

Chapter 2

Methods for Culturing Mouse and Human Embryonic Stem Cells

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Abstract

Mouse embryonic stem cells (mESCs) were first derived and cultured almost 30 years ago and ever since have been valuable tools for creating knockout mice and for studying early mammalian development. More recently (1998), human embryonic stem cells (hESCs) have been derived from blastocysts, and numerous methods have evolved to culture hESCs in vitro in both complex and defined media. hESCs are especially important at this time as they could potentially be used to treat degenerative diseases and to access the toxicity of new drugs and environmental chemicals. For both human and mouse ESCs, fibroblast feeder layers are often used at some phase in the culturing protocol. The feeders – often mouse embryonic fibroblasts (mEFs) – provide a substrate that increases plating efficiency, helps maintain pluripotency, and facilitates survival and growth of the stem cells. Various protocols for culturing embryonic stem cells from both species are available with newer trends moving toward feeder-free and serum-free culture. The purpose of this chapter is to provide basic protocol information on the isolation of mouse embryonic fibroblasts and establishment of feeder layers, the culture of mESCs on both mEFs and on gelatin in serum-containing medium, and the culture of hESCs in defined media on both mEFs (hESC culture medium) and Matrigel (mTeSR). These basic protocols are intended for researchers wanting to develop stem cell research in their labs. These protocols have been tested in our laboratory and work well. They can be modified and adapted for any relevant user's particular purpose.

Key words: Embryonic fibroblasts, Mouse embryonic stem cells, Human embryonic stem cells, Culture methods, Feeder layer

1. Introduction

The first derivation of mouse embryonic stem cells (mESCs) was reported independently in 1981 by laboratories in the USA (1) and in England (2). Success in deriving and culturing mESCs grew from prior experience with teratocarcinoma cells, which required a feeder layer for survival and growth, and the use of fibroblast feeder layers became a standard for the derivation and

growth of mESCs (3). Feeder layers, which are still often used today with mESCs, have been valuable in providing conditions that support survival, proliferation, and the maintenance of pluripotency in stem cell populations. Since their initial isolation in 1981, numerous labs derived new mESC lines and used them to study mammalian development and to create knockout mice (4). Various protocols are now available for culturing mESCs, and to some extent, specific protocols work best with lines derived from specific strains of mice (3).

In 1998, Dr. Thomson's laboratory at the University of Wisconsin reported, for the first time, that embryonic stem cells can be derived from human blastocysts and propagated in vitro, opening the possibility of creating pluripotent cell lines with the potential to treat and cure numerous human diseases (5). Initially, culturing protocols for human embryonic stem cells (hESCs) involved the use of feeder layers as substrates and media containing serum and animal proteins. However, feeder layers add complexity to stem cell cultures and have the potential to introduce animal viruses and unwanted immunogens into the stem cell populations, which would preclude the use of hESCs grown on feeders in therapeutic applications. Subsequently, various protocols have been developed for feeder-free culturing of hESCs. The first of these replaced the feeders with Matrigel (6) and replaced serum with medium conditioned by murine embryonic fibroblasts (mEFs) (7). While Matrigel is readily available and easy to use, it is not strictly defined, may vary from lot to lot, and contains animal proteins. There has, thus, been interest in refining the substrate, and other options, such as laminin and fibronectin, have been successful (7, 8).

Media conditioned by fibroblasts or media containing serum have been valuable in initial work on embryonic stem cells, but there is a trend to eliminate these undefined components, especially from hESC culture protocols. Serum is highly variable from lot to lot and in some instances may promote differentiation rather than pluripotency of embryonic stem cells. Likewise, media conditioned by fibroblasts is not defined and contributes animal proteins to the culture milieu. New defined media formulations (containing some animal proteins) that work well with embryonic stem cells, such as Knockout Serum Replacement (SR or KSR) (Invitrogen) and mTeSR (StemCell Technologies), have been developed recently (9, 10) and are commercially available at affordable prices. More refined xeno-free media can also be purchased at a higher cost for hESC work demanding animal-protein-free media. Recently, three-dimensional culture systems, which would be beneficial for differentiation of hESCs, have also been introduced (11–13). Culture methods for ESCs are continually evolving, and protocols can be expected to improve and become more accessible in the future.

While many different protocols have been developed for both mESCs and hESCs and while the exact choice of media and culture conditions will be determined by the needs of individual investigators and purpose of their work, we describe in this review the fundamental methods needed for isolating mouse embryonic fibroblasts (mEFs) that can be used as feeder layers for both mouse and human ESCs. We also present a standard method for culturing mESCs in a serum-containing medium. It is possible to grow mESCs in serum-free knockout medium, and this would be an alternative strategy that might be preferable depending on the research goal. Finally, we present methods for growing hESCs on both mEFs and Matrigel in defined conditions using hESC medium in which serum is replaced with KSR or a new medium, mTeSR, developed by Ludwig and Thomson (9). We have had good success with hESC culture medium and mTeSR medium, both of which can be used with induced pluripotent stem cells (iPSCs) as well as hESCs. This set of protocols is intended for those who have not worked with ESCs before and need a starting point for accomplishing basic steps that would lead to setting up embryonic stem cell experiments in their laboratory. We present protocols as we often perform them in our laboratory. Amounts and sizes of preparations can be scaled up or down as needed. Once these methods have been mastered, numerous other techniques can be used in conjunction with ESCs, or more advanced methods of culturing can be added to this starting repertoire.

2. Materials

2.1. General Considerations

All methods described in this chapter need to be done using strict sterile technique. For more information on sterile technique, see the reviews by Phelan and by Cote (14, 15). For more information on contamination, how to prevent it, and how to deal with it when it occurs, see the excellent technical bulletin by Ryan (16). Testing for Mycoplasma infection of cultures should be done routinely (16). We recommend that all methods be done in a clean room, which is accessed only by users of the room. Individuals entering the clean room should step onto a sticky mat and then put on lab coats, booties, and masks, all of which are always kept in the clean room (disposable sticky mats, lab coats, latex gloves, face masks, and booties can be purchased from internet sources at considerable savings).

Floors should be cleaned at least once a week by users. Friday is a good time for this so that the clean rooms are ready for use at the beginning of the next week. All items that are removed from the sterile hoods (e.g. centrifuge tubes) should be sprayed with 75% ethanol, which is allowed to evaporate before returning them

to the hood. Controlling contamination will be easier if air entering the rooms is HEPA-filtered. The need for fastidious sterile technique is especially important for hESCs, which are usually cultured without antibiotics.

2.2. Culturing Mouse Embryonic Fibroblasts

2.2.1. Isolating mEFs

1. Ethanol spray bottle (75%). All items going into the sterile hoods should be sprayed with ethanol, which will help kill microorganisms upon evaporation.
2. Stereoscopic (dissecting) microscope.
3. Pipet-Aid (i.e. Drummond).
4. Sterile plastic pipettes (10 and 25 mL, individually wrapped).
5. Trypsin/Ethylenediaminetetraacetic acid (EDTA): 40 mL of 0.25% trypsin is used for isolating mEFs, while 0.05% trypsin/EDTA solution is used for passaging cells.
6. Sterile stir bar and stir plate. Sterilize by allowing 75% ethanol to evaporate from their surfaces.
7. Autoclaved 100-mL Erlenmeyer flask (for use with one to two mice for isolating mEFs).
8. Autoclaved aluminum foil (small piece to cover Erlenmeyer flask).
9. Sterile deionized water (dH_2O).
10. Phosphate-buffered saline (PBS) without Mg^{2+} and Ca^{2+} (2,600 mL dH_2O , 8.3 mg $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$, 28.4 mg Na_2HPO_4 , pH = 7.4). Autoclave and store at 4°C .
11. 0.2% gelatin (i.e. Sigma, tissue culture grade) in PBS.
12. T75- cm^2 tissue culture flasks: When isolating mEFs, generally 4–5 T75- cm^2 flasks are needed for each pregnant mouse. When passaging mEFs or mESCs, we normally use T25- cm^2 flasks.
13. One or two pregnant female mice: for isolating mEFs, mice should be used between day 12.5 and 13.5 of pregnancy (day 1 = first day after mating) (see Note 1).
14. Autoclaved paper towels.
15. Sterile dissecting tools (scissors, two pairs of forceps, scalpel). Sterilize by autoclaving.
16. Sterile 60-mm diameter petri dishes (i.e. Falcon, Fisher Scientific).
17. DNase (100 $\mu\text{g}/\text{mL}$) (i.e. Sigma, 10 mg/mL at 10,000 total units).
18. Heat-inactivated and filtered fetal bovine serum (FBS): thaw FBS in a 37°C water bath (if frozen) until serum is liquid. Gently shake and then incubate at 56°C with whole volume of serum immersed in water bath for 30 min (do not immerse cap).

Swirl every 5–10 min and when done, cool in an ice bath. Do not overheat. Serum should be filtered (0.2- μ m filter), aliquoted, and stored frozen at -20°C until needed.

19. Stericup filter flask (0.22 μ m, 150 mL or larger depending on volume of medium being made).
20. mEF medium: 500 mL of high-glucose Dulbecco's Modified Eagle's Medium (DMEM), 6 mL of 200 mM L-glutamine (Invitrogen), 6 mL of penicillin/streptomycin (5,000 units of penicillin + 5 mg/mL streptomycin), and 55 mL of heat-inactivated FBS. After combining ingredients, sterilize using a 0.22- μ m filter flask and store at 4°C for up to 2 weeks. Medium can also be made in smaller batches.
21. 100- μ m cell strainer (BD Falcon, 100 μ m nylon).
22. Sterile 50-mL conical tubes.
23. Inverted microscope (phase contrast or Hoffman modulation).

2.2.2. Freezing and Passaging mEFs

1. Ethanol spray bottle (75%). All items going into the sterile hoods should be sprayed with ethanol, which will help kill microorganisms upon evaporation.
2. Cryovials (1.8 mL).
3. mEF freezing medium: 20% dimethyl sulfoxide (DMSO) and 80% mEF medium (see Note 2).
4. 0.25% trypsin/EDTA (Invitrogen).
5. mEF medium (see above).
6. Sterile 50-mL conical tubes.
7. T25-cm² Nunc flasks (Fisher).
8. 0.2% Gelatin (i.e. Sigma, tissue culture grade) in PBS (see Note 3).
9. Sterile 15-mL conical tubes.
10. Pipettes (P1000, P200, P20) and sterile tips appropriate for each pipette.

2.2.3. Preparing mEF Feeder Layers for Subsequent Culture of mESCs

1. mEFs Isolated from these animals or mEFs purchased from commercial sources (e.g. ATCC, StemCell Technologies) which will be used to make feeder layers for mESCs and hESCs.
2. 0.2% Gelatin (i.e. Sigma, tissue culture grade) in PBS.
3. Ethanol spray bottle (75%). All items going into the sterile hoods should be sprayed with ethanol, which will help kill microorganisms upon evaporation.
4. mEF medium (see above).
5. Sterile 50-mL conical tubes.
6. T25-cm² Nunc flasks (Fisher).

7. Sterile 15-mL conical tubes.
8. Pipettes (P1000, P200, P20) and sterile tips appropriate for each pipette.
9. Radiation source that can produce 8,000 rads.
10. Leukemia inhibitory factor (LIF, Millipore), see Note 4.
11. Low LIF mESC medium: 125 mL DMEM, 22.5 mL FBS (various sources), 1.5 mL of 100 mM sodium pyruvate (Invitrogen), 1.5 mL of 100× nonessential amino acids (ATCC, catalog no. 30-2116), 1.5 mL of 200 mM L-glutamine, 750 μ L penicillin/streptomycin (5,000 units of penicillin + 5 mg/mL streptomycin), 1 μ L of 2-mercaptoethanol (99%, tissue culture grade, Sigma, Catalog no. M7522), and 0.4 μ L of LIF from the stock bottle (10^6 units/mL).
12. Mitomycin C: reconstitute mitomycin C in PBS (without Mg and Ca) and 5% DMSO to reach a final concentration of 1 mg/mL (e.g. 0.1 mL DMSO + 1.9 mL PBS + 2 mg mitomycin C). Mix ingredients carefully until all of mitomycin C powder has dissolved (see Notes 5 and 6).
13. mEF medium + mitomycin C: for each T25-cm² flask of mEFs, 4 mL of mitomycin C containing medium is used. The final concentration of mitomycin C is 10 μ g/mL (e.g. for every 10 mL of mEF medium, add 100 μ L of aliquoted mitomycin C solution).
14. Waste beaker (500 mL).
15. 0.05% trypsin/EDTA solution.
16. Sterile 15-mL conical tubes.

2.3. Culturing Murine Embryonic Stem Cells

1. Mitotically inactivated mEF feeder layers growing in T25-cm² flasks.
2. Murine embryonic stem cells (e.g. the D3 line from ATCC).
3. Low LIF mESC medium (see above).
4. Ethanol spray bottle (75%).
5. Sterile 15-mL conical tubes.
6. Pipettes (P1000, P200, P20) and sterile tips appropriate for each pipette.
7. mESC freezing medium: mESC medium plus 10% FBS, and 10% DMSO. For one T25-cm² flask, 4 mL of medium should be sufficient. (The amount can be scaled up if more than one flask is used or if a larger flask is used).
8. 0.2% gelatin (i.e. Sigma, tissue culture grade) in PBS.
9. PBS without Mg²⁺ and Ca²⁺ (see above).
10. 0.05% trypsin/EDTA solution.

11. High LIF mESC medium: as described above for low LIF mESC medium, except for LIF, make a small amount of working solution by diluting stock solution (10^6 units/mL) tenfold, and add 1 μ L of working solution for each mL of high LIF mESC medium (e.g. use 10 μ L of working solution for 10 mL of mESC medium).

2.4. Culturing Human Embryonic Stem Cells

1. 0.05% trypsin/EDTA solution.
2. Gelatin-coated T25-cm² tissue culture flasks and six-well plates (see Subheading 3.1.1, steps 5 and 23).
3. hESCs (e.g. H9 line from WiCell). This could be a frozen vial or live culture.
4. Human basic fibroblast growth factor (hbFGF, i.e. Peprotech): Stock solution is made by dissolving 10 μ g of hbFGF in 5 mL of PBS with 0.1% BSA (Fraction V). hbFGF is very sticky, so when using, prewet all pipette tips, tubes, and the filter with PBS + 0.1% BSA. Stock solution should be aliquoted (250 μ L) and stored at -20°C (short term) or at -80°C (long term). Once thawed, aliquots can be stored at 4°C for a month.
5. hESC medium: 400 mL DMEM/F-12, 100 mL Knockout Serum Replacement (KSR, Invitrogen), 5 mL nonessential amino acids (NEAA, Invitrogen, catalog no. 11140-050), 5 mL L-glutamine-2-mercaptoethanol mix (7 μ L 2-mercaptoethanol plus 5 mL of 200 mM L-glutamine), and 1 mL of hbFGF (10 μ g/5 mL of PBS with 0.1% BSA) to a final concentration of 4 ng/mL. Filter through a 0.22- μ m filter and store at 4°C (it is stable for 2 weeks). It can be made in smaller batches.
6. Ethanol spray bottle (75%).
7. Sterile 15-mL conical tubes.
8. MatrigelTM.
9. DMEM/F-12.
10. mTeSR culture medium kit (includes basal medium plus supplements) (StemCell Technologies). Medium is made by combining 400 mL of mTeSR basal medium with 100 mL of mTeSR supplement. Complete medium can be made in smaller aliquots. Complete medium is stable at 4°C for 2 weeks.
11. PBS without Mg^{2+} and Ca^{2+} (see above).
12. Accutase (eBioscience) or collagenase IV or trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA): Collagenase IV is made by warming DMEM/F12 in a 37°C water bath, then dissolving 0.01 mg of collagenase per mL of DMEM/F12. The solution is passed through a 0.22- μ m filter before use.

13. Glass beads (3 mm diameter, i.e. Fisher Scientific). To prepare beads, place a bottle of glass beads in a beaker (beaker should be 1/3 to 1/2 full). Rinse the beads three times with dH₂O, then cover the beads with 10 N HCl and soak overnight at room temperature. Add an equal volume of 10 N NaOH (carefully) to neutralize HCl and then run dH₂O over the beads overnight. The next day, remove all water from the beaker and wash four times with dH₂O. Dry off the glass beads with an autoclaved paper towel or in a drying oven and put the beads back in beaker and cover with aluminum foil. Autoclave the beaker/beads and glass test tubes with plastic snap on caps. Allow the beads to dry overnight and then aliquot the beads into the glass test tubes. Autoclave the test tubes with the beads and allow them to dry overnight, after which they will be ready for use in passaging hESCs.
14. Sterilized inverted light microscope: Sterilize with 75% ethanol and UV light in hood.
15. Sterile scalpel.
16. Sterile 10-mL Pasteur pipettes.

3. Methods

3.1. Culturing mEFs

3.1.1. Procedure for Isolating mEFs

1. Place 75% ethanol-sterilized dissection microscope in the sterile hood.
2. Wipe down all bench tops that will be used with 75% ethanol.
3. Place 40 mL of 0.25% trypsin and a sterile stir bar in the Erlenmeyer flask, cap the flask with autoclaved aluminum foil, and preheat trypsin solution in a 37°C water bath.
4. Preheat 40 mL of mEF medium in a 50-mL conical tube in the 37°C water bath.
5. Coat T75-cm² culture flasks with 6 mL of 0.2% gelatin (see Note 7) and incubate at 37°C for a minimum of 15 min or until needed (see Note 3).
6. Sacrifice female mice (one at a time) using CO₂ gas.
7. Place sacrificed female mouse on her back on autoclaved paper towels.
8. Spray the mouse with 75% ethanol.
9. Open the peritoneal cavity by making a Y-incision (Fig. 1).
10. Dissect out embryos from the uterus and remove all tissue surrounding each embryo using sterile forceps.
11. Carefully transfer dissected embryos into 60-mm petri dishes containing sterile PBS.

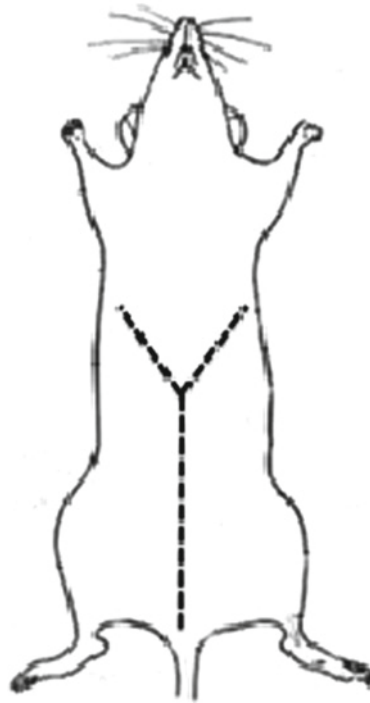


Fig. 1. Diagram showing where to make incisions on the ventral surface of a mouse during isolation of mEFs.

12. Rinse embryos in clean PBS twice and place them in new dish with fresh PBS for dissection.
13. Remove the head and internal organs from the embryos carefully with sterile forceps and scissors.
14. Place all dissected embryos in a fresh 60-mm dish of PBS and cut tissue into approximately 1 mm pieces with the scalpel.
15. Transfer all tissue pieces into the sterile Erlenmeyer flask containing 40 mL of preheated (37°C) 0.25% trypsin/EDTA solution and sterile stir bar.
16. Stir cell solution for 40 min at room temperature on a stirring plate.
17. Observe solution periodically. Add 200 μ L of 100 μ g/mL DNase if solution appears viscous and clumpy (add additional DNase in 200 μ L increments if necessary).
18. After 40 min, add 40 mL of prewarmed (37°C) mEF medium and swirl the solution gently.
19. Strain the solution through a 100 μ m cell strainer into a 50-mL conical tube in the sterile hood. Repeat this procedure twice. Use a new strainer each time.
20. Centrifuge the cell suspension in the 50-mL conical tube at $270 \times g$ for 4 min.

21. Decant supernatant into a fresh sterile 50-mL conical tube (save the supernatant in case there are not enough cells for plating).
22. Resuspend the pellet using fresh mEF medium (1 mL for each T75-cm² flask, e.g. if using four flasks, break the pellet with 4 mL of medium).
23. Aspirate excess gelatin out of T75-cm² flasks and replace it with 8 mL/flask of mEF medium.
24. Add 1 mL of cell suspension to each T75-cm² flask and rock the flask back and forth gently to distribute cells evenly across the bottom of the flask.
25. Observe cells with the inverted microscope to make sure that they look normal, for example, round with smooth surfaces and not apoptotic (see Note 8).
26. Incubate cells in the 37°C incubator.
27. Change medium after 24 h of plating and thereafter on alternate days.
28. Allow cells to reach 90–95% confluency (which should take 3–4 days) before passaging or freezing (Fig. 2b) (see Notes 9 and 10).

*3.1.2. Freezing and Storing
Passage 1 mEFs*

1. Label cryovials with cell line, passage number, date, and initials (see Note 11).
2. Aspirate mEF medium and wash the cells twice with 5 mL of room temperature PBS.
3. Aspirate PBS and add 5 mL of 0.05% trypsin/EDTA to each T75-cm² flask and incubate at 37°C for 1 min.
4. Remove flasks from incubator and gently tap the sides of each flask. Do not leave cells in trypsin for more than 5 min (see Note 12).

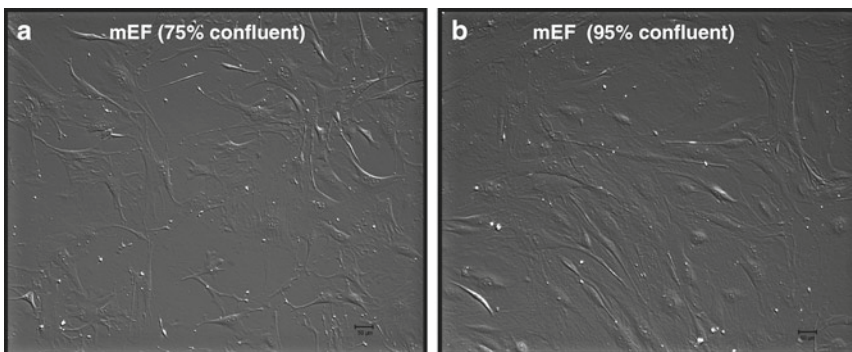


Fig. 2. mEFs that have been plated and inactivated using mitomycin C at 75% confluency (**a**) and 95% confluency (**b**). These cells are now ready to use as feeder layers for culturing mESCs (**b**) or hESCs, respectively (**a**). Note that the density of the cells is greater when they are prepared for mESCs.

5. Add 10 mL of mEF medium to each flask and mix gently. Amount of mEF medium added should be double the amount of trypsin solution that is used.
6. Aspirate the cell suspension from each flask and transfer it to one sterile 50-mL conical tube.
7. Centrifuge the cells at $270\times g$ for 4 min and then decant off the supernatant.
8. Break the pellet with 5 mL of freezing medium and then add the remaining 5 mL of the freezing medium and mix by gently inverting the tube (e.g. for five T75-cm² flasks, the total volume of freezing medium will be 50 mL).
9. Add 1 mL of the cell suspension in freezing medium to each sterile cryovial.
10. Transfer the cryovials to a -80°C freezer and leave it overnight.
11. Remove cryovials from freezer, place on dry ice, and transfer to liquid nitrogen for long-term storage (see Note 13).

*3.1.3. Preparing and
Freezing Passage
2 mEFs*

1. It is convenient to grow and freeze a number of passage 2 vials of mEFs for eventual use as feeder layers (see Notes 14 and 15).
2. Coat 3–4 T25-cm² flasks with 0.2% gelatin and incubate for at least 15 min.
3. Get a passage 1 vial of mEFs from the liquid nitrogen storage and bring the vial to a 37°C water bath on dry ice (see Note 16).
4. Thaw the cryovial in the water bath for no longer than 1.5 min or until a small ice crystal is left (see Note 17).
5. Spray the cryovial with 75% alcohol and transfer it to the sterile hood. Allow the alcohol to evaporate off before opening the vial.
6. Place 5 mL of fresh mEF medium into a 15-mL conical tube.
7. Transfer thawed cells into the conical tube using the P1000 pipette.
8. Rinse cryovial with 1 mL of mEF medium and transfer to the conical vial.
9. Cap the tube and centrifuge for 4 min at $270\times g$. Spray the tube with 75% ethanol before transferring it to the sterile hood.
10. Decant supernatant and break the pellet with 3–4 mL of fresh mEF medium. Use 1 mL of mEF medium per T25-cm² flask.
11. Aspirate gelatin from the T25-cm² flasks.

12. Add 3 mL of fresh mEF medium into each T25-cm² flask.
13. Plate 1 mL of mEFs into each T25-cm² flask, rock flasks gently to distribute cells, and incubate at 37°C. Observe flasks after 20 min to verify that cells are attaching.
14. Change the medium 24 h after plating and then change the medium on alternate days.
15. When 90–95% confluency is reached, a flask may be used to prepare the feeder layer (Fig. 2b). Cells in the remaining flasks can be frozen as has been done for passage 1 cells (Subheading 3.1.2).

*3.1.4. Preparing mEF
Feeder Layers
for Subsequent
Culture of mESCs*

1. Coat T25-cm² flasks with 0.2% gelatin and incubate for at least 15 min (use two flasks for each vial of mEFs).
2. Get stock vial of passage 2 mEFs from liquid nitrogen storage or use a T25-cm² flask containing a live culture of passage 2 mEFs (see Subheading 3.1.3 and Notes 14–16).
3. If frozen, thaw cells in the 37°C water bath for 1.5 min or until only a small crystal of ice remains. Keep cap above water during thawing.
4. Before transferring to the sterile hood, spray vial with 75% ethanol.
5. In a sterile hood, transfer thawed cells (1 mL) to a 15-mL conical tube containing 5 mL of mEF medium using a P1000 pipette. Rinse cyrovial with 1 mL of mEF medium and add this to the conical tube.
6. Centrifuge at 270×*g* for 4 min to create a loose pellet of cells.
7. Decant supernatant from the conical tube and gently break the pellet with 1–2 mL of mEF medium per pellet.
8. Aspirate excess gelatin from the T25-cm² flasks.
9. Pipette 0.5–1.0 mL of mEF medium containing the cell suspension into each of two T25-cm² flasks containing 3 mL of fresh medium and transfer flasks to the incubator.
10. After about 15–20 min, check to be sure that mEFs are attaching to the bottom of the flasks, using an inverted microscope. Then, observe the flasks daily for cell growth, normal appearance, and absence of contamination.
11. Cells will double about every 24 h. For use with mESCs, let mEFs grow until they are about 90–95% confluent.
12. mEF medium should be changed every other day.

*3.1.5. Mitotic Inactivation
of mEFs Using Irradiation*

1. To prevent mEFs from dividing when stem cells are plated on them, they must be inactivated with either irradiation (for example cesium exposure) or mitomycin C (see Note 18).

2. To irradiate mEFs, place flasks in a cesium source irradiator and irradiate at 8,000 rads for approximately 2 h. It may be necessary to empirically determine the length of exposure and dosage for your particular instrument.
3. Remove flasks promptly and return them to the cell culture room.
4. Upon return to the lab, immediately replace mEF medium with 4 mL of room temperature low LIF mESC medium and incubate at 37°C for 30–60 min, after which mEFs can be used as feeder layers (see Notes 19 and 20).

*3.1.6. Mitotic Inactivation
of mEFs Using Mitomycin C*

1. If an irradiator is not available, mEFs can be inactivated using mitomycin C.
2. Label a 50-mL conical vial to dispose of any waste containing mitomycin C.
3. Aspirate mEF medium out of T25-cm² flasks containing mEFs, add 4 mL of mEF medium + mitomycin C into each flask, and incubate at 37°C for 2–2.5 h.
4. For each T25-cm² flask of mitomycin-treated mEFs, coat one T25-cm² flask with gelatin and incubate for at least 15 min.
5. After treatment, aspirate the mitomycin C-containing mEF medium out of the T25-cm² flask (see Note 5).
6. Wash mitomycin C-treated mEFs with 5 mL of PBS for three times and discard the wash into the waste bottle containing mitomycin C medium.
7. Add 2 mL of 0.05% trypsin/EDTA to each T25-cm² flask and incubate at 37°C for 1 min.
8. Remove the flasks from the incubator and gently tap the sides of the flasks to loosen the cells. Check the flasks using the inverted microscope periodically to make sure that mEFs detach from the bottom of the flask. Do not leave mEFs in trypsin for more than 5 min (see Note 12).
9. Add 4 mL of mEF medium to each flask to inactivate the trypsin.
10. Transfer the 6 mL of cell solution from each flask into a single sterile 15-mL conical tube and cap the tube.
11. Centrifuge the cell solution at 270 × *g* for 4 min. Spray conical tube with 75% ethanol before returning it to the sterile hood.
12. Decant the supernatant and gently break pellet with 1–2 mL of fresh mEF medium by pipetting repeatedly with a P1000 pipette. Use 1 mL of mEF medium per flask being plated.
13. Aspirate excess gelatin in T25-cm² flasks and add 3 mL of mEF medium to each flask.

Table 1
Schedule for mEF preparation

Day of week	Action
Monday	Plate passage 2 mEFs on gelatin coated T25-cm ² flasks
Tuesday	Change medium for newly plated mEFs
Wednesday	Observe mEFs using a inverted light microscope for confluency and contamination
Thursday	Mitomycin C-treat or irradiate mEFs. Irradiated mEFs can be used on Thursday for plating mESCs
Friday	Mitomycin C-inactivated mEFs can be used for plating ESCs; change medium if not used

- 14. Plate mitomycin C-treated mEFs on two fresh 0.2% gelatin-coated T25-cm² flasks. Observe plated cells after 20 min (see Note 21).
- 15. mEFs freshly treated with mitomycin C can be used as feeders after overnight incubation (see Note 22).
- 16. mEF medium should be changed 24 h after mitomycin C treatment and every other day thereafter.

*3.1.7. Schedule
for Preparing mEFs*

- 1. It is a good idea to set up a schedule for harvesting mEFs so that you will have them ready when needed for your stem cells. An example of a possible schedule is given in Table 1.
- 2. All mitotically inactivated mEFs should be fed on alternative days, and they are good for 2 weeks.

3.2. Culturing mESCs

*3.2.1. Thawing
and Expanding
mESCs on mEFs*

- 1. Prepare mEF feeder layer as described in Subheadings 3.1.4–3.1.6.
- 2. Aspirate mEF medium off of mEFs and add fresh low LIF mESC medium (4 mL to a T25-cm² flask) and then incubate for at least 30–60 min (see Note 23).
- 3. Get a vial of frozen mESCs from liquid nitrogen storage and transport it on dry ice to the cell culture room.
- 4. Immediately put the frozen vial in the 37°C water bath without submerging the cap and thaw for no more than 90 s or until a small ice crystal is left.
- 5. Remove vial and spray down with 75% ethanol before placing it in the sterile hood.
- 6. Put 5 mL of fresh low LIF mESC medium in a 15-mL conical tube.

7. Transfer the mESCs into the conical tube using the P1000 pipette and then wash the vial with 1 mL of low LIF mESC medium and add wash to the tube. Cap the tube before removing it from the hood.
8. Spin down cells in centrifuge at $270\times g$ for 4 min and then spray the conical tube with 75% ethanol before returning it to the hood.
9. Decant the supernatant into the waste beaker and add 1 mL of low LIF mESC medium to the tube. Pipette gently with the P1000 pipette to break the pellet. Be sure that the cells are fully dispersed, but do not pipette too hard or else the cells may be damaged.
10. Set the cells aside and aspirate the low LIF mESC medium out of the T25-cm² flask and replace it with fresh low LIF mESC medium (4 mL).
11. Add 1 mL of mESC suspension to the T25-cm² flask using a P1000 and then rock the solution in the flask back and forth gently to evenly distribute the cells. Observe using the inverted microscope and be sure that the cells appear normal and are not apoptotic.
12. Incubate at 37°C and check the next day for evidence of mESC attachment.
13. Observe cultures every day to be sure that there is no evidence of contamination or differentiation (see Note 24).
14. Change the mESC medium every day (replace 5 mL of old medium with 5 mL of fresh low LIF mESC medium).
15. For vials that are freshly thawed, it will generally take 3–4 days for mESCs to become 50–60% confluent (adjacent colonies should not be touching each other).
16. Figure 3a and b show examples of pluripotent mESCs growing in colonies. These colonies are round, three-dimensional, and have defined edges. Colonies in Fig. 3b have been labeled to show alkaline phosphatase activity, a marker for pluripotency. Figure 3c and d, in contrast, show mESC colonies in which differentiation has begun. Colonies are flatter, and cells (arrow) have begun to migrate out of a colony.

3.2.2. Passaging and Freezing mESCs Using mEFs

1. Aspirate mEF medium from a new flask of mitotically inactivated mEFs, add 4 mL of low LIF mESC medium to the flask, and incubate at 37°C for 30–60 min.
2. Coat a 60-mm tissue culture dish with 0.2% gelatin for at least 15 min and incubate at 37°C until ready for use.
3. Aspirate the low LIF mESC medium out of the T25-cm² flask containing mESCs.

4. Add 5 mL of PBS to wash any serum out of the flask.
5. Aspirate out PBS and add 2 mL of 0.05% trypsin/EDTA to the flask with mESCs, then incubate for 1 min at 37°C (see Note 12).
6. Remove the flask from the incubator and gently tap its sides to loosen the cells. Check using the inverted microscope to be sure that the cells have detached.
7. Add 4 mL of low LIF mESC medium to the flask with mESCs and rock back and forth (flask contains a total of 6 mL).
8. Aspirate out 1 mL of solution containing mESCs using a P1000 pipette and transfer to a 15-mL conical tube and cap tube.
9. Aspirate out the remaining 5 mL of solution containing mESCs and transfer to a second 15-mL conical tube and cap tube.
10. Centrifuge the two conical tubes for 4 min at $270\times g$. Spray down with 75% ethanol before placing tubes back in the hood.
11. Decant medium from conical tube containing 5 mL and gently break the pellet with 4 mL of freezing medium.
12. Add 1 mL of cell suspension in freezing medium per cyrovial (four vials total for one T25-cm² flask).
13. Transfer to a -80°C freezer for 24 h and then transfer to liquid nitrogen (use dry ice to carry vials from freezer to liquid-nitrogen tank).
14. Decant the medium from conical tube containing 1 mL of solution.
15. Break the pellet gently with 1 mL of low LIF mESC medium and set aside in the hood.
16. Aspirate out excess gelatin from a 60-mm culture dish and add 3 mL of fresh low LIF mESC medium.
17. Add 1 mL of the mESC suspension to the 60-mm culture dish using a P1000 pipette and mix solution carefully with the pipette.
18. Place the 60-mm culture dish in the incubator and wait 20–30 min to allow mEFs to attach (see Note 25).
19. After 20–30 min, aspirate the cell suspension containing mESCs from the 60-mm dish and transfer to a 15-mL conical tube and cap tube (see Note 26).
20. Centrifuge the cell suspension for 4 min at $270\times g$.
21. While centrifuging, change the medium in the T25-cm² flask containing mEFs by replacing 4 mL of mESC medium with an equal amount of low LIF mESC medium. Spray the conical tube before returning it to the hood.
22. Decant the medium from the conical tube and break the pellet using 1 mL of low LIF mESC medium.

23. Transfer all of the cell suspension to a T25-cm² flask containing mEFs in fresh low LIF medium and rock the flask back and forth to distribute the cells.
24. Incubate the flasks at 37°C.
25. Routinely check and change medium everyday as described in Subheading 3.2.1.

3.2.3. Plating mESCs on Gelatin

1. Add 3 mL of 0.2% gelatin to T25-cm² flasks and incubate at 37°C for at least 15 min (one T25-cm² flask of mESCs can be passaged to three new flasks).
2. Decant medium in the T25-cm² flask containing mESCs and wash the flask using 5 mL of PBS.
3. Aspirate PBS and add 2 mL of 0.05% trypsin/EDTA to the T25-cm² flask and incubate for 1 min at 37°C.
4. Remove the flask from the incubator and the tap sides of the flask gently. Check cells with the inverted light microscope to see if all mESCs have detached.
5. Once all mESCs have detached (which should be no longer than 5 min), add 4 mL of high LIF mESC medium to neutralize the trypsin (see Notes 12 and 27).
6. Aspirate cell suspension and transfer to a sterile 15-mL conical tube and cap tube.
7. Centrifuge mESCs at 270 × *g* for 4 min.
8. While centrifuging, remove gelatin-coated flasks from incubator and decant excess gelatin in sterile hood.
9. Add 3 mL of high LIF mESC medium to the flask and set in hood (see Note 27).
10. Decant supernatant and break pellet gently with 3 mL of high LIF mESC medium.
11. Add 1 mL of cell suspension to each flask and rock flasks back and forth for even distribution. Observe cells using an inverted light microscope for normal cell morphology (round, no apoptosis).
12. Incubate mESCs at 37°C and change medium every day.
13. mESCs should be ready for passaging once the flask reaches 70–75% confluency (Fig. 2a).
14. Figure 3e shows pluripotent mESCs plated on gelatin. The colony is flat and has the characteristic cobblestone appearance of mESCs on gelatin. Figure 3f shows mESCs that are starting to differentiate on gelatin. Cells have begun migrating out of the colony.
15. Once a frozen vial of mESCs has been expanded and passaged to produce sufficient frozen stock, experiments can be set up with the mESCs.

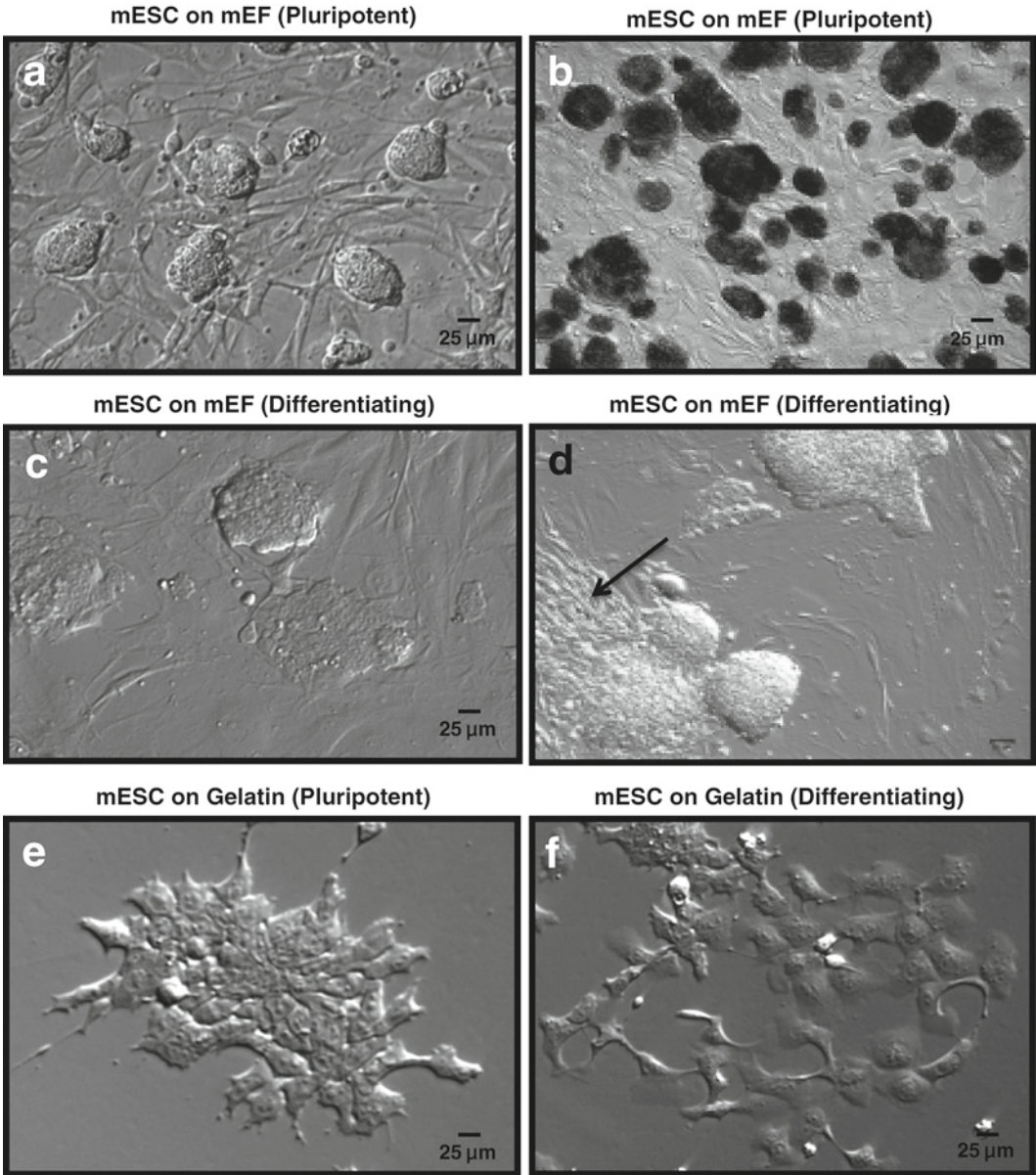


Fig. 3. Well-formed colonies of mESCs (**a**, **b**) growing on mEFs. The edges of the colonies are sharp, and colonies are round and three-dimensional, indicative of pluripotency. The colonies in (**b**) appear *dark* because they have been stained for alkaline phosphatase activity which shows the sharply defined edges of the colonies and indicates their pluripotent nature. mESC colonies that have begun differentiating on mEFs are shown in (**c**) and (**d**). In (**c**), the colonies have become flat, an early indicator of differentiation, while in (**d**), the colony at the lower left has lost its sharp edges, and differentiating cells are moving away from the colony (*arrow*). mESC colonies appear cobblestoned when grown on gelatin (**e**). When mESC differentiate on gelatin, they move away from the colony (**f**).

3.3. Culturing Human Embryonic Stem Cells

3.3.1. Prepping mEFs for hESC Culture Using Mitomycin C

1. Plate a vial of frozen mEFs (passage 2) in a gelatin-coated T25-cm² flask (see Subheading 3.1.1 and Note 14).
2. Allow the mEFs to grow to 95–100% confluency (Fig. 2b).
3. Mitomycin C-treat the T25-cm² flask of mEFs (see Subheading 3.1.6).
4. After treatment, wash the flask twice with PBS and then remove the mEFs using trypsin/EDTA (see Subheading 3.1.6).
5. Centrifuge the cell suspension, remove the supernatant, and break pellet with fresh mEF medium as described in Subheading 3.1.6.
6. One T25-cm² flask of mitomycin C-treated mEFs can be replated into four to five wells of a six-well plate (this will give about 70% confluency in each well) (Fig. 2a).
7. Alternatively, the mitomycin C-treated mEFs can be placed back into a T25-cm² flask if desired.
8. Put the plate or flask in the incubator for 24 h (check after 20 min to be sure that cells begin to attach).
9. After 24 h, the mEFs are ready to be used for plating hESCs.

3.3.2. Prepping mEFs for hESC Culture Using Irradiation

1. Plate two vials of frozen passage 2 mEFs in a gelatin-coated six-well plate. Two vials contain enough cells for six wells.
2. Allow mEFs to grow to 70–75% confluency in the incubator (Fig. 2a).
3. Irradiate as described in Subheading 3.1.5.

3.3.3. Thawing and Plating hESCs on mEFs

1. For the mitomycin C-treated or the irradiated mEFs, replace mEF medium with hESC medium and incubate for 30–60 min at 37°C. Use 1 mL for each well of a six-well plate or 3 mL into a T25-cm² flask.
2. Obtain a frozen vial of hESCs from liquid-nitrogen storage (see Notes 16 and 28). One frozen vial of hESCs is usually plated in one well of a six-well plate or in one T25-cm² flask.
3. Thaw the vial in 37°C water bath for no more than 1.5 min or until a small crystal is left (do not submerge the cap). Spray vial with 75% ethanol before transferring to sterile hood.
4. Put 5 mL of fresh hESC medium into a 15-mL conical tube.
5. Add hESCs to the conical tube dropwise by running drops down the sides of the tube.
6. Centrifuge the tube at 200×*g* for 3 min at room temperature. Spray the tube with 75% ethanol before returning it to the sterile hood.
7. Decant the supernatant and gently break the pellet using 1 mL of hESC medium.

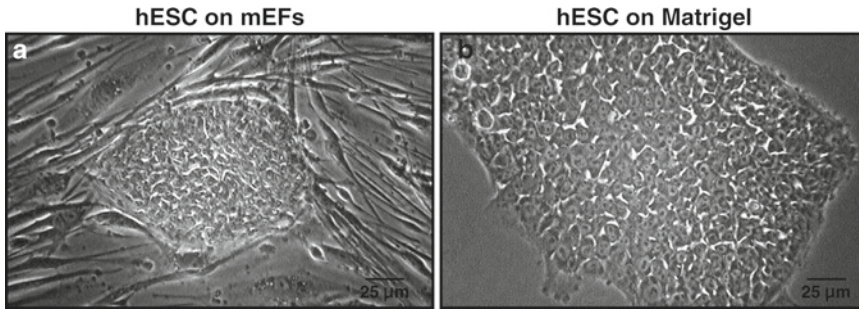


Fig. 4. Examples of hESCs growing on mEFs (a) and Matrigel (b). In both cases, colonies appear flatter than the mESC colonies. hESCs grow between the mEFs, while mESCs grow on mEFs. Note that the edges of the colonies are sharply defined.

8. Replace the hESC medium in the six-well plate (1 mL) or flask (3 mL) with fresh hESC medium.
9. Add the 1 mL of hESC suspension dropwise into one well of a six-well plate or one T25-cm² flask.
10. Routinely check and change medium every day.
11. Figure 4a shows hESCs growing on mEFs. The hESCs actually grow in between the mEFs and form a colony. The colony is flat and has a cobblestone appearance and well-defined edges.

3.3.4. Preparing Matrigel Plates for hESCs

1. Thaw a 5-mL bottle of stock Matrigel in the refrigerator overnight. Do not thaw at room temperature because it may clump and not be usable.
2. Add 5 mL of DMEM/F12 (at 4°C) to the Matrigel bottle and mix thoroughly by pipetting up and down gently (avoid formation of bubbles at the surface of the solution).
3. Aliquot 500 μL of Matrigel solution to twenty 15-mL conical tubes. Aliquots that will not be used should be stored at -20°C. When using a frozen aliquot, it should be thawed for 1–2 h in the refrigerator (4°C) before use.
4. When making Matrigel plates, add 7 mL of DMEM/F12 (at 4°C) to a 500-μL aliquot of Matrigel prepared as described above and pipette gently without making bubbles.
5. Add 1 mL of Matrigel working solution to each well in a six-well plate and rock to evenly distribute the solution.
6. Leave the plate at room temperature for 1–2 h or leave it in the refrigerator overnight (see Note 29).

3.3.5. Thawing and Plating hESCs on Matrigel

1. One frozen vial of hESCs is usually plated in one well of a six-well plate or in one T25-cm² flask.
2. Thaw a vial of hESCs at 37°C for 1.5 min in a water bath or until a small ice crystal is left.
3. Add 5 mL of mTeSR to a 15-mL conical tube.

4. Transfer the cells in the thawed vial to the conical tube by adding cells dropwise to the sides of the tube and cap the tube.
5. Centrifuge for 3 min at $200\times g$ and spray the tube with 75% ethanol before placing it in the sterile hood.
6. Decant the supernatant from the tube and gently break the pellet using 1 mL of mTeSR medium. Do not pipette too many times; cells should remain in colonies.
7. Aspirate out excess Matrigel from the six-well plate and add 1 mL of fresh mTeSR to each Matrigel-coated well.
8. Add 1 mL of cell suspension to the Matrigel-coated well dropwise, keeping drops near the surface of the well.
9. Incubate the plate at 37°C overnight.
10. Change the mTeSR medium every day.
11. Passage when hESCs are 70–75% confluent.
12. Figure 4b shows a hESC colony plated on Matrigel. The colony is flat with a cobblestone appearance. The cells are tightly joined to each other in a colony.

*3.3.6. Passaging hESC
on Either mEFs or
Matrigel-Coated Dishes
with Glass Beads*

1. If plating hESC on mEFs, remove mEF medium and replace with 1 mL of hESC medium and incubate at 37°C for 30–60 min before using. If plating on Matrigel, prepare Matrigel-coated dishes 1–2 h before they are needed.
2. Wash hESCs with 1 mL of PBS per well and then aspirate out PBS.
3. Add 1 mL of Accutase or collagenase IV to each well and incubate at 37°C for 1 min (see Note 30).
4. Remove plate from incubator and observe with an inverted microscope. Edges of the colonies will start to curl.
5. Add 10–12 sterile glass beads to each well (see Notes 28 and 29).
6. Rock plate back and forth gently. Observe the plate from the bottom. hESC colonies should become detached and float in solution.
7. Do not leave hESC in Accutase or collagenase for more than 3 min.
8. If plating on mEFs, add 2 mL of hESC medium to the well. If plating on Matrigel, add 2 mL of mTeSR medium to the well.
9. Aspirate the cell suspension and transfer to a 15-mL conical tube and cap the tube.
10. Centrifuge the cell suspension at $200\times g$ for 3 min. Spray the conical tube with 75% ethanol before placing in the hood.
11. Decant supernatant and gently break the pellet using either hESC medium (for mEF plating) or mTeSR medium (for

Matrigel plating). If two wells of hESCs will be passaged into six new wells, then break pellet with 3 mL of medium, or 500 μ L per well.

12. Do not pipette too many times to prevent colonies from breaking up (hESCs survive better in colonies and not as single cells).
13. If plating onto mEFs, aspirate hESC medium from the wells and add 1 mL of fresh medium. If plating on Matrigel, aspirate out Matrigel from the wells and add 1 mL of mTeSR medium.
14. Replate hESCs by adding 500 μ L of cell suspension dropwise to each well. Add drops right above the surface of the solution. Rock the plate in all four directions gently.
15. Clumps of hESCs should be observed using the inverted microscope.
16. Incubate the cells at 37°C overnight and change the medium (hESC medium or mTeSR) every day.
17. Passage again when colonies reach 70–75% confluency.
18. Once a frozen vial of hESCs has been expanded and passaged to produce sufficient frozen stock, experiments can be set up with the hESCs.

3.3.7. “Cut and Paste” Passaging

1. Instead of using the beads, the “cut and paste” method of passaging may be used. For this alternative, place a sterilized inverted light microscope in the sterile culture hood.
2. Place a six-well plate of hESCs on the stage of the microscope and open the lid.
3. Observe hESC colonies and choose those that look pluripotent and healthy (Fig. 4a, b).
4. Use a scalpel to gently cut the colonies into quadrants (Fig. 5a).

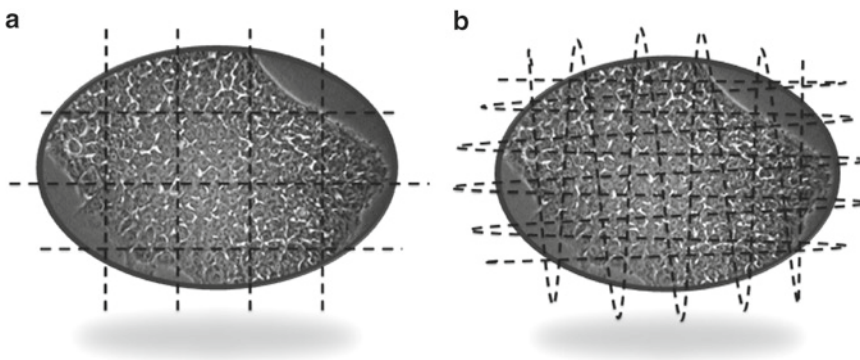


Fig. 5. Diagrams showing how to score colonies for passaging with the cut-and-paste (a) and the mechanical (b) method.

5. Gently scrape off the desired portions of the colonies.
6. Once all the pieces have been mechanically removed from the plate, transfer the solution in each well to a 15-mL conical tube.
7. Centrifuge the cell suspension and break pellet with appropriate media (mTeSR if plating on Matrigel and hESC medium if plating on mEFs).
8. Replate hESC colonies as described in Subheading 3.3.6, steps 8–18.

3.3.8. Passaging hESCs Mechanically

1. Instead of using the beads, the mechanical method can also be used to passage cells. For this alternative, place the hESC plate in the sterile hood.
2. Add 1 mL of Accutase or collagenase IV to each hESC well and incubate for 1 min at 37°C.
3. Return the plate to the sterile hood.
4. Unwrap a 10-mL Pasteur pipette in sterile hood. Do not allow the tip to touch any surfaces.
5. Scrape the colonies off from the bottom of the wells in the directions shown in Fig. 5b. You should see colonies floating in medium.
6. Add 2 mL of hESC medium or mTeSR medium into each well.
7. Aspirate all cell suspensions into one 15 mL conical tube and cap tube.
8. Centrifuge the cell suspension at $200\times g$ for 3 min.
9. Break pellet and replate hESCs on Matrigel or mEFs as described in Subheading 3.3.6, steps 8–18.

4. Notes

1. Although mEFs from various strains of mice have been used successfully in stem cell culture, we have found that mEFs from NIH Swiss mice work very well with both mESCs and hESCs.
2. Make 10 mL of freezing medium per T75-cm² flask of cells that will be frozen (e.g. for five flasks, make 50 mL of freezing medium).
3. When plating mEFs or mESCs, T25-cm² and T75-cm² flasks can be used with or without gelatin coating. We have found that mEFs and mESCs stick better and are healthier with a gelatin coating, and therefore we routinely coat flasks for mEF isolation or passaging.

4. LIF is expensive. It can be made in small batches so as not to waste it. The stock bottle is stored at 4°C. Check expiration date of each bottle.
5. Mitomycin C is a potent chemical that inhibits cell division. It should be handled carefully under a hood while wearing gloves. Be sure to discard mitomycin C waste in the appropriate toxic waste bin.
6. Aliquots of mitomycin C stock solution can be stored at -20°C for 6 months. The unconstituted powder is stable at -20°C for 1 year.
7. Cells from about two to three embryos can be plated on one T75-cm² flask.
8. Observe cells with the inverted microscope at 20 min after plating. If mEFs are healthy, they will have begun attaching by this time.
9. It normally takes the mEFs about 3–4 days to become 90% confluent. If it takes longer than this, the cells may be unhealthy.
10. We refer to these cells as passage 1 mEFs (some labs call this stage passage 0).
11. One 90–95% confluent T75-cm² flask of passage 1 mEFs will usually yield 10–12 frozen vials.
12. It is important not to overtrypsinize the cells. To determine if cells have completely dislodged, you can examine the flask carefully with naked eye or look at it with the inverted microscope. Do not leave mESCs in trypsin for more than 4–5 min. Staying in trypsin too long will reduce plating efficiency.
13. Although it is customary to store mEFs in liquid nitrogen, we have also stored mEFs at -80°C for up to 6 months with success.
14. Passages 3 or 4 are the best for use as feeder layers. We have found that younger or older passages do not work well.
15. Our frozen vials of mEFs normally contain $3\text{--}5 \times 10^6$ cells/vial.
16. It is important to keep cells frozen until you thaw them in the water bath. If necessary, they can be transferred from the liquid-nitrogen storage site to the water bath on dry ice.
17. Do not submerge the cap.
18. The use of a cesium source machine may require special training at your institution. Try to receive training and gain access to the cesium source before beginning your work.
19. Irradiation can also be done when cells are in 15-mL conical tubes before plating on flasks as feeders.

20. Once the mEFs have been mitotically inactivated, they are only good for 10 days.
21. mEFs should begin to attach by 15 min after plating.
22. Irradiation of cells in flasks has the advantage that cells are ready to use after the relatively short irradiation period. Mitomycin C-treated cells have the disadvantage of needing to be removed from their flasks and replated which requires an extra night of culture before they can be used. We have used both methods with success. If mitomycin C-treated mEFs have less than 70% confluency when replated, the mitomycin C treatment may have damaged some of the mEFs.
23. FBS is highly variable from batch to batch. Even embryonic-stem-cell-qualified batches may not support pluripotency well in ESC populations. It is important to screen batches of FBS to ascertain which are suitable for your work. Often companies will hold specific lots of FBS in reserve for you if you commit to purchase them in the future.
24. Be sure to also observe the morphology of the mEFs. If mEFs start to die or detach, mESCs will not be properly supported.
25. Check dish after 15 min to see if most mEFs have already attached; if so, the mEFs are healthy.
26. Do this step one time if passaging mESCs or two times if preparing mESCs for an experiment.
27. High LIF medium should be used when plating mESC on gelatin to keep them from differentiating.
28. A number of lines of hESCs are available for culture. Some lines require their own particular protocols. The protocol described here works well with the WiCell H9 line.
29. Matrigel plates are good for 2 weeks if refrigerated, although fresh plates are preferable.
30. Accutase will give mainly single cells, while collagenase IV will give mainly colonies.

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References

1. Martin, G. R. (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.
2. Evans, M. J., and Kaufman, M. H. (1981) Establishment in culture of pluripotential cells from mouse embryos. *Nature* **292**, 154–156.
3. Tremml, G., Singer, M., and Malavarca, R. (2008) Culture of mouse embryonic stem cells. *Curr. Protoc. Stem Cell Biol.* Chapter 1, Unit 1C 4.
4. Downing, G. J., and Battey, J. F., Jr. (2004) Technical assessment of the first 20 years of research using mouse embryonic stem cell lines. *Stem Cells* **22**, 1168–1180.
5. Thomson, J. A., Itskovitz-Eldor, J., Shapiro, S. S., Waknitz, M. A., Swiergiel, J. J., Marshall, V. S., et al. (1998) Embryonic stem cell lines derived from human blastocysts, *Science* **282**, 1145–1147.
6. Kleinman, H. K., and Martin, G. R. (2005) Matrigel: basement membrane matrix with biological activity. *Semin. Cancer Biol.* **15**, 378–386.
7. Xu, C., Inokuma, M. S., Denham, J., Golds, K., Kundu, P., Gold, J. D., et al. (2001) Feeder-free growth of undifferentiated human embryonic stem cells. *Nat. Biotechnol.* **19**, 971–974.
8. Amit, M., Shariki, C., Margulets, V., and Itskovitz-Eldor, J. (2004) Feeder layer- and serum-free culture of human embryonic stem cells. *Biol. Reprod.* **70**, 837–845.
9. Ludwig, T., Bergendahl, V., Levenstein, M. E., Yu, J., Probasco, M. D., and Thomson, J. A. (2007) Defined, feeder-independent medium for human embryonic stem cell culture. *Curr. Protoc. Stem Cell Biol.* Chapter 1, Unit 1C 2.
10. Ludwig, T. E., Levenstein, M. E., Jones, J. M., Berggren, W. T., Mitchen, E. R., Frane, J. L., et al. (2006) Derivation of human embryonic stem cells in defined conditions. *Nat. Biotechnol.* **24**, 185–187.
11. Hanjaya-Putra, D., and Gerecht, S. (2009) Vascular engineering using human embryonic stem cells. *Biotechnol. Prog.* **25**, 2–9.
12. Hewitt, K. J., Shamis, Y., Carlson, M. W., Aberdam, E., Aberdam, D., and Garlick, J. (2009) Three-dimensional epithelial tissues generated from human embryonic stem cells. *Tissue Eng. Part A* **15**(11), 3417–3426.
13. Wang, X., and Ye, K. (2009) Three-dimensional differentiation of embryonic stem cells into islet-like insulin-producing clusters. *Tissue Eng. Part A* **15**(8), 1941–1952.
14. Cote, R. J. (2001) Aseptic technique for cell culture. *Curr. Protoc. Cell Biol.* Chapter 1, Unit 1.3.
15. Phelan, M. C. (2006) Techniques for mammalian cell tissue culture. *Curr. Protoc. Hum. Genet.* Appendix 3, Appendix 3G.
16. Ryan, J. Understanding and Managing Cell Contamination. http://catalog2.corning.com/Lifesciences/en-US/TDL/techInfo.aspx?categoryname=cell_culture|Contamination.

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