

# 4

## Derivation of human embryonic stem cell lines

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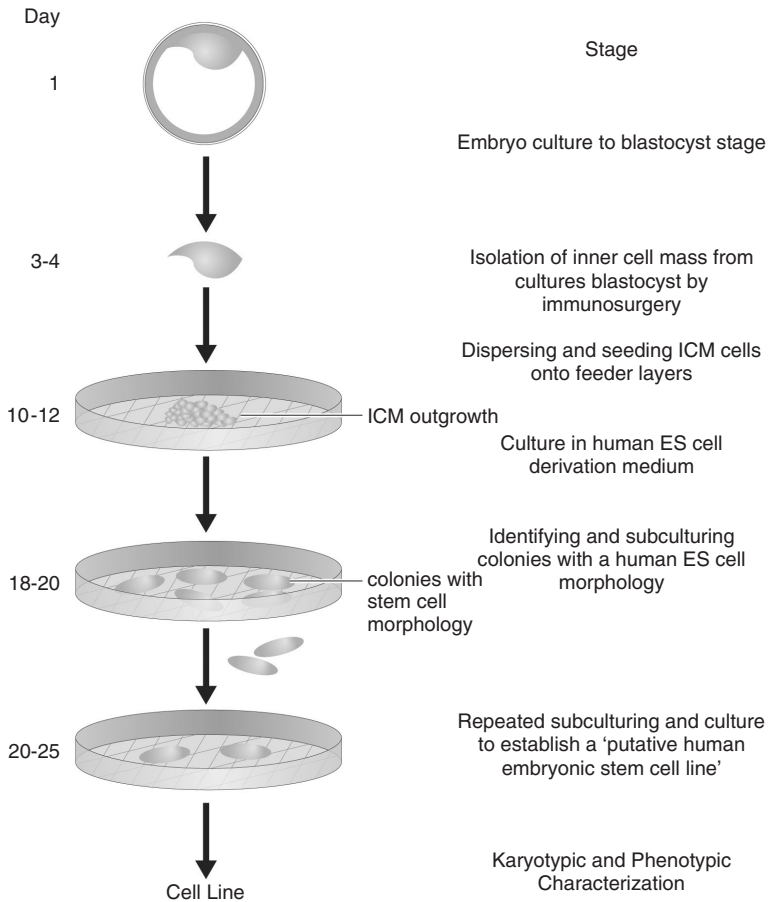
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### Introduction

Human embryonic stem cells are culture artifacts derived from ICM cells placed in a culture system selecting for the capacity to proliferate. They are an important new experimental system for studying human development and modelling human disease (Trounson, 2005). There is, and will continue to be, a need for deriving new human ES cell lines for both basic research and clinical applications due to loss of genetic and epigenetic stability during human ES cell culture and manipulation (Cowan *et al.*, 2004; Ludwig *et al.*, 2006; Buzzard *et al.*, 2004; Maitra *et al.*, 2005) and the considerable variance within the human genome between individuals (Redon *et al.*, 2006).

It is still not known why specific lines have different levels of robustness and proclivities to differentiate into specific lineages, although variables in culture history and genetic background of the lines will likely play a large part (Allegrucci and Young, 2006). How such variables affect these innate characteristics of the lines is currently a focus of interest in the field. There is also interest in how ICM cells change in culture to become human ES cells and how these cells can change in culture. The more lines that are generated, the clearer an idea we will have as to what is happening.

Traditionally, human ES cells have been derived from day 5/day 6 blastocysts and propagated on mouse feeder layers. Using the method presented here, components of animal origin are present in the culture; however, this is likely only to be a concern for clinical applications. At present, this derivation method is still a robust method



**Figure 1** Overview of human embryonic stem cell derivation. Deriving new human ES cell lines requires more than 3 weeks uninterrupted and focused effort. Human embryos are cultured to blastocyst stage as described in **Chapter 3**. The ICM is removed from the blastocyst by immunosurgery and large clumps of ICM cells are transferred to a good quality feeder layer. After several days, ICM outgrowths will appear. Those with a human ES cell morphology (i.e. cells with prominent nucleoli tightly packed together, growing in a flat 2-D morphology) are isolated and subcloned. Successive culture and subcloning produce 'putative human ES cell lines'. These should be extensively characterized (see **Chapters 7, 8, 9 and 10** for details) to verify they are new human ES cell lines.

for deriving new human ES cell lines, requiring fewer embryos than for derivations under xeno-free conditions (Lee *et al.*, 2006). Given the scarcity of human embryos available to most laboratories worldwide, this method will likely be the most used protocol for some time to come. This process of derivation consists of several steps that are highlighted in **Figure 1**.

## Overview of protocol

This chapter assumes the human embryos have been cultured to blastocyst stage as described in **Chapter 3**. The derivation process lasts typically between 3 and 6 weeks and involves preparation of mitotically-inactivated feeder layers, isolation of the inner cell mass (ICM) from cultured blastocysts, seeding the ICM clumps on feeders layers, culturing these explants in human ES cell derivation medium and subculturing resulting outgrowths bearing a human ES cell morphology.

## Materials, reagents and equipment

Sterile phosphate buffered saline without  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  (PBS), pH 7.2 (INVITROGEN/GIBCO, Cat#1404-133)

Gelatin, Type A from porcine skin (SIGMA Cat#G1890)

10 and 15 cm tissue culture dishes (FALCON, Cat#35-3005 and Cat#35-3025 respectively)

Industrial single-edged razor blades (VWR, Cat#55411-050)

Knockout Dulbecco's modified Eagle medium (Knockout DMEM; INVITROGEN/GIBCO, Cat#10829)

KO-Serum Replacement (INVITROGEN/GIBCO, Cat#10828-018)

Plasmanate (BAYER, Cat#0026-0613-20)

Fetal bovine serum (HYCLONE, Cat#SH30070.03)

Glutamax-I (INVITROGEN/GIBCO, Cat#35050-061)

Non-essential amino acids (INVITROGEN/GIBCO, Cat#11140050)

50 units/mL penicillin and 50  $\mu\text{g}/\text{mL}$  streptomycin (INVITROGEN/GIBCO, Cat#15070-063)

$\beta$ -mercaptoethanol (INVITROGEN/GIBCO, Cat#21985-023)

12 ng/mL recombinant hLIF (CHEMICON INTERNATIONAL, Cat#LIF1010)

basic Fibroblast Growth Factor (bFGF, INVITROGEN/GIBCO, Cat#13256-029)

0.05% trypsin/EDTA (INVITROGEN/GIBCO, Cat# 25300-054).

Acid tyrodes (SPECIALTY MEDIA, Cat#MR004-D)

Derivation media (SIGMA, Cat#S1-1939)

Collagenase IV (INVITROGEN/GIBCO, Cat#21985-023)

Rabbit anti-human RBC antibodies (INTERCELL TECHNOLOGIES, Cat#AG28840)

Guinea pig sera complement (SIGMA, Cat#S1639)

Isopropanol

0.22  $\mu$ m 500 mL steritop filter units (MILLIPORE Cat#SCGPS05RE)

Mitomycin C (Sigma Cat#M-0503)

Mycoalert Mycoplasma Detection Assay (Cambrex, Cat#LT07-218)

## Equipment

37°C water bath

Flame pulled thin capillaries

Mouth-controlled suction device or Stripper micropipettor (MID-ATLANTIC DIAGNOSTICS Cat#MXL3-STR) with 175  $\mu$ m capillary tubes (MID-ATLANTIC DIAGNOSTICS Cat#MXL3-175)

Dissection microscope

Inverted microscope

Heated microscope stage

Laminar air hood with a HEPA filter

Tissue culture incubator

CO<sub>2</sub> monitor

Thermometer

Hygrometer

Sterile door strips/ gloves/

## Media/Solutions

MEF Medium (90% Dubelbecco's Modified Eagles Medium, 10% Fetal Bovine Serum, 50 units/mL penicillin and 50  $\mu$ g/mL streptomycin).

Freezing Medium (90% Fetal Bovine Serum, 10% DMSO)

Human ES cell derivation medium (80% Knockout DMEM, 10% KO-Serum Replacement, 10% Plasmanate, 2 mM Glutamax-I, 1% non-essential amino acids, 50 units/mL penicillin and 50  $\mu$ g/mL streptomycin, 0.055 mM beta-mercaptoethanol, and 5 ng/mL bFGF).

Human ES cell growth medium (80% Knockout DMEM, 20% Serum Replacement, 4 ng/mL bFGF, 2 mM Glutamax-I, 0.055 mM beta-mercaptoethanol)

Complement solution (dilute sera complement 1:10 in human ES cell derivation medium prior to use. Aliquot prior to use.)

0.1% Gelatin (1 g gelatin in 1 L MilliQ quality water, followed by sterile filtering)

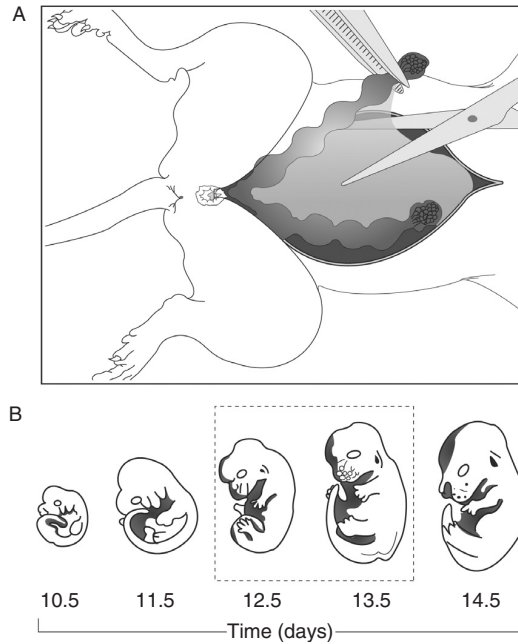
Mitomycin C solution (1 mg mitomycin C powder is dissolved in 100 mL KO-DMEM and filter sterilized. Aliquot prior to use.)

## Protocols

### Derivation of mouse embryonic fibroblasts (MEFs)

Cull a pregnant ICR mouse (12.5–13.5 days post coitum) in accordance with local animal welfare guidelines. Use two methods of culling (e.g. CO<sub>2</sub> or avertin exposure followed by cervical dislocation) to verify the animal is dead prior to dissection.

1. Place the mouse facing upward on the bench and soak its fur with 70% ethanol. Soaking the fur minimizes the risk of contamination with mycoplasma and other organisms and also prevents fur getting in the way of the dissection.
2. Dissect the abdomen, unravelling and pushing the intestinal tract to one side to reveal the uterine horns. Extract the uterine horns by snipping above the genitalia and just below the ovaries as shown in **Figure 2(A)**.
3. Transfer the uterine horns to a fresh dish of 1× PBS supplemented with 1× Pen/Strep (PBS+P/S). Cut the uterine wall between individual embryos and using a forceps, tease the embryos into the PBS+P/S by tearing the mesometrium with two sets of sharp tipped forceps. Day 12.5–13.5 embryos should resemble those shown in **Figure 2(B)**.
4. Transfer the embryos to a fresh 10 cm Petri dish with 10 mL PBS+P/S. Hold the head of the embryo with one pair of blunt forceps, and carefully remove all the red viscera using sharp forceps with a pecking motion.
5. Cut off the limbs and tail of the embryo using the forceps. Finally, remove the head of the embryo and transfer the remaining trunk to a new Petri dish with PBS+P/S.
6. Mince the eviscerated embryo sections very well with two razor blades and transfer the pieces to several milliliters of prewarmed 1× trypsin EDTA (2 mL/embryo). Triturate the cells continuously until the trypsin solution becomes cloudy with liberated cells. Be careful not to over-trypsinize the cells as this will lead to lysis, clumping and loss of cells. About 5 minutes continuous vortexing of the embryo pieces should be sufficient.
7. Neutralize the protease with MEF medium (1 mL/embryo) (serum in the medium contains trypsin inhibitor and so stops the reaction).
8. Let the cell suspension sit for 5 minutes. Adipose tissue will generally float to the top of the supernatant, and cell clumps will fall down to form a pellet. Remove



**Figure 2** Murine embryo extraction for feeder preparation. (A) The uterus from a 12.5–13.5 dpc pregnant mouse is removed as follows: After a longitudinal section is made from above the diaphragm to the genitalia, the intestinal tract is unwound and pushed to one side, revealing the uterine horns behind. The uterine horns are held up with forceps and a sharp scissors is used to snip the mesometrium so they can be separated from the abdominal cavity. Subsequently, the embryos are removed from the uterine horns. (B) Typical embryos from day 12.5 to 13.5: the embryos should be similar in size and development to those shown.

the fat with a pipette, and carefully transfer the cell suspension to a fresh 15 mL centrifugation tube without disturbing the pellet.

9. Pellet the cells by centrifugation (300  $g$ /~1,000 rpm for 5 min).
10. Resuspend the cells in MEF medium (1 mL/embryo). Measure cell number by counting an aliquot of the cells using a haemocytometer and seed the flask or dish to be used for generating feeder layers with  $1 \times 10^5$  MEFs/cm<sup>2</sup>.
11. Add additional MEF medium to the dishes/plates so the medium is at least 0.5 cm in depth.

12. Leave the cells to sit down overnight in the incubator (37°C, 5% CO<sub>2</sub>) before expanding these cultures and mitotically inactivating them.

Note: Remember MEFs and other primary cell cultures are a common source of microbial contamination in the cell culture facility. To reduce the risk of contamination, always soak euthanized mice in 70% ethanol prior to embryo extraction, dip uterine horns in 70% ethanol before embryo removal, and culture the MEFs in medium supplemented with 1× Pen/Strep for 2 days after the MEFs are derived.

Note: MEFs should be specifically screened for mycoplasma (See **Chapters 1 and 2** for more on mycoplasma) before being used as feeders for human ES cell culture. We recommend an enzymatic assay [Mycoalert mycoplasma detection kit made by Cambrex [www.Cambrex.com](http://www.Cambrex.com)] as a quick and straightforward way to screen cultures for mycoplasma. To increase the sensitivity of this assay, MEFs should have been cultured without antibiotics for at least 48 hr prior to the mycoplasma screening.

### Generation of mitotically-inactivated feeder layers

Proliferating MEFs will compete with human ES cells for media nutrients and space so feeder layers are mitotically-inactivated before human ES cells are seeded on them. There are two commonly-used ways to mitotically-inactivate MEFs: treatment with drug mitomycin C or exposure to  $\gamma$  irradiation.

#### *Mitomycin C treatment*

1. Culture the MEFs in 10  $\mu$ g/mL mitomycin C for 2 hr at 37°C, 5% CO<sub>2</sub>.
2. It is important to wash as much mitomycin C away after the MEFs have been inactivated to form feeder layers, otherwise the human ES cells may themselves be affected by the drug. Aspirate the mitomycin C solution and wash the feeders thoroughly four times with PBS.
3. Disaggregate feeders with warm 0.25% trypsin/EDTA solution. It is important to minimize the exposure of the feeders to trypsin/EDTA as the cells will begin to senesce or lyse. After about 1 min, the cells will 'round up'. Repeatedly tap the flask off the bench to help dislocate the feeders and closely monitor them under the microscope to identify when most have been dislodged. All in all this process should take less than 2 min.
4. Add 0.5–1 volumes of serum-containing MEF medium to stop the trypsinization reaction. Pellet the feeders down by centrifugation (300 g/ $\sim$ 1,000 rpm, 5 min).
5. Seed flasks, dishes or wells with 1–2  $\times$  10<sup>5</sup> cells/cm<sup>2</sup>. Wash the cells three times in PBS to remove serum that will cause differentiation of human ES cells. Grow the cells in human ES cell medium overnight.

- If feeders are not immediately required or an excess has been generated, the cells should be frozen and stored in liquid nitrogen as described in **Chapter 5**.

### *$\gamma$ irradiation*

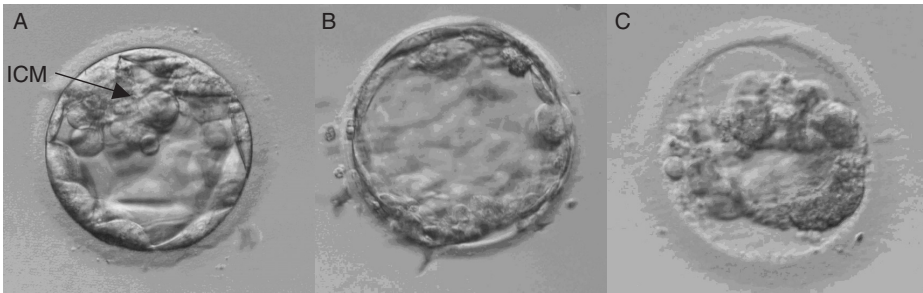
- Culture freshly isolated MEFs to confluence and split 1:5 (generating passage 2 MEFs).
- Once these passage 2 MEFs are confluent, trypsinize and resuspend them in a few milliliters MEF media, (we routinely resuspend a pellet generated from a confluent T175 flask of MEFs into 3 mL MEF medium). Irradiate the MEF pellet for 25 min at 200–250 Rad/min for a total exposure of 5,000–6,250 Rad.
- Pellet the irradiated cells (300 g/~1,000 rpm, 5 min).

### Seeding feeder layers

- Treat the culture vessel with 0.1% gelatin and allow the gelatin to sit for at least 5 min before aspirating it off. This gelatine preparation prevents ‘rolling-up’ of confluent feeder layers.
- Mitotically inactivated MEFs should be seeded onto gelatinized culture vessels with a density of ~50,000 cells/cm<sup>2</sup>.

### Isolation of the inner cell mass from cultured blastocysts

**Figure 3** shows high and low quality blastocysts prior to immunosurgery. Derivation efficiency is at a maximum when high-quality blastocysts are used. Immunosurgery



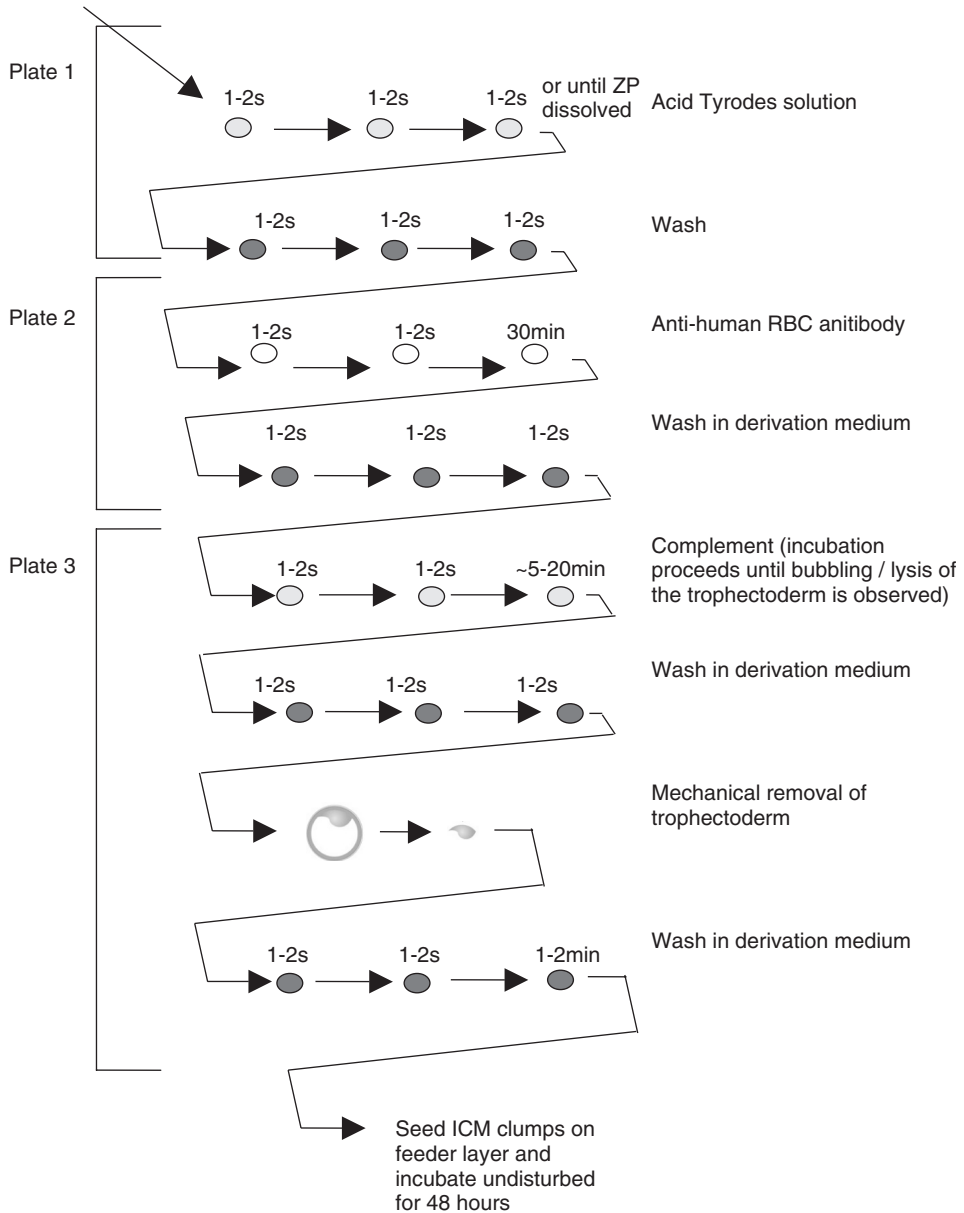
**Figure 3** Day 1 Embryos cultures to blastocyst stage. (A) A good quality blastocyst (there is obvious cavitation, and large numbers of cells in the ICM, no graininess or lysis). (B) shows a blastocyst with few ICM cells (this can still produce a human ES cell line). (C) shows a necrotic embryo (extensive graininess and lysis).

allows the removal of the ICM from individual blastocysts and consists of three main steps:

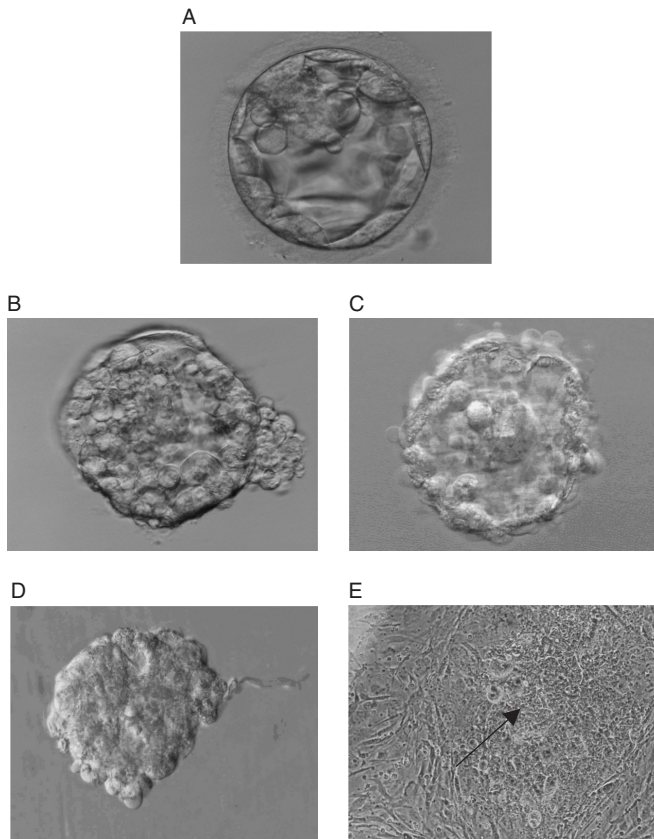
- (a) Dissolving the zona pellucida with acid tyrodes solution.
- (b) Selectively killing the trophoctoderm by complement-mediated lysis. The blastocyst is incubated with anti-human RBC antibody and subsequently complement. The cells of the trophoctoderm layer have tight junctions between them providing a physical barrier which protects the ICM cells such damage.
- (c) Removal of the ICM from the lysed trophoctoderm cells.

**Figure 4** shows an overview of the immunosurgery procedure and **Figure 5** shows blastocysts undergoing immunosurgery.

1. As the success of this procedure is dependent on exposing the blastocyst for the correct duration, and thus requires constant observation under the microscope, it is best to perform immunosurgery on one embryo at a time.
2. Prepare Petri dishes for embryo immunosurgery as follows: Make a series of 30  $\mu\text{L}$  microdrops across three plates (see **Figure 4**): three of acid Tyrodes solution, three of derivation medium in the first dish (Plate 1); three of antibody solution, three of derivation medium, and three of Complement in the second (Plate 2), and six of derivation medium for the third (Plate 3). The drops are covered with embryo-tested mineral oil (to prevent evaporation) and while Plate 1 is left at room temperature, Plates 2 and 3 are placed in an incubator (5%  $\text{CO}_2$ , 37°C) for at least 1 hr and only removed directly prior to use.
3. Place a heating plate onto the stage of the dissection microscope and set the heating plate temperature to 37°C.
4. Prepare the mouth controlled suction device (assemble mouthpiece, rubber tubing, holder and glass pipette) and triturate  $\sim 200 \mu\text{L}$  FBS using the pipette. Coating the pipette with serum prevents the embryo sticking to the inside of the glass pipette during transfer. Avoid the aspiration of mineral oil into the glass pipette.
5. Remove dishes containing the embryo cultured to blastocyst stage, and the dish with microdrops from the incubator (See **Chapter 3** for details of human embryo culture). Locate the blastocyst under the microscope and transfer the embryo to the first microdrop of acid Tyrodes solution (AT) using a mouth pipette. Move the blastocyst from drop to drop as shown in **Figure 4**. The embryo should spend 1–2 s in each drop of AT. Watch the embryo carefully under the microscope as it is transferred from drop to drop. When the embryo has been transferred to the third drop of AT, the zona pellucida should have become thinner and be nearly digested. Transfer the embryo through the second row of microdrops containing derivation medium to neutralize the AT and then to the third row containing microdrops of the human antibody solution.



**Figure 4** Extracting ICM by immunosurgery. This schematic shows the various steps and incubation times for extracting the ICM from the blastocyst. The small ovals represent microdrops in the dishes between which the embryo is transferred. Acid Tyrodes treatment and initial washing of the embryo is done in Plate 1, antibody incubation, second washing and complement incubation is carried out in Plate 2. Additional washing and trophoblast removal is carried out in Plate 3 after which the ICM is seeded on a feeder layer.



**Figure 5** Day 3–4 Isolation of inner cell mass from cultured blastocyst by immunosurgery. (A) Embryo before incubation acid tyrosides (AT) solution. (B) Embryo after AT incubation, see the thinning of the zona pellucida. (C) Collapsed embryo after immunosurgery, note the trophoblast ‘bubbling’. (D) ICM after stripping of lysed trophoblast layer. (E) Clump of ICM cells attached to feeder layer.

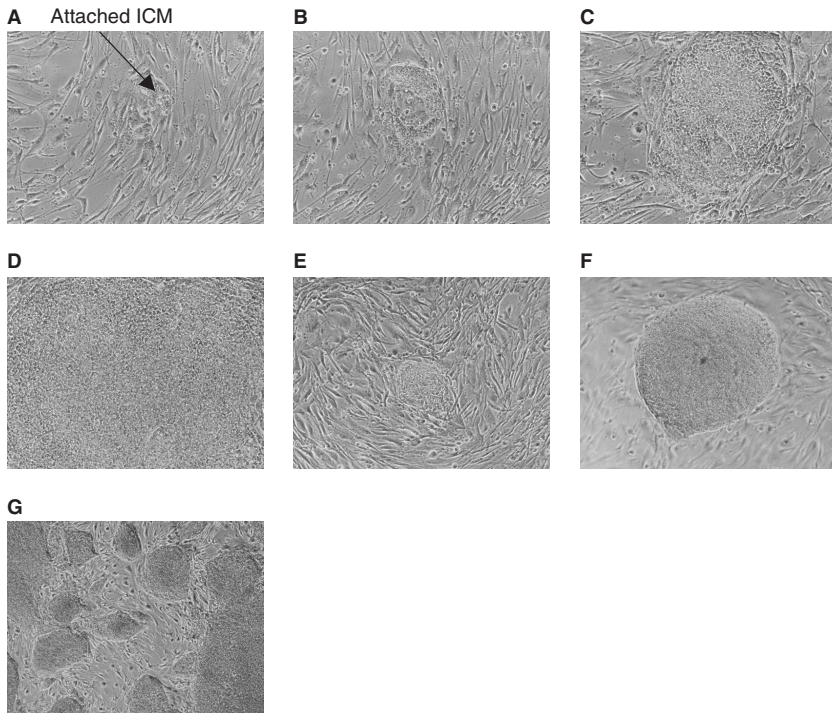
6. Transfer the embryo from the first to the third drop of antibody solution (1–2 s in the first and second microdrops). Once the embryo is in the third drop of antibody solution, the dish is placed back in the incubator for 30 min.
7. Remove the dish and place it back on the heated stage. Resume transferring the embryo from drop to drop using the mouth pipette initially through the fourth row of derivation medium (to wash off excess antibody) and then the fifth row of complement (1–2 s in the first and second drops of complement). Once the embryo has been transferred into the third drop of complement, place the dish back in the incubator. Leave for 15 min.

8. Place the dish back on the heated stage and observe the embryo for lysis of the trophoctoderm cells and the collapse of the embryo in on itself. This Complement-mediated lysis is commonly described as trophoctoderm ‘bubbling’ as the cells become large, transparent and bubble-like (See **Figure 5**). If no such ‘bubbling’ is evident, place the dish back in the incubator. Extend the incubation by intervals of 5 min until the embryo has collapsed and all trophoctoderm cells are lysed. The incubation time for the embryo in complement should not be longer than 30 min.
9. After washing the embryo a third time by transferring through all drops of the sixth row, it is carefully drawn through a thinly drawn pipette, the bore of which is just smaller than the diameter of the embryo. The ICM should detach from the lysed trophoctoderm cells with 1–2 draws up the pipette.
10. Transfer the detached ICM cell cluster to microdrops of human ES cell derivation medium and proceed as shown in **Figure 4**. Finally, transfer the ICM to a well of a four-well plate seeded with mitotically-inactivated feeders and containing pre-warmed human ES derivation medium. Place this plate back into the incubator and do not disturb for at least 48 hr, after which attachment and outgrowth should be evident.

### Breaking up the ICM, growing explants and analyzing colony morphology

At this stage of the derivation process, the goal is to increase ‘putative human ES cell’ number, and not to be too much concerned with removing differentiated from the outgrowths. However, explants should not be allowed to become over-confluent as all cells will begin to differentiation. Here now are some general notes on maximizing derivation efficiency:

- During the initial dispersion of the ICM-derived outgrowth, a part of the initial outgrowth should be left intact, in case the reseeded clumps fail to attach and/or grow after transfer to new wells.
- Do not be tempted to expand the cultures too quickly, or to treat the cells too harshly, breaking them into clumps that are too small to sustain proliferation.
- Remember not to seed the feeder layers too densely (should seed at  $>50,000$  cells/cm<sup>2</sup>) - MEFs form cell clusters that can easily be mistaken for early human ES cell outgrowths.
- Partially change medium when the original medium on the cells becomes acidic (medium becomes light pink/yellow). If changed too often, medium will have less auto-stimulatory factors secreted from the human ES cells themselves that encourage their proliferation.
- Mechanical disaggregation of human ES cell colonies is described in detail in **Chapter 5 (Routine Protocols)**.
- Colonies displaying a human ES cell morphology, are most readily identifiable as such, by their prominent and numerous nucleoli and their flattened shape of



**Figure 6** Identifying and subculturing colonies with a human cell morphology. (A–D) show the same colony with human ES cell morphology at days 5, 7, 10, 13. The colony is ready to mechanically disaggregate at day 13. (E) shows a P1 colony from this mechanical disaggregation. (F) shows a P2 colony. (G) shows a P5 culture successfully adapted to trypsinization.

their colonies, and the ability to distinguish individual cells within the colony (See **Figure 6**). Other morphologies that also arise from outgrowths include fibroblast-like cells (which are indistinguishable from feeders), spread out trophoblast cells, and epitheloid-like cells.

The protocol outlined using mouse feeder layers has been described in detail here as it has been independently used to repeatedly derive human ES cells in several laboratories over three years and so we consider it our most established protocol.

The use of animal feeders has some disadvantages, such as risks of pathogen transmission and viral infection (Richards *et al.*, 2002; Amit and Itskovitz-Eldor, 2003; Amit *et al.*, 2004; Rosler *et al.*, 2004). Also, foreign sugars may be transferred from feeders to the glycoproteins of the human ES cells (Martin *et al.*, 2005). These concerns, however, will probably only be significant if cells or their derivatives are to be used therapeutically. For purposes of using human ES cells as a basic *in vitro* tool, this protocol has proved sufficient. While some laboratories are actively seeking alternatives to mouse feeders (human fibroblast feeders, human fallopian feeders, feeder conditioned medium, chemically defined medium), we feel these protocols are still

being optimized and have yet to be tested repeatedly in our hands. Should you wish to try one of these alternative protocols, we direct you to the papers cited.

## Adapting newly derived human ES cells to trypsin

Freshly derived lines can be adapted to trypsin as early as passage 3, when you have two wells of a six-well plate confluent with human ES cell colonies. Here are guidelines for successful adaptation of lines to trypsin:

- Initial splits with trypsin should be low i.e. 1:2–3. We trypsinize one of the two wells with trypsin and seed it into two or three new wells, while continuing to mechanically disaggregate cells the other well, in case there is a problem with enzymatic disaggregated cultures.
- Remember that cells are extremely prone of lysis by forces induced by rough pipetting when the cells are in serum or serum derivative containing medium. Do not make the mistake of scraping the cell cultures after the trypsin reaction has been stopped with serum or serum containing medium. Only scrape the wells when the trypsinization reaction is still proceeding, otherwise a large proportion of cells will lyse and seeding efficiencies will be very low.
- Ensure good plating efficiencies by gently pipetting the cells, and by carefully observing the cells under the microscope as they round up after trypsin has been added. As soon as all human ES cells have rounded up and become apparent as individual cells in the colony, wells should be scraped and the reaction stopped with serum or serum containing medium.
- We only trypsinize the cultures to clumps of ten cells or more during these early passages. If you have trouble with trypsinization, disaggregation with accutase or collagenase is recommended. When performing the second and third trypsinization on the culture, we gradually raise the split ratio to 1:2 and 1:4 respectively.
- Some have suggested that enzymatic disaggregation leads to a higher incidence of genetic abnormalities in lines of equivalent passage number [Mitalipova *et al.* 2005]. However, we point out that cultures can be expanded much more readily with enzymatic disaggregation and that comparisons between mechanically and enzymatically disaggregated cultures of equivalent passage is misleading (i.e. cells in trypsinized cultures will have undergone a substantially greater number of population doublings than mechanically disaggregated cultures of equivalent passage).

### Protocol for trypsinization

Routine trypsinization of human ES cells is covered in the Extended Protocols section of **Chapter 5**. For initial adaptation of human ES cell lines to trypsin we proceed as follows:

1. Warm aliquots of 0.05% trypsin EDTA solution and serum containing medium to 37°C in a waterbath.

2. Remove the medium from the well containing the culture to be disaggregated. Rinse the well twice with 1×PBS (1 mL per well of a four-well plate).
3. Add 1 mL warm 0.05% trypsin EDTA solution. Immediately transfer the plate to the heated platform of an inverted microscope and watch carefully as the human ES cells' morphology changes and these cells become refractile and spherical. When about 90% of the human ES cells round up, scrape the well until cell clusters (>10 cells or more) are floating in the medium. Do not wait until the feeders become refractile before stopping the reaction. Add 0.5 mL medium to stop the trypsin reaction.
4. Carefully remove the cell suspension with a P1000 pipette and transfer to a 15 mL centrifuge tube.
5. Pellet the human ES cells by centrifugation (300 g/~1,000 rpm for 5 min).
6. Carefully resuspend the pellet and seed the newly prepared wells according to the appropriate split ratio.
7. Half-change the medium in the well with fresh medium 24 hr after trypsinization. The human ES cell colonies should have adhered to the feeder layers and show signs of proliferation. Some cell death may also be evident and this medium change should remove most of the floating dead cells.

## Troubleshooting

***When performing immunosurgery, the trophoctoderm fails to exhibit bubbling and the embryo does not collapse? What has happened?***

There is a problem with the antibody and/or the complement. Ensure the correct dilutions were made when making up the solutions and that fresh preparations of both solutions are used in the next derivation.

***The ICM fails to attach or proliferate. Why?***

This could be due to overexposure the blastocyst to complement, thereby damaging the ICM cells. It could also be due to use of poor quality feeder layers or medium.

***After trituration through a drawn glass capillary, some trophoctoderm is still attached to the ICM. Should I triturate further at risk of damaging the ICM?***

No, do not triturate further. Removal of all trophoctoderm is not essential. In fact, having some trophoctoderm still present can facilitate attachment of the ICM to the feeder layer and promote outgrowth.

***I have an ICM outgrowth but after mechanical disaggregation it stopped growing. What happened?***

This may be due to the cells being manipulated too roughly, breaking the colonies into two small clumps to support initial proliferation. Be more gentle, pipetting the cells more slowly next time.

***None of my outgrowths produced a line displaying human ES cell morphology that continued to proliferate. What can I change to improve derivation frequencies in the next attempt.***

You should probably change serum replacement/FBS batches. Screening serum batches for human ES cell growth is very important. Seed previously established human ES cell lines at different densities using media supplemented with different batches. Culture the cells for two weeks and assess the cultures morphologically and by immunoFACS (see **Chapter 9** for characterizing human ES cell cultures in this way). Use the serum batch that supports proliferation (but not differentiation or cell death) of human ES cells.

- Feeders provide a matrix and growth factors for your ICM outgrowths. Poor feeder support of the outgrowth can also be the reason the putative human ES cells cease proliferating, always ensure feeders are seeded at the appropriate density ( $\sim 50,000$  cells/cm<sup>2</sup>) are of high quality (e.g. with a passage number  $\leq 3$ , an upright, slender cell morphology, and no evidence of senescence).
- Poor embryo development and explant outgrowth can be due to microbial contamination. Filter and screen all solutions and media prior to using them for derivation of new lines. Microbial contamination and the avoidance of it are discussed in detail in **Chapters 1 and 2**. The media used to derive and culture human ES cells are extremely rich and so are susceptible to becoming contaminated with microbes. Routine use of antibiotics masks contamination which slows cell growth and as these cells are very sensitive the presence of antibiotics can be toxic to the cells. Remember feeders are a common source of contamination. Feeders should always be screened for microbes including mycoplasma as outlined in **Chapter 1**.
- The problem could also be due to physical changes in the cell culture, i.e. temperature, humidity, pH of medium. Make sure your incubator and heated plates are functioning and are set to the correct temperature (37°C). Make sure to keep the water tray in the incubator full of autoclaved water. Change the medium on the cells before it becomes too acidic (i.e. yellow).
- Remember organic solvents and other toxic aerosols are not introduced to the derivation facility. Other harsh chemicals like PFA also should not be used within the derivation facility, refrain from using perfume/aftershave. Human embryonic cultures are very sensitive to such aerosols and they can perturb cell proliferation if present in the atmosphere.

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