

Aggregate and Microcarrier Cultures of Human Pluripotent Stem Cells in Stirred-Suspension Systems

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Abstract

Pluripotent stem cells can differentiate to any cell type and contribute to damaged tissue repair and organ function reconstitution. The scalable culture of pluripotent stem cells is essential to furthering the use of stem cell products in a wide gamut of applications such as screening of candidate drugs and cell replacement therapies. Human stem cell cultivation in stirred-suspension vessels enables the expansion of stem cells and the generation of differentiated progeny in quantities suitable for use in animal models and clinical studies. We describe methods of culturing human pluripotent stem cells in spinner flasks either as aggregates or on microcarriers. Techniques for assessing the quality of the culture and characterizing the cells based on the presentation of pertinent markers are also presented. Spinner flask culture with its relatively low capital and operating costs is appealing to laboratories interested in scaling up their production of stem/progenitor cells.

Keywords: Pluripotent stem cells, Differentiation, Scalable culture, Xeno-free culture, Spinner flasks, Cell aggregates, Microcarriers

1 Introduction

Pluripotent stem cells with their capacity to differentiate into a broad range of cell types [1] are a promising source of cellular material for clinical applications and screening platforms in drug development. Human pluripotent stem cells (hPSCs) are successfully used in cell replacement studies and animal models after their differentiation to retinal pigment epithelium [2] in humans, spinal cord cells [3], neural cells to treat ischemia stroke in rats [4], and endothelial-like cells to treat hind limb ischemia in mice [5]. Realization of stem cell-based therapies will require the development of scalable systems for the expansion of stem/progenitor cells and their differentiation to desired phenotypes. For example, a minimum of $1\text{--}2 \times 10^9$ cells is required for replacing damaged cells in the myocardium after infarction [6]. Given also the $<100\%$ differentiation efficiencies, it becomes obvious that typical dish cultures are not ideal for generating differentiated cells for studies in animal models and eventually for clinical therapies.

Stirred-suspension bioreactors are an attractive modality for stem cell cultivation. While a fully automated benchtop bioreactor system affords tight control and real-time monitoring of several culture variables, the associated capital, maintenance, and operation costs as well as the required knowledge basis for its use make it a less suitable option for many laboratories. In this chapter, we detail methods for the culture of hPSCs (embryonic—hESCs and induced pluripotent stem cells—hiPSCs) in spinner flasks, which require minimal investment, are simple to operate, and provide a reasonable approximation of larger stirred-suspension systems.

Human stem cells can be cultured in spinner flasks as aggregates [7, 8], on microcarriers [9, 10] or after encapsulation [11]. In this chapter, we describe methods developed in the lab for the culture of stem cells in spinner flasks either as aggregates or on microcarriers. Aggregate cultures are simpler to set up and require less downstream processing than microcarrier cultures. The beads however provide a larger surface area-to-volume ratio, which may lead to more efficient use of culture media and factors. Moreover, the presence of concentration gradients is typically more pronounced in aggregate cultures, which may impose challenges, particularly for directed differentiation of stem cells in a spinner flask following their expansion. The characterization of cultured cells includes the detection of expressed pluripotency and/or differentiation markers by flow cytometry, immunohistochemistry, and quantitative polymerase chain reaction (qPCR). Karyotyping is also carried out to assess gross abnormalities caused during the course of successive passages. The differentiation capacity of the cells is tested by coaxing cell specification toward the three germ layers.

2 Materials

2.1 Static Culture of Pluripotent Stem Cells Using Vitronectin or Matrigel

1. Human embryonic or induced pluripotent stem cells [e.g., H9 hESCs (WiCell Research Institute) or any other hPSC line of interest].
2. Biosafety hood.
3. Tissue culture microscope with epifluorescence attachment and camera.
4. Tissue culture plates, e.g., 6-cm dishes, 6-well and 12-well plates (Corning).
5. Pipettes, pipet aids, and 1 ml, 200 μ l, and 10 μ l pipet tips.
6. E8 medium: Mix basal medium with 20 \times and 500 \times supplements along with penicillin/streptomycin (optional: if using, add 5 ml per 500 ml total medium) (Stemcell Technologies; available as 100 \times solution) (*see Note 2*).

7. Water bath (37 °C).
8. A tissue culture CO₂ incubator (37 °C; 5 % CO₂). A stirring table is placed in the incubator for spinner flask culture (Fig. 1a).

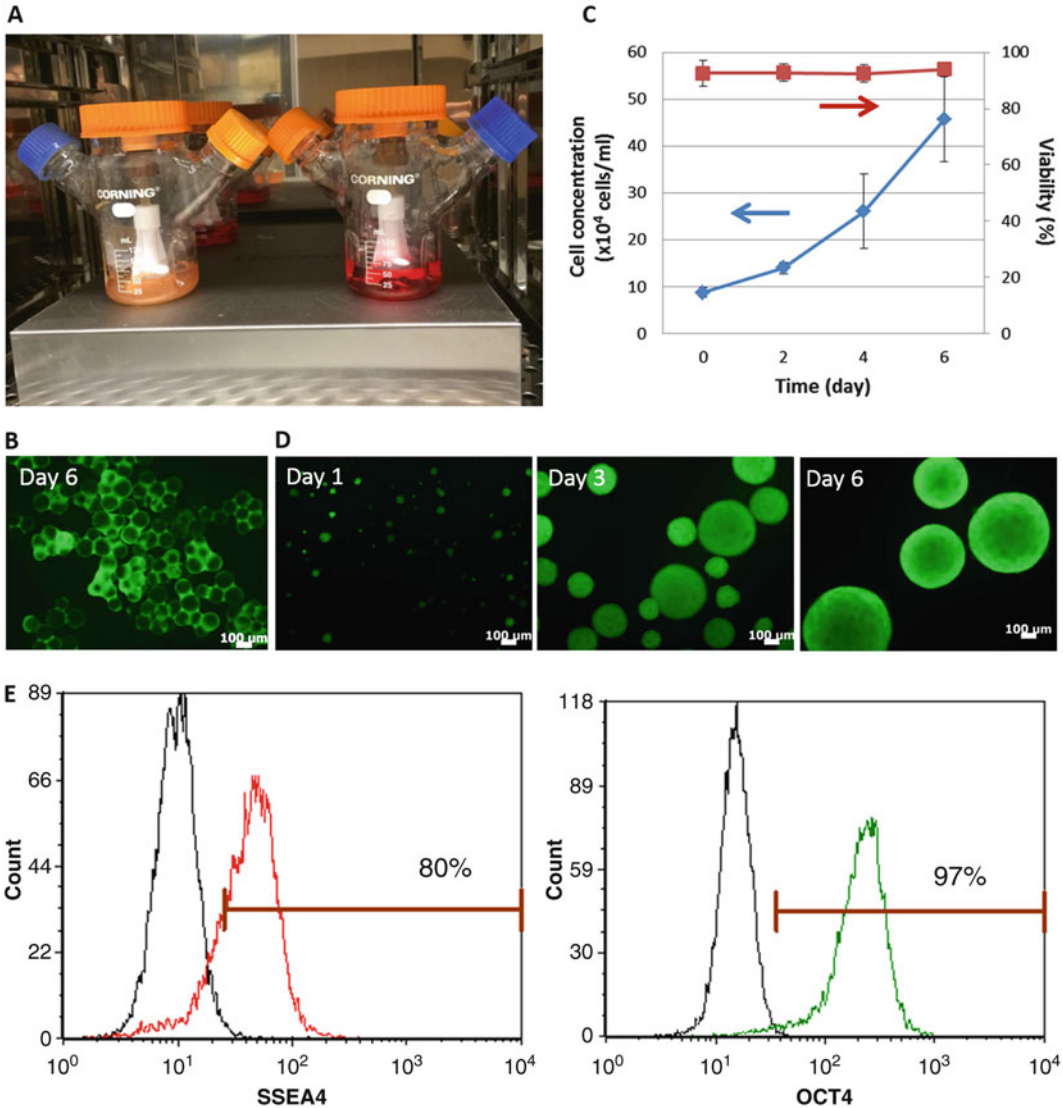


Fig. 1 Human pluripotent stem cell culture in spinner flasks. (a) Spinner flasks in a CO₂ incubator. Human H9 embryonic stem cells are cultured as aggregates in E8 medium (*left*). Spinner flasks shown on the right are prepared for passaging these cells. (b) Human H9 cells grown on Matrigel-coated polystyrene beads in a spinner flask. Bar: 100 μm. (c) Typical growth profile and viability of hPSCs grown as aggregates in a spinner flask. Each point is shown as mean ± standard deviation from triplicates. (d) Cell aggregates stained with FDA on days 1, 3, and 6 of culture. Bar: 100 μm. (e) Determination of the expression of pluripotency markers SSEA4 and OCT4 using flow cytometry for hPSCs after 6 days of stirred suspension culture. *Black curves*: Isotype control. *Colored curves*: Antigen. The fractions of positive cells may vary between experiments

9. Matrigel (Corning) (*see Note 1*)
10. Recombinant human vitronectin, truncated (Life Technologies)
11. Dispase (Life Technologies)
12. DMEM/F-12 (Life Technologies)

2.2 Microcarrier Culture of Stem Cells in Spinner Flasks

1. Phosphate buffer saline, free of Ca^{+2} and Mg^{+2} (PBS; Life Technologies).
2. Accutase (Innovative Cell Technologies).
3. SoloHill Fact III collagen-coated polystyrene microcarriers (Pall Corp.) for use after being coated with Matrigel.
4. SoloHill plastic microcarriers (Pall Corp.) for use after being coated with vitronectin.
5. ProCulture spinner flasks (125 or 250 ml; Corning).
6. Multiposition magnetic stirring plate (Thermo Scientific) with controller.
7. Matrigel solution.
8. Recombinant human vitronectin, truncated.
9. Human serum albumin (HSA; Sigma Aldrich).
10. Y-27632 Rho-associated protein kinase (ROCK) inhibitor (Enzo Biochem; 10 mM solution).
11. Pluronic F-68 (Life Technologies).
12. Fluorescein diacetate (FDA; Sigma Aldrich): Stock solution is prepared by dissolving the FDA powder in dimethylsulfoxide (DMSO) to a concentration of 10 mM; the stock solution is diluted to 10 μM (working concentration) with PBS.
13. 100- μm mesh strainer (BD Falcon).
14. Trypan Blue (Life Technologies).
15. Lactate dehydrogenase (LDH) cytotoxicity detection assay (Roche).

2.3 Differentiation to Definitive Endoderm and Mesoderm Cells in Spinner Flask Cultures

1. RPMI 1640 medium (Life Technologies).
2. CTS B-27 supplement, xeno-free (Life Technologies).
3. Recombinant human Activin A (R&D Systems).
4. Recombinant human BMP4 (R&D Systems).
5. Knockout serum replacer (KSR; Life Technologies).

2.4 Characterization

2.4.1 Total RNA Isolation, Reverse Transcription, and Quantitative PCR

1. Trizol (Life Technologies).
2. Isopropanol (100 % pure; HPLC grade).
3. Ethanol (200 proof; HPCL grade).
4. Chloroform (HPLC grade).
5. Nuclease-free water.

6. OligodT (Thermo Fisher).
7. ImProm-II 5× Buffer (Promega).
8. MgCl₂ (Promega).
9. dNTP mix (Thermo Fisher).
10. ImProm-II Reverse Transcriptase (Promega).
11. Dynamo SYBR Green mix set (Thermo Scientific).

2.4.2 Flow Cytometry

1. 4 % Paraformaldehyde solution: To make a 100 ml solution, mix 4 g paraformaldehyde (J.T. Baker) in 100 ml PBS and heat the mixture to 65 °C while stirring using a magnetic stirrer with heating option. Increase pH by adding NaOH until the solution clears (allow 2–4 h for solution to clear). The final pH is to be around 6.9 (*see Note 4*).
2. PBS.
3. Normal donkey serum (NDS; Jackson ImmunoResearch Laboratories, Inc.) or other blocking agent depending on the antibodies used.
4. TrypLE (Life Technologies).
5. Cytonin (Trevigen).

2.4.3

Immunohistochemistry

1. Paraformaldehyde (4 %) solution.
2. Bovine serum albumin (BSA; Sigma Aldrich).
3. Labtek 8-well microscope glass slides with surface area 0.7 cm²/well (Thermo Scientific).
4. Deionized (DI) water.
5. Octylphenylpolyethylene glycol or Triton X (J.T. Baker).

2.4.4 Karyotyping

1. T-75 flasks (Corning).
2. KaryoMAX Colcemid Solution (Gibco from Life Technology).
3. Cell hypotonic solution (CHS) is made by making a solution of 40 mM KCl, 20 mM HEPES, 0.5 mM EDTA, and 9 mM NaOH in DI water.
4. Acetic acid.
5. Methanol.
6. GelMount aqueous mounting medium (Sigma-Aldrich).
7. Wright-Giemsa stain, modified (Sigma-Aldrich).

2.4.5 Differentiation into Mesoderm

1. DMEM (Life Technologies).
2. Sodium selenite (Sigma Aldrich).
3. RPMI 1640 vitamin solution (100×) (Sigma-Aldrich).
4. Chemically defined (CD) lipid concentrate (Life Technologies; sold as 1000× solution).

5. RPMI 1640 amino acid solution (50×) (Sigma-Aldrich).
6. Holo-transferrin (Sigma-Aldrich).
7. MEM nonessential amino acids (100×) (Life Technologies).
8. Sodium pyruvate (Life Technologies; sold as 100× solution).
9. Penicillin/streptomycin.
10. Carrier-free recombinant human BMP4 (R&D Systems).
11. Carrier-free recombinant human WNT3A (R&D Systems).
12. KY02111 (Xcessbio Biosciences, Inc).
13. Matrigel or recombinant human vitronectin as substrate.

2.4.6 Differentiation to Ectoderm Cells

1. DMEM/F12 medium.
2. Neurobasal medium (Life Technologies).
3. B-27 supplement minus vitamin A (50×) (Life Technologies).
4. Glutamax (100×) (Life Technologies).
5. N2 supplement (100×) (Life Technologies).
6. FGF2 (R&D Systems).
7. Collagenase IV (Life Technologies).
8. Low binding non-treated polystyrene 6-cm dishes (BD Biosciences).

2.5 Antibodies

1. Anti human-NANOG (BD Pharmingen).
2. Anti-human OCT4 (Santa Cruz Biotechnology, Inc.).
3. Anti-human SSEA4 (Abcam).
4. Antihuman TRA-1-60 (BD Pharmingen).
5. Anti-human KDR (R&D Systems).
6. Anti-human PDGFR α (R&D Systems).
7. Anti-human C-KIT (R&D Systems).
8. Anti-human Nestin (R&D Systems).
9. Anti-human β III-Tubulin (Sigma-Aldrich).
10. Appropriate secondary antibodies (e.g., from Jackson ImmunoResearch Laboratories, Inc.).

3 Methods

3.1 Static (Dish) Culture

Aseptic conditions must be maintained during cell culture and manipulations.

1. Two days before thawing cells, thaw Matrigel at 4 °C overnight. Prepare 400- μ l aliquots in the biosafety hood taking care to use icepacks to keep Matrigel cold. Freeze the aliquots at -80 °C.

2. If using vitronectin, thaw the vial at room temperature, prepare 10- μ l aliquots, and store them at -80°C .
3. Prepare E8 medium aliquots in 50 ml tubes and store them at -20°C .
4. On the day before thawing cells, thaw aliquots of Matrigel and E8 medium at 4°C overnight.
5. Using ice-cold DMEM/F12, make a solution of Matrigel in DMEM/F12 (1:70). Coat each dish to be used immediately with the Matrigel solution. If using vitronectin, a solution of vitronectin in PBS at the ratio 1:100 should be prepared for coating dishes.
6. Warm E8 medium at room temperature. Remove the cryovial with the frozen cells from the liquid nitrogen tank and place it in the 37°C water bath. Remove the vial from the bath when the last crystal of ice is remaining, spray with 70 % ethanol, and swipe with a paper towel.
7. Transfer the contents of the vial to a 15-ml tube in the biosafety hood using a 5-ml pipet.
8. Using a 10-ml pipet, add 9 ml of E8 medium to the tube dropwise while shaking so that the cells do not go under osmotic shock.
9. Centrifuge the tube at $200 \times g$ for 5 min. Meanwhile, aspirate the Matrigel solution from the dish and replace it with a volume of E8 medium that is 1 ml less than the total volume to be used.
10. Aspirate the medium from the centrifuge tube, and use a 1-ml pipette tip to flush the pellet at the bottom with E8 medium exactly once. Remove the contents using the pipette tip and add it slowly to the dish. Gently shake the dish to ensure even distribution of the colonies.
11. To passage cells on Matrigel, incubate them in dispase for 1–2 min. Wash them twice with DMEM/F12 and scrape them using a 10-ml glass pipet. Add the scraped colonies to a coated dish splitting the cells at ratios ranging between 1:4 and 1:6. If cells are on vitronectin, incubate them in 0.5 M EDTA solution for 1–2 min at room temperature. Wash the cells twice with PBS. Using a 1-ml pipette tip, flush the dish with medium. Seed the cells at ratios between 1:4 and 1:6.

3.2 Stirred-Suspension Culture in Spinner Flasks

3.2.1 Microcarrier Culture Coated with Vitronectin, HSA, and UV Radiation

1. Wash and autoclave the spinner flasks before use.
2. Weigh 0.5 g of plastic microcarriers suspended in PBS and autoclave them. The amount of beads can vary depending on the total surface of beads needed.
3. Remove as much of the PBS as possible without disturbing the beads.

4. Mix 200 μ l vitronectin in 2 ml PBS, and add it to the beads. Mix the beads with the vitronectin solution. Incubate the beads in vitronectin for at least 1 h; resuspend the beads every 20 min.
5. Dissolve HSA in PBS at a concentration of 0.01 g/ml. Filter sterilize the solution. Add 2 ml of the albumin solution to the beads with vitronectin so that the final concentration of albumin is 0.005 g/ml. Incubate the beads in the solution for exactly 30 min.
6. Meanwhile, treat the cells intended for seeding with 10 μ M ROCK inhibitor for 1 h.
7. Transfer the beads and the solution to a 35-mm sterile culture dish. Remove as much as 3 ml supernatant leaving behind just enough solution to allow the beads to move around. Tape the dish to a shaker, and gently shake the dish without the lid under UV light (254 nm) for 40 min.
8. Use E8 medium with 10 μ M ROCK inhibitor to transfer the beads to a 15-ml tube. Wash the dish with the medium until you collect all the beads.
9. Let the beads settle down and aspirate as much of the medium as possible. Wash once more with the wash medium. Let the beads incubate in the wash medium until ready for use.
10. Add appropriate volume of Accutase (e.g., 2 ml Accutase for a 6-cm dish) to the cells ready to be seeded and incubate them for 10 min at 37 °C to disperse them into single cells. Harvest the cells by adding an equal volume of medium containing ROCK inhibitor. Spin down the cells at $200 \times g$ for 5 min. Count the cells using a cell counter.
11. Aspirate the wash medium without disturbing the beads and add fresh E8 medium with ROCK inhibitor so that the total volume including the cells and medium with beads equals 8 ml. Transfer the beads to a 10-cm petri dish.
12. Add ten million cells to the top of the beads.
13. Manually shake the dish a few times to distribute the cells among the beads and allow them to stick. Place them in the incubator, and manually shake the dish every 15 min for the first hour, and then every 30 min for the next 3 h. Add 4 ml of medium with 10 μ M ROCK inhibitor and let the beads remain with the cells overnight in the incubator (37 °C, 5 % CO₂).
14. The next morning, transfer the beads to the spinner flask and add another 38 ml (total of 50 ml) medium containing 0.02 % Pluronic F-68 (diluted 1:500 from a 10 % Pluronic F-68 stock) (*see Note 3*).
15. Take a 1 ml sample and let the microcarrier with cells settle. Remove the supernatant, add 1 ml of 10 μ M FDA solution, and incubate for 1 min. Check for cell attachment under a fluorescence microscope.

16. Take another 1 ml sample and after the microcarriers settle remove the supernatant for measurement of the LDH activity. Incubate the cells/beads with 1 ml of TrypLE to detach the cells. After 10 min, pipette the sample vigorously to separate all cells from microcarriers. Add equal amount of Trypan Blue to count live and dead cells. This number can be used to calculate the cell seeding efficiency and viability.
17. The supernatant from the last step can be used for LDH activity determination according to the manufacturer's instruction and thus assess the level of cell lysis transpiring in the medium.

3.2.2 Microcarrier Culture Using Matrigel

1. Equilibrate FACT III collagen-coated polystyrene microcarriers (0.5 g microcarriers for five million cells seeding) in PBS for 5 min and autoclave them. Allow the beads to cool to 4 °C.
2. Wash the microcarriers with cold DMEM/F12 and mix well with cold liquefied Matrigel (200 µl of Matrigel solution for 0.5 g of microcarriers).
3. Leave the microcarrier/Matrigel mix at room temperature for 1 h. Pipette up and down every 10 min to get all microcarriers suspended.
4. After 1 h, equilibrate the coated microcarriers in culture medium containing 10 µM ROCK inhibitor for 1 h before cell seeding. Beads at 0.5 g (~180 cm² surface area)/50 ml medium were used.
5. For initial seeding on beads, treat the stem cells on Matrigel-coated dishes with 10 µM ROCK inhibitor for 1 h and dissociate the colonies into single cells by incubating with Accutase for 10 min.
6. Transfer the dispersed hPSCs with microcarriers (cell-to-bead ratios as stated) to Petri dishes and place in 5 % CO₂ at 37 °C.
7. Gently shake the dish to better mix the microcarriers with cells every 15 min.
8. Subsequently, the cell-laden beads are transferred to spinner flasks (Corning) and the total medium (with Y-27632) volume is brought to 50 ml.
9. The agitation rate is set to 45 rpm.
10. After the first day, the medium is replaced with the same medium but without Y-27632.
11. Subsequent medium changes are performed at half-volume every day.
12. The cultures are maintained at 37 °C in 5 % CO₂. Cells on microcarriers are shown in Fig. 1b after staining with FDA.
13. In preparation for passaging cells between spinner flasks, Y-27632 is added to the culture at a final concentration of 10 µM for about 1 h prior to harvesting.

14. Collect cells on microcarriers from spinner flasks into 50 ml centrifuge tubes.
15. Wash the cells once with PBS: allow the microcarriers to settle and remove as much of the medium as possible. Add 10 ml PBS to the beads and resuspend them. Incubate the beads with Accutase for 10–15 min in the incubator and gently mix them occasionally.
16. Complete detachment from microcarriers as single cells can be verified by microscopy. Pass the cell/bead suspension through a 100- μ m mesh strainer. The harvested cells can be characterized and/or inoculated onto fresh microcarriers repeating the steps described above.

3.3 Aggregate Culture of hPSCs in Spinner Flasks

1. Wash and autoclave the spinner flasks to be used.
2. Incubate five million cells in dishes with 10 μ M ROCK inhibitor for 1 h.
3. After 1 h, dissociated the colonies into single cells by incubating them in Accutase for 10 min.
4. Centrifuge the dispersed hPSCs at $200 \times g$ for 5 min and remove the supernatant.
5. Resuspend the cell pellet in 2 ml of E8 medium supplemented with 10 μ M ROCK inhibitor and 0.1 ml cell coating matrix (Life Technologies) for 10 min.
6. Transfer the cell suspension to ProCulture spinner flasks (Corning). The total medium volume supplemented with ROCK inhibitor is brought to 50 ml.
7. The agitation rate is set to 45 rpm.
8. After the first day, replace the medium with medium not containing Y-27632.
9. Subsequent medium changes are performed at half-volume every day.
10. The cultures are maintained at 37 °C in 5 % CO₂.
11. In preparation for passaging cells between spinner flasks, Y-27632 is added to the culture at a final concentration of 10 μ M about 1 h prior to harvesting.
12. Then, cell aggregates are collected from spinner flasks into centrifuge tubes and incubated with Accutase for 10–15 min while gently mixing occasionally.
13. Images of aggregates as well as the growth profile, viability, and expression of pluripotency markers of a 6-day culture of H9 hESCs are shown (Fig. 1c–e).

3.4 Differentiation to Definitive Endoderm and Mesoderm Cells

1. After the microcarriers become confluent with cells (usually in 6 days of culture), differentiation can be initiated.
2. Before changing to differentiation medium, let the beads with cells settle for 10 min.
3. Remove as much culture medium as possible from the flask.
4. Add 20 ml of warm DMEM/F12 medium to the spinner flask and shake gently to resuspend the beads.
5. Let the beads settle again for another 10 min.
6. Remove as much medium as possible while being careful not to aspirate cells with beads.
7. Add 50 ml of fresh differentiation medium to the spinner flask and set the stirring rate to 45 rpm.
8. Change medium every day for 4 days by letting the beads settle, aspirating carefully most of the existing medium and replacing it with equal volume of fresh medium.
9. For definitive endoderm differentiation, the differentiation medium is composed of RPMI 1640 medium containing 100 ng/ml activin A and 1 % CTS-B27 (xeno-free).
10. For mesoderm differentiation, the differentiation medium is composed of RPMI 1640 medium containing 10 ng/ml activin A, 10 ng/ml BMP4, and no KSR (day 1 of differentiation), 0.2 % KSR (day 2), or 2 % KSR (days 3–4).

3.5 Characterization of Cultured Cells

3.5.1 Reverse Transcription-Quantitative PCR

All procedures must be done on RNase-free surfaces. Wipe the benchtop, and all relevant equipment with RNase Away solution (Thermo Scientific). Use RNase/DNase-free tubes and filtered pipet tips only. Use a fume hood for RNA extraction. Work with all samples on ice for the cDNA synthesis and qPCR steps.

1. Isolate RNA from the harvested cells ($1-10 \times 10^6$ cells) using 1 ml Trizol. Pellet the harvested cells by centrifuging at $200 \times g$ for 5 min. Add 1 ml Trizol to the cells. Mix the cell pellet with the Trizol using a 1-ml pipet tip, and triturate vigorously to remove any signs of the pellet. Allow the solution to incubate at room temperature for 5 min.
2. Add 200 μ l chloroform to the mixture, and allow it to incubate at room temperature for 2–3 min. Shake the tube vigorously for 15 s.
3. Centrifuge at $12,000 \times g$ and 4 °C for 15 min. There will be three layers: a lower (pink) organic phase, a middle (white) phase, and a top (clear) aqueous phase. Using a 200- μ l pipet tip, remove the aqueous phase and transfer it into a fresh tube taking care not to disturb the other phases.
4. Add 500 μ l of isopropanol to the aqueous phase. Add 0.5 μ l glycogen to the mixture to visualize better the RNA pellet.

Shake the tube vigorously for 10 s. Allow to incubate at room temperature for 10 min, and centrifuge at $12,000 \times g$ and 4°C for 10 min. A white pellet will be visible at the bottom of the tube (if using a swinging bucket rotor; for a fixed-angle rotor, it will be visible at the outer lower edge of the tube).

5. Remove the isopropanol by upending the tube and add 75 % ethanol to the pellet. Vortex the pellet so that it floats in the ethanol (this is a washing step). Centrifuge at $7500 \times g$ and 4°C for 5 min.
6. Upend the tube to remove the majority of the ethanol, and allow the rest of it to air-dry taking care not to let the pellet dry completely.
7. Resuspend the pellet in 30–200 μl nuclease-free water depending on the size of the pellet and measure the concentration using a UV spectrometer. Measure the 260 nm-to-280 nm absorbance ratio to ensure the purity of the RNA (ideally this should be above 1.8).
8. Aliquot 1 μg RNA in a nuclease-free PCR tube. Add 1 μl oligodT primers, and enough nuclease-free water to make the volume up to 5 μl .
9. Program the thermal cycler to hold the samples at 70°C for 5 min and 4°C for at least 10 min, and place the samples in the cycler.
10. Add 2.4 μl MgCl_2 , 4 μl $5\times$ reaction buffer, 1 μl dNTP mix, 6.4 μl nuclease-free water, 0.5 μl RNasin RNase inhibitor, and 1 μl reverse transcriptase to the samples to make a total volume of 20 μl .
11. Program the thermal cycler to hold the samples at 25°C for 5 min to anneal the samples, 42°C for 1 h to extend the chain, 70°C for 15 min to inactivate the reverse transcriptase, and 4°C to cool the samples and hold them until they can be used for qPCR (store samples at -20°C if not performing qPCR on them immediately). Add a melting curve cycle to check for nonspecific products. Place the samples in the cycler.
12. Place the 96-well qPCR plate on a plate holder. Each sample would contain 10 μl SYBR Green master mix, 8 μl nuclease-free water, 0.5 μl of the forward primer, 0.5 μl of the reverse primer (forward and reverse primers are specific for the genes the expression of which will be probed), 0.2 μl of the ROX dye (for Applied Biosystems qPCR platforms), and 1 μl of the cDNA. Prepare aliquots of the mixture for running triplicate samples. Each sample will be probed for *ACTB* (b-actin), *NANOG*, *POU5F1* (OCT4), and *SSEA4*.
13. Seal the plate using sealing film, and insert in the qPCR instrument.

3.5.2 Flow Cytometry

Use a no-primary antibody control (NPAC) or an isotype control (for conjugated primary antibodies).

1. Make a 1 % NDS solution for washes. Make a 3 % NDS solution for blocking (*see Note 5*).
2. Use one million of the harvested cells per sample, preparing samples to be tested for NANOG, OCT4, SSEA4, and TRA-1-60.
3. Add 1 ml of 4 % paraformaldehyde solution to each sample. Incubate at room temperature for 10 min tapping every 2 min to keep cells in suspension. Do not vortex or pipet.
4. Centrifuge the sample at $1500 \times g$ for 5–10 min. Remove the paraformaldehyde solution in the fume hood. Add 1 ml of 1 % NDS solution and resuspend the pellet by tapping and vortexing. Centrifuge the samples at $1500 \times g$ for 5–10 min to be able to collect all the cells. Remove the NDS solution.
5. Add 100 μ l cytonin to each pellet and tap tubes several times to resuspend cells. Incubate at room temperature for 1 h. Tap occasionally to keep the cells in suspension.
6. Remove cytonin by first adding 1 ml of 1 % NDS to the samples and then spinning down at $1500 \times g$ for 5–10 min.
7. Block the samples by adding 600 μ l of 3 % NDS. Keep cells in suspension during the wait by tapping them at regular intervals. Incubate for 1 h at room temperature. Spin down at $1500 \times g$ for 5–10 min.
8. Prepare primary antibody solutions in 1 % NDS at dilutions according to the manufacturers' instructions. Incubate for 40 min to an hour at room temperature. For an NPAC add 1 % NDS only. Keep the samples in suspension by tapping them every 10 min.
9. Wash each sample three times with 1 % NDS.
10. Make a solution of appropriate secondary antibodies in 1 % NDS. Add 100 μ l secondary antibody to the samples and resuspend them. Incubate the samples at room temperature for 1 h in a dark place.
11. Wash three times with 1 % NDS.
12. Add 250–500 μ l PBS to each pellet and resuspend the samples.
13. Examine the samples using a flow cytometer.

3.5.3

Immunohistochemistry

1. Coat microslides with vitronectin or Matrigel following the steps above.
2. Seed the harvested single cells onto the slides such that each sample has two wells: one experimental well and one NPAC well.

3. After 1 or 2 days of culture, add PBS to the cells. Aspirate the PBS and add 4 % paraformaldehyde solution to the cells in a fume hood. Incubate for 15–20 min.
4. Meanwhile, make a blocking/permeabilizing solution by adding 10 μ l Triton X and 100 mg BSA to PBS to make a 0.1 % Triton-X and 1 % BSA solution.
5. Wash the cells three times with PBS. Incubate the cells for 5 min in PBS before aspirating it and adding fresh PBS. This constitutes one wash step.
6. Add the blocking/permeabilizing solution to the cells and incubate for 30 min.
7. Wash three times with PBS, incubating the cells for 5 min in PBS.
8. Prepare the primary antibody solution in 1–2 % serum (*see Note 6*) at dilutions recommended by the manufacturer. NANOG, OCT4, SSEA4, and TRA-1-60 are some of the markers tested.
9. Incubate the cells in primary antibody solution for 1 h at room temperature or overnight at 4 °C. Take care not to add the primary antibody solution to the wells with no primary control.
10. Wash the cells three times with PBS.
11. Prepare the secondary antibody solution by adding an appropriate amount of secondary antibody to 1–2 % serum solution.
12. Incubate the cells in the secondary antibody solution for 1 h at room temperature.
13. Wash the cells three times in DI water. Prepare a DAPI solution according to the manufacturer's instructions by adding an appropriate amount of DAPI to DI water.
14. Incubate in DAPI for 5 min.
15. Wash three times in DI water. Aspirate all the DI water, and remove the cover and gasket with the wells from the slide.
16. Add one to two drops of gel mounting solution to the slide. Carefully add the cover slip. Incubate for 1 h in the dark at room temperature.
17. Seal the cover slip using nail polish or other appropriate sealant.

3.5.4 Karyotyping

1. Cells harvested from spinner flask cultures are re-plated on T-75 flasks and allowed to grow until 70 % confluence.
2. Cells are treated with 30 ng/ml of KaryoMAX Colcemid Solution (Gibco) for 4 h at 37 °C.

3. Cells are then collected, transferred to 15-ml conical tubes, centrifuged for 5 min at $250 \times g$, and gently resuspended in cell hypotonic solution (CHS: 40 mM KCl, 20 mM HEPES, 0.5 mM EGTA, and 9 mM NaOH) for 1 h of incubation at 37°C .
4. After centrifugation of the cell/CHS suspension at $250 \times g$, the supernatant is removed and the cells are fixed with 1:3 (v/v) acetic acid:methanol solution. Resuspend by pipetting up and down.
5. Spin at $200 \times g$ for 5 min and remove supernatant. Repeat twice the fixing wash with methanol/acetate.
6. Resuspend the cells in 2 ml methanol/acetate. Apply one drop of the cell suspension to a glass slide. Keep the pipette at a considerable distance from the slide so that the drop can spread on the slide.
7. Allow the slide to dry.
8. Dip the slide in staining solution (Wright-Giemsa stain modified) or add the solution drop by drop to the slide until all the slide surface is covered. Transfer to PBS to wash and let the slide air-dry.
9. Alternatively, the samples can be stained with DAPI (instead of the Wright-Giemsa stain solution) and GelMount solution is applied before covering each slide with a cover slip [12].
10. Slides are viewed under a microscope and at least 20–25 metaphase spreads are examined per preparation. This analysis reveals only gross karyotypic abnormalities or polyploidy.

3.5.5 Differentiation of Harvested Cells into Mesoderm

1. Coat a 12- or 6-well plate with Matrigel or vitronectin.
2. To make 50 ml of the differentiation medium, add 500 μl RPMI vitamins, 500 μl nonessential amino acids, 500 μl sodium pyruvate, 500 μl penicillin/streptomycin, 1000 μl RPMI amino acids, 50 μl chemically defined lipids, 50 μl sodium selenite solution (made at a concentration of 5 $\mu\text{g}/\text{ml}$), and 12.5 μl holo-transferrin (made at a concentration of 2 mg/ml) to DMEM.
3. Use ROCK inhibitor-supplemented E8 medium to seed cells. Count the harvested cells using a cell counter or hemocytometer. Seed cells at a density of $4\text{--}8 \times 10^4$ cells/ cm^2 .
4. Expand the cells for 6 days to a density of around 0.5×10^6 cells/ cm^2 .
5. On day 0 of differentiation (day 6 of expansion) add 30 ng/ml BMP4 and 100 ng/ml Wnt3a to the differentiating medium. Incubate the cells for exactly 24 h.

6. Aspirate the medium and add fresh differentiation medium after 24 h (day 1) of differentiation.
7. On day 2 of differentiation, or exactly 24 h after medium change, add KY0211 at a ratio of 1:1000 to the differentiating cells. This medium can be used henceforth and replaced daily.
8. On day 5 of differentiation, test for KDR, PGDFR α , and C-KIT using flow cytometry and qPCR.
9. Mesodermal cells are KDR⁺/PGDFR α ⁺ as well as C-KIT^{low} [13, 14].

3.5.6 Differentiation of Harvested Cells into Ectoderm

1. Coat a 6-cm dish with Matrigel or vitronectin. Seed the harvested cells onto the dish.
2. Make neural induction medium (NIM) and neural proliferation medium (NPM) [15]. To make 50 ml NIM, add 500 μ l N2 supplement, 500 μ l glutamax supplement, 500 μ l B27 supplement, and 24.25 ml neurobasal medium to 24.25 ml of DMEM/F12. To make 50 ml NPM, add 250 μ l N2 supplement, 250 μ l B27 supplement, 500 μ l glutamax supplement, 20 ng/ml FGF2, and 24.25 ml neurobasal medium to DEMD/F12.
3. When the cells have reached confluence, use collagenase to dissociate the cells into aggregates: incubate cells in 2 ml of collagenase (2 mg/ml) for 5 min. Scrape the colonies using a 10 ml glass pipet. Add an equal volume of NIM to the aggregates and centrifuge the suspension at $200 \times g$ for 5 min. Using a 1 ml pipette tip, seed the cells on a low-binding 6-cm dish.
4. Culture the cells in NIM for 6 days, changing medium every day, and then culture them in NPM for 7 days. Test for upregulation and translation of Nestin and β III-tubulin using qPCR and immunohistochemistry.

4 Notes

1. Matrigel is routinely used for the culture of hPSCs in dishes or suspension. This substrate however is a chemically undefined matrix derived from mouse sarcoma cells. And while Matrigel provides cues for maintaining the pluripotency of stem cells [16], its use is limited to the culture of stem cells intended for in vitro use. Recombinant vitronectin has recently been shown to support cultured stem cells.
2. Although E8 is the medium described here, other media such as the mTeSR (Stemcell Technologies) can be utilized for the same procedures.

3. If cells do not attach properly the following can be considered: (1) Cells need to be in the exponential growth phase. If the colonies are too large, or the dishes are too confluent, the cells may not attach and grow properly. (2) If the cells are not gently seeded onto the beads, cell viability may be reduced by bead damage to the cells. (3) Shaking the dish at regular 15-min intervals during the seeding phase is important as cells may stick to the dish surface instead of the microcarriers.
4. Paraformaldehyde solution preparation along with pH measurement should be carried out in a fume hood wearing personal protective equipment such as lab coat, gloves, and goggles.
5. For flow cytometry and immunostaining, the blocking serum depends on the species in which the secondary antibody is synthesized. We use NDS with donkey-generated secondary antibodies.
6. Although a general protocol for immunostaining is given, following the manufacturers' instructions for the blocking and cell permeabilization steps is recommended. The given procedure can serve as a starting point for most antigen detection. However, in some cases such as flow cytometry detection of surface markers, live-cell staining can also be implemented.

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