culture well, rinse each well with 1 mL TrypLE Select, and aspirate the TrypLE Select. Residual TrypLE should be sufficient to dissociate the cells. Incubate the plate at 37 °C for 3 min, then add 1 mL of hESC Wash Medium to each well and mechanically dissociate the cells from the tissue culture surface by pipetting with a P1000 micropipettor. Transfer the cells (in hESC Wash Medium) to a 15 mL conical tube.

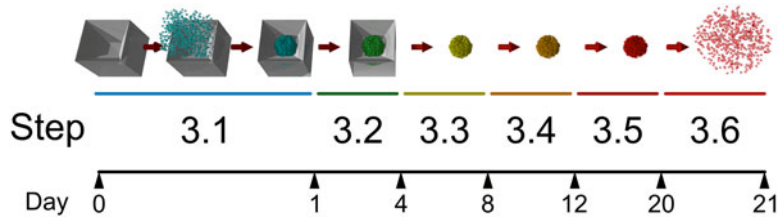

Fig. 1 Schematic of cardiac differentiation protocol. The sequence of steps in Subheadings 3.1–3.6 is diagrammed, along with an indication of the timescale over which they occur

Optionally, if the dissociation appears to be incomplete and cell clumps remain, the suspension may be passed through a strainer at this point.

3. Perform a cell count: Take a 10 μL sample of the cell suspension collected in the previous step. Add 30 μL of Trypan Blue and mix the suspension well by pipetting up and down. Transfer 10 μL of Trypan Blue-stained cells to each chamber of a hemocytometer and visualize under the inverted microscope with the 10× objective. Count the cells.
4. From the cell count results, calculate how many wells can be seeded at 1.2×10^6 cells per well. Resuspend the dissociated hESCs in Aggregation Medium at a density of 1.2×10^6 cells/mL (*see* **Note 4**).
5. Aspirate the PBS wash solution from each well on the Aggrewell™ Plate. Using a P1000 micropipettor, evenly distribute 1 mL of the cell suspension to each well on the Aggrewell™ Plate. If seeding a large numbers of wells, periodically mix the container of cell suspension to prevent settling. Centrifuge the plate at $200 \times g$ for 5 min. Visualize the plate under the microscope to confirm cells have spun to the bottom of each microwell (*see* **Note 5**).
6. Incubate the plate for 24 h at 37 °C in a 5 % CO_2, 5 % O_2 (*hypoxic*) incubator.

3.2 Cardiac Induction Stage 1 (24 ± 2 h Following Aggregation)

1. Prepare the required volume of Stage 1 Induction Medium (for a 24 well plate, volume = 1 mL × number of wells). Place the medium in a 37 °C water bath for at least 15 min.
2. Remove the Aggrewell™ plate from the incubator. Inspect the aggregates under the microscope. Compared to immediately post-centrifuge aggregation, they should appear intact with smooth edges (more round and less square than the previous day).
3. To remove the supernatant but retain the aggregates in their individual microwells: Use a P1000 micropipettor. For each well on the Aggrewell™ plate, place the tip of the micropipettor at the surface of the culture medium in the well and against the edge of the well (keep the plate level horizontally). Slowly remove the medium, being careful not to disturb the aggregates at the bottom of the microwells. Once the medium level has been reduced to about 1–2 mm from the surface of the textured microwell surface, the plate can be slowly tilted to collect medium at one side of the well (once the volume is this low, fluid motion is greatly reduced and it is easier to avoid aggregates getting lifted out of their individual microwells). Slowly pipette out the remaining medium in the well.
4. To add fresh Stage 1 Induction Medium while ensuring the aggregates remain in their individual microwells: Take up 1 mL

of Stage 1 Induction Medium with a P1000 micropipettor. Hold the pipette tip against the inside edge of the well and very slowly dispense the medium against the inside wall of the well. Repeat for the remaining wells.

5. Return the plate to the incubator under *hypoxic* conditions for 4 days.

3.3 Cardiac Induction Stage 2 (Day 4: 96 ± 2 h After Aggregate Formation)

Stage 2 Induction directs mesoderm differentiation. Therefore at this time-point it is very important to remove all residual Activin A from the Stage 1 Induction Medium. Activin A is a very potent signaling molecule and at this stage of differentiation, even trace levels would promote differentiation to the endoderm lineage at the expense of cardiac induction. Therefore, at this stage the aggregates are removed from the Aggrewell™ plates, washed well, and transferred to bulk aggregate cultures in 24 well ULA plates.

1. Place hESC Wash Medium (volume = number of wells × 2 mL) in a 37 °C water bath for 10–15 min.
2. Prepare the necessary volume of Stage 2 Induction medium (volume = number of wells × 1 mL) and place in a 37 °C water bath for 10–15 min.
3. Using a 5 mL serological pipette, harvest the aggregates from each well of the Aggrewell™ plate, and collect the aggregate suspension in a 15 mL conical tube (up to 10 wells per tube).
4. Allow the aggregates to settle for 15 min in hypoxic (5 % O_2) conditions. This step is performed to separate single cells and cellular debris from the intact aggregates. Aspirate the supernatant carefully and resuspend the aggregates in 10 mL hESC Wash Medium to wash out residual inductive cytokines (especially Activin A which is a potent signaling molecule even at very low concentrations).
5. Centrifuge the aggregates at 50 × *g* for 2 min and aspirate the supernatant.
6. Resuspend the pelleted aggregates in the Induction 2 Medium prepared in **step 2** above.
7. Dispense the aggregate suspension into a 24 well ULA plate at 1 mL per well. Visualize the aggregates under the microscope. They should appear as uniform, tight cell clusters.
8. Incubate under *hypoxic* conditions until day 8.

3.4 Cardiac Induction Stage 3 (Day 8: 192 ± 2 h After Aggregate Formation)

Typically, in a successful cardiac induction, spontaneously contracting aggregates will be observed under the microscope between day 8 and day 12.

1. Prepare the necessary volume of Stage 3 Induction Medium (volume = number of wells × 2 mL) and place in a 37 °C water bath for 10–15 min.

2. Use a 5 mL serological pipette to transfer the aggregates to 15 mL conical tubes, pooling up to 10 mL of aggregates per tube. Allow 10 min for the aggregates to settle. Aspirate the supernatant and resuspend the aggregates in Stage 3 Induction Medium. Using a 5 mL serological pipette, redistribute the aggregates into a 24 well ULA plate at 1 mL per well.
3. Incubate under *hypoxic* conditions until day 12.

3.5 Cardiac Induction Stage 3 (Day 12 to Harvest)

1. Prepare the necessary volume of Stage 3 Induction Medium (volume = number of wells × 1 mL) and place in a 37 °C water bath for 10–15 min.
2. Use a 5 mL serological pipette to transfer the aggregates to 15 mL conical tubes, pooling up to 10 mL of aggregates per tube. Allow 10 min for the aggregates to settle. Aspirate the supernatant and resuspend the aggregates in Stage 3 Induction Medium. Using a 5 mL serological pipette, redistribute the aggregates into a 24 well ULA plate at 1 mL per well.
3. Incubate the cells at *normoxic* oxygen levels for the remainder of the culture period (37 °C, 20 % O_2, 5 % CO_2). *After this time point, cells are no longer cultured under hypoxic conditions.*
4. Repeat this complete medium exchange (**steps 1–3**) at day 16.
5. Cardiac aggregates are ready for harvest on day 20.

3.6 Flow Cytometry Analysis of Cardiac Troponin T Expression Frequency of Aggrewell™ Culture Output

For flow cytometry analysis of cardiac Troponin T (cTnT) expression frequency, it is recommended that 4 wells of day 20 cardiac aggregates be harvested.

1. Using a 5 mL serological pipette, transfer 1 well of aggregates to a 15 mL conical tube. Centrifuge the aggregates at 50 × *g* for 2 min and carefully aspirate the supernatant.
2. Add 1 mL of freshly dissolved 1 mg/ml collagenase Type II solution to the aggregates, transfer the aggregate suspension to a 1.5 mL microcentrifuge tube, and incubate the aggregates in collagenase Type II overnight at room temperature.
3. The next day, using a P1000 micropipettor, gently pipette the aggregates to dissociate them into a homogenous single cell suspension. If the aggregates do not readily dissociate, settle the aggregates, aspirate the supernatant, and incubate the aggregates in 700 μL TrypLE for 1–2 min at room temperature. Gently pipette the aggregates 1–2 times with a P1000 micropipette to dissociate. Add 700 μL hESC Wash Medium containing 14 μL of 1 mg/mL DNAse stock solution to dilute the TrypLE. Take a 10 μL sample for cell counting, and centrifuge the remaining suspension using a bench-top microcentrifuge for 2 min at 300 × *g*.
4. Stain the 10 μL counting sample with an equal volume of Trypan Blue and count using a hemocytometer.

5. Remove the 1.5 mL microcentrifuge tube containing the remaining cells from the microcentrifuge. Aspirate the supernatant and resuspend the cells in HF at a concentration of 200,000–500,000 cells per 100 μL.
6. Transfer 100 μL per well to 2 wells of a 96 well plate (*see* **Note 6**) per experimental condition or replicate (one well will be for cTnT staining and the other will be the unstained control).
7. Pellet by centrifuging the plate at 300 × *g* for 2 min in a swinging bucket centrifuge.
8. Fix the cells in 200 μL of Intraprep Reagent 1 per well for 15 min at room temperature.
9. Centrifuge the plate at 300 × *g* for 2 min. Carefully aspirate the supernatant and dispose of in a paraformaldehyde waste container.
10. Wash the fixed cells twice: Add 200 μL HF to each well. Centrifuge the plate at 300 × *g* for 2 min, aspirate the supernatant, and repeat the wash step once more. Fixed cells can be stored for up to 1 week in HF at 4 °C.
11. Permeabilize the cells: Centrifuge the plate at 300 × *g* for 2 min. Add 100 μL of Intraprep Reagent 2 per well and incubate the plate for 5 min at room temperature. Centrifuge the plate at 300 × *g* for 2 min and aspirate the supernatant.
12. Prepare a staining solution of anti-cTnT (Neomarkers MS-295) in HF at the optimal concentration for the given lot number (determined by titration: typically ranges from 1:500 to 1:2,000 dilution).
13. Add 100 μL staining solution to one well of cells and 100 μL of plain HF to the other well (negative control). Incubate the cells for 30 min at 4 °C.
14. Centrifuge the cells at 300 × *g* for 2 min. Aspirate the supernatant and add 200 μL HF per well. Repeat the wash step one more time.
15. Prepare a master mix of the secondary antibody: Transfer a volume of HF that corresponds to 100 μL for every well (control and cTnT-stained) being treated. Add 1 μL of goat anti-mouse-APC secondary antibody per 200 μL of HF (1:200 dilution).
16. Stain the samples: Add 100 μL of staining solution to each well (negative and anti-cTnT-stained wells). Incubate cells for 30 min at 4 °C (keep the plate covered or in the dark after adding fluorescent secondary antibody to avoid photobleaching).
17. Centrifuge the cells at 300 × *g* for 2 min. Aspirate the supernatant and add 200 μL HF per well. Repeat the wash step one more time.

18. Transfer the samples to 5 mL round-bottom flow cytometer analysis tubes and analyze on a flow cytometer using the red laser.

4 Notes

1. If 500 mL of complete StemPro-34 medium (with supplement) will not be used within 30 days, it is recommended that the basal medium and supplement be aliquoted as follows: prepare ten 50 mL aliquots of the basal medium and store at 4 °C, prepare ten 1.3 mL aliquots of the supplement and store at −20 °C. When preparing complete medium from the aliquots, completely thaw 1 aliquot of supplement in a 37 °C water bath and then add the supplement to a 50 mL aliquot of basal medium.
2. To prepare feeder-depleted hESCs, passage the cells onto Growth Factor Reduced Matrigel (BD 354230) coated 6 well plates at a split ratio that will produce $1–2 \times 10^6$ cells per well after 1–2 days. Use the same medium that was being used to maintain the hESCs on feeders.
3. Depending on the volume of Induction Medium to be prepared, the volume of some of the growth factor stock solutions to be added on a given day may be less than 1 μL. To ensure accurate volumes of growth factor stock solutions are being added to the medium preparation, the stock may first be diluted 1:10 in StemPro-34 basal medium, and then the appropriate volume of diluted stock may be added to the medium preparation.
4. At this seeding density, aggregates containing 1,000 cells will be formed. This is based on our observations with cardiac induction from the HES-2 cell line maintained on feeders [12]. The ideal seeding density may vary and should be optimized for the specific cell lines and culture conditions being used.
5. If the cells do not appear to form a tight pellet at the bottom of the microwells, try eliminating air bubbles in the microwells by spinning some medium into the wells prior to adding the cells: Add 0.4 mL of medium per well and centrifuge the plate at $200 \times g$ for 5 min. Then add the appropriate number of cells to each well in 0.6 mL medium per well.
6. If the total number of samples being prepared is small, staining can also be performed in 1.5 mL microcentrifuge tubes. If microcentrifuge tubes are used, supernatant is removed by aspiration and it is recommended that minimum wash volumes of 0.5 mL and centrifuge settings of $900 \times g$ (in a bench-top microcentrifuge) for 3 min be used for the wash steps.

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