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Feeder-Dependent Culture of Human Induced Pluripotent Stem Cells (hiPSCs)

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Introduction

Induced pluripotent stem cells (iPSCs) are derived from adult cell types such as fibroblasts using various methods. iPSCs are generally maintained on a layer of inactived murine embryonic fibroblast (MEF) feeder cells. Cells can be maintained under these conditions for several passages without compromising the cells' proliferation or pluripotency and differentiation potential. This protocol describes how to culture established iPSCs on feeder cells.

Materials Needed

- Dulbecco's Modified Eagle Medium (D-MEM) (Cat. no. 10569-010)
- Fetal Bovine Serum (FBS), ESC-Qualified (Cat. no. 10439-024)
- D-MEM/F-12 (1X) with GlutaMAX™-I (Cat. no. 10565-018)
- KnockOut™ Serum Replacement (KSR) (Cat. no. 10828-028)
- MEM Non-Essential Amino Acids Solution, 10 mM (Cat. no. 11140-050)
- β-mercaptoethanol, 1000X (Cat. no. 21985-023)
- Gibco® Mouse Embryonic Fibroblasts (Irradiated) (Cat. no. S1520-100)
- Basic Fibroblast Growth Factor (bFGF) (Cat. no. PHG0264)
- GlutaMAX™-I (100X) (Cat. no. 35050-079)
- Collagenase Type IV (Cat. no. 17104-019) for enzymatic passaging or StemPro® EZPassage™ Tool (Cat. no. 23181-010) for mechanical passaging
- Cell Scraper (Falcon, Cat. no. 353085)
- Dulbecco's PBS (DPBS) with Calcium and Magnesium (Cat. no. 14040-133)
- Dulbecco's PBS (DPBS) without Calcium and Magnesium (Cat. no. 14190-144)
- Attachment Factor (Cat. no. S006100)
- 37°C water bath
- Appropriate tissue culture plates and supplies

Note: As an alternative to D-MEM/F-12 (1X) with GlutaMAX™-I, KSR, and bFGF, you can use the KnockOut™ ESC/iPSC Media Kit (Cat. no. A1412901).

Preparing Media and Materials

10 µg/mL bFGF Solution (1000 µL)

1. To prepare 1 mL of 10 µg/mL bFGF solution, aseptically mix the following components:

bFGF	10 µg
DPBS without Calcium and Magnesium	980 µL
10% KSR	10 µL

2. Aliquot and store at –20°C for up to 6 months.

1 mg/mL Collagenase Type IV Solution

1. Add D-MEM/F-12 to Collagenase Type IV to make a 10 mg/mL stock solution. Gently vortex to suspend and filter sterilize the solution. This solution can be aliquoted and frozen at –20°C until use.
2. Make a working solution of 1 mg/mL Collagenase Type IV in D-MEM/F-12. The working solution can be used for 2 weeks if properly stored at 2–8°C (store in aliquots to avoid repeated warming).

MEF Medium (for 100 mL of complete medium)

1. To prepare 100 mL of complete MEF medium, aseptically mix the following components:

D-MEM	89 mL
FBS, ESC-Qualified	10 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
β-mercaptoethanol, 1000X	100 µL

2. Complete MEF medium can be stored at 2–8°C for up to 1 week.

Pluripotent Stem Cell (PSC) Culture Medium (for 100 mL complete medium)

1. To prepare 100 mL of complete PSC Culture Medium, aseptically mix the following components:

D-MEM/F-12	79 mL
KSR	20 mL
MEM Non-Essential Amino Acids Solution, 10 mM	1 mL
bFGF (10 µg/mL)*	40 µL
β-mercaptoethanol, 1000X	100 µL

*Add bFGF at the time of medium change (final concentration 4 ng/mL).

2. Complete MEF medium can be stored at 2–8°C for up to 4 weeks.

Preparing MEF dishes

1. Cover the whole surface of each new culture vessel with Attachment Factor (AF) solution and incubate the vessels for 30 minutes at 37°C or for 1 hour at room temperature.
2. Using sterile technique in a laminar flow culture hood, completely remove the AF solution from the culture vessel by aspiration just prior to use. Coated vessels may be used immediately or stored at room temperature for up to 24 hours.

Note: It is not necessary to wash the culture surface before adding cells or medium.

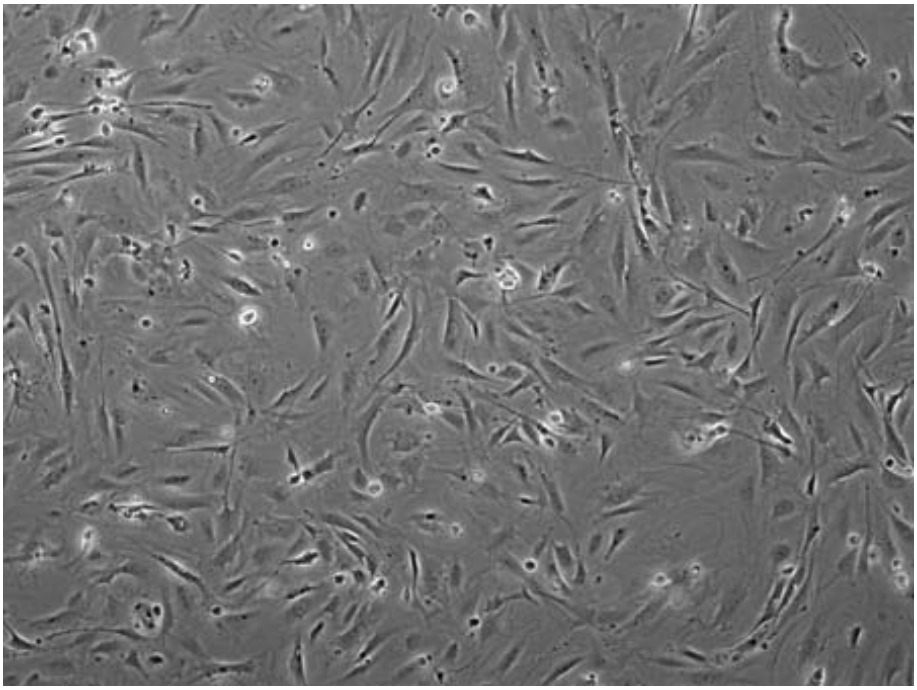
3. One to two days before initiating or passaging iPSC culture, plate 30,000/cm^2 of mitotically inactivated MEFs on an Attachment Factor-coated culture vessel in MEF medium (refer to Table 1)
4. Place MEF dishes into a 37°C, 5% CO_2 incubator.

Note: MEF dishes can be used up to 3–4 days after seeding.

Table 1 Amount of Inactivated MEFs Needed

Culture Vessel	Surface Area	Number of MEFs	Optimum Volume (mL)
6-well plate	10 cm^2/well	3.0×10^5	2.0 mL per well
12-well plate	4 cm^2/well	1.5×10^5	1.0 mL per well
24-well plate	2 cm^2/well	0.8×10^5	0.5 mL per well
35-mm dish	10 cm^2	3.0×10^5	2.0 mL
60-mm dish	20 cm^2	6.0×10^5	4.0 mL
100-mm dish	60 cm^2	1.8×10^6	10.0 mL

Figure 1 Mitotically inactivated MEFs on an Attachment Factor-coated culture plate in MEF medium



Thawing and Plating iPSCs

1. Aspirate the MEF medium from a dish containing inactivated MEFs and add pre-warmed PSC Culture Medium to the dish, 3–4 hours before plating iPSCs.
2. Label the dish containing inactivated MEF cells with the passage number from the vial, the date, and user initials.
3. Remove the vial of iPSCs from liquid nitrogen storage using metal forceps.

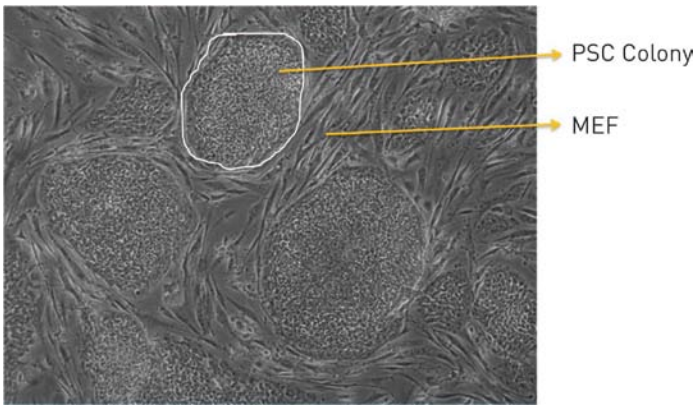
Note: If the vial is exposed to ambient temperatures for more than 15 seconds between removal and thawing, transfer the vial into a container containing a small amount of liquid nitrogen.

4. Roll the vial between your gloved hands until the outside is free of frost. This should take ~10–15 seconds.
5. Immerse the vial in a 37°C water bath without submerging the cap. Swirl the vial gently.
6. When only an ice crystal remains, remove the vial from the water bath.
7. Spray the outside of the vial with 70% ethanol and place it in hood.
8. Pipet cells gently into a sterile 50-mL conical tube using a 5-mL sterile pipette.
9. Slowly add 10 mL of PSC Culture Medium drop-wise to cells in the 50-mL conical tube. While adding the medium, gently move the tube back and forth to mix the iPSCs. This reduces osmotic shock to the cells.
10. Rinse the vial with 1 mL of PSC Culture Medium and add to the 50 mL conical with cells.
11. Transfer cell suspension to a 15 mL conical tube and centrifuge the cells at 200 × *g* for 5 minutes.
12. Aspirate and discard the supernatant.
13. Resuspend the cell pellet in sufficient volume of PSC Culture Medium according to Table 2 by gently pipetting the cells up and down in the tube a few times.
14. Aspirate the spent PSC Culture Medium from the MEF dish and slowly add the thawed colonies onto the dish. Place dish gently into the 37°C, 5% CO_2 incubator and move the dish in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the dishes.
15. Incubate the cells overnight.
16. The next day, remove the spent medium with debris using a sterile serological pipette and transfer it into a prepared MEF dish. You can use this dish as a backup in case there is a problem with the main dish.
17. Add fresh PSC Culture Medium to each dish according to the volumes in Table 2. Place both plates gently into a 37°C, 5% CO_2 incubator overnight.
18. Examine cells under the microscope and replace spent medium daily from both plates. If feeding more than one plate, use a different pipette for each well to reduce risk of contamination. Colonies may not be visible for up to a week.

Table 2 Volume of PSC Culture Medium Required

Culture Vessel	Surface Area	Volume (mL)
6-well plate	10 cm^2/well	2.0 mL per well
12-well plate	4 cm^2/well	1.0 mL per well
24-well plate	2 cm^2/well	0.5 mL per well
35-mm dish	10 cm^2	2.0 mL
60-mm dish	20 cm^2	4.0 mL
100-mm dish	60 cm^2	10.0 mL

Figure 2 iPSCs cultured on mitotically inactivated MEF feeder layer in PSC Culture Medium containing KSR



Passaging iPSCs

When to Split Cells

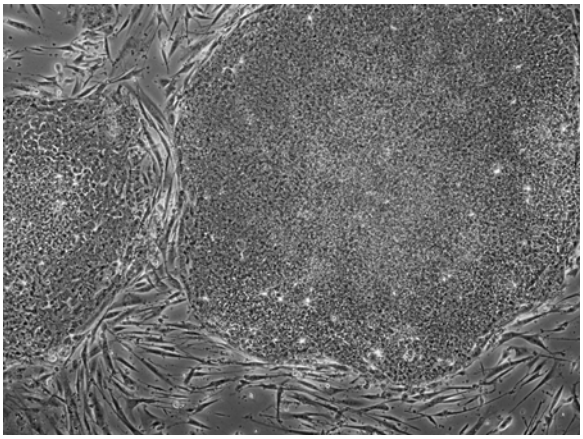
In general, split cells when one of the following occurs:

- The MEF feeder layer is 2 weeks old.
- iPSC colonies are becoming too dense or too large.
- Increased differentiation occurs.

Split Ratio

- The split ratio can vary, though it is generally between 1:2 and 1:4. Occasionally, cells will grow at a different rate and the split ratio will need to be adjusted. A general rule is to observe the last split ratio and adjust the ratio according to the appearance of the iPSC colonies.
- If the cells look healthy and colonies have enough space, split using the same ratio. If they are overly dense and crowding, increase the ratio. If the cells are sparse, decrease the ratio. Cells will need to be split every 4–10 days based upon appearance.
- iPSCs do well in iMEF plates that have been conditioned with PSC Culture Medium. It is common practice to condition new feeder plates before passaging iPSCs into them.

Figure 3 PSC colonies ready to be passaged. Note the large colony and the close proximity of the colonies to each other.



Enzymatic Passaging Using Collagenase

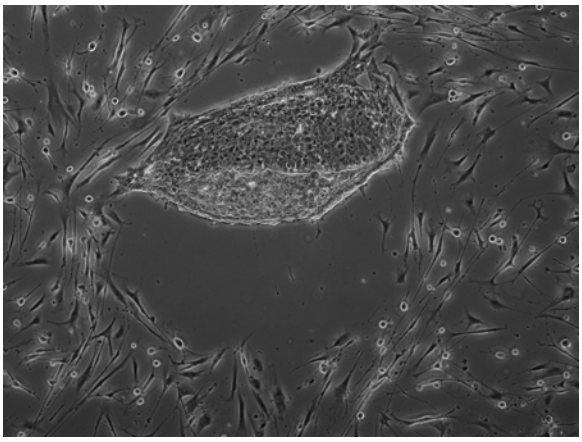
You may passage cells via the enzymatic method as described below, or mechanically as described in the following section.

1. Aspirate the MEF medium from a dish containing inactivated MEFs and add pre-warmed PSC Culture Medium to the dish, 3–4 hours before plating iPSCs.
2. Label the new MEF dish with the cell line name, the new passage number, the date, the split ratio, and user initials. Return the plate to the incubator.
3. Under a dissecting microscope, remove differentiated colonies from the dish to be passaged.
4. Aspirate the spent medium from the dish with a Pasteur pipette.
5. Add Collagenase Type IV (1 mg/mL) solution to the dish containing iPSCs. Adjust the volume of Collagenase Type IV for various dish sizes (e.g., 35-mm dishes require 1 mL of Collagenase IV).
6. Incubate the dish(es) for 30–60 minutes in a 37°C, 5% CO_2 incubator. Note that the incubation times may vary among different batches of collagenase; therefore, examination of the colonies is needed to determine the appropriate incubation time.

Note: As an alternative to Collagenase Type IV, you may use Dispase at a concentration of 2 mg/mL and incubate the dish(es) for 2–3 minutes in a 37° C, 5% CO_2 incubator.

7. Stop the incubation when the edges of the colonies are starting to pull away from the plate (see Figure 4).

Figure 4 PSC colony pulling away from iMEF layer after treatment with enzyme



8. Aspirate the Collagenase Type IV Solution with a Pasteur pipette. Remove the collagenase carefully without disturbing the attached cell layer.
9. Add PSC Culture Medium to each dish. Use a 5-mL pipette to gently blow the cells off the surface of the dish while pipetting up and down. Make sure to pipet gently to minimize the formation of bubbles.
10. After the iPSCs have been removed from the surface of the well, pool the contents of the wells into a 15-mL conical tube.
11. Using a 5-mL pipette, add PSC Culture Medium to the dish to wash and collect any residual cells. Pipet up the medium and cells, and then add the collected cells to the 15-mL tube.
12. Pipet cells up and down gently a few times in the 15-mL tube to further break up cell colonies. Pipet carefully to reduce foaming.

Note: Avoid making a single cell suspension.

13. Centrifuge at 200 × *g* for 5 minutes, and then aspirate the supernatant from the iPSC pellet.
14. Resuspend the pellet with an appropriate amount of PSC Culture Medium (refer to Table 2). This is dependent on the split ratio and the number of dishes used.
15. Mix the cell suspension well with a 10-mL pipette. Be careful not to break up the colonies too much or cause bubbles in the media.

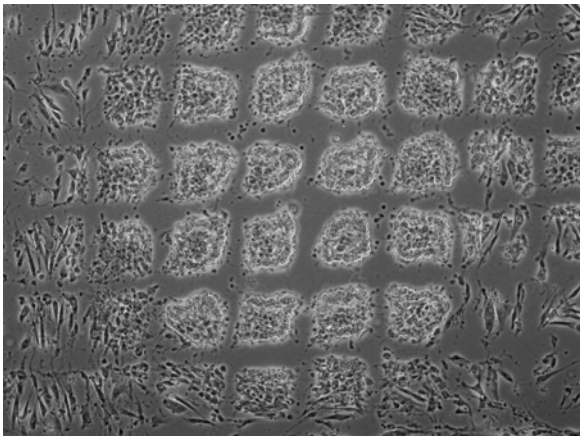
16. Add appropriate volume of cell suspension to each dish. Return the dish to the incubator.
17. Move the dish(es) in several quick, short, back-and-forth and side-to-side motions to disperse cells across the surface of the dishes.
18. Incubate cells overnight to allow colonies to attach. Replace spent medium daily.

Note: While cells are attaching, be careful when opening and closing the incubator doors to avoid disturbing the even distribution of cells on the surface of the wells.

Mechanical Passaging Using the StemPro® EZPassage™ Disposable Cell Passaging Tool

1. Replace the medium in the dish containing the cells with fresh PSC Culture Medium.
2. Under a laminar flow hood, open the package containing the EZPassage™ tool and remove the tool.
3. Hold the culture vessel in one hand and pull (roll) the EZPassage™ tool across the entire dish in one direction. Apply gentle but firm pressure so that the entire roller blade touches the dish and maintains uniform pressure during the rolling action.
4. Keep rolling the EZPassage™ tool parallel to the first pass until the entire dish has been covered.
5. Rotate the culture dish 90°, and then repeat rolling the cell layer as described above.
6. When you are finished, discard the EZPassage™ tool and do not reuse. Use a cell scraper to lift cell clusters off the plate, if necessary.
7. Using a serological pipette, rinse the dish with medium so that the cut colonies are suspended in the medium.
8. Transfer the medium containing the colonies to a 15-mL sterile tube.
9. Seed the cell colonies on dishes plated with mitotically inactivated MEFs at an appropriate density.
10. Place the plates into a 37°C, 5% CO_2 incubator. Shake the plates gently to evenly spread out cells.

Figure 5 PSC colony after being cut with the StemPro® EZPassage™ Disposable Cell Passaging Tool



APPENDIX

A. In the PSC Culture Medium, DMEM/F12 containing GlutaMAX™-I (Cat. no 10565-018) can be substituted with the following alternatives**:**

i. KnockOut™ DMEM/F-12 (Cat. no. 12660-012)

To prepare 100 mL of complete PSC Culture Medium using KnockOut™ DMEM/F-12, aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
KnockOut™ DMEM/F-12 (Cat. no. 12660-012)	—	1X	78 mL
KnockOut™ SR (Cat. no. 10828-028)	—	20%	20 mL
GlutaMAX™-I (Cat. no. 35050-061)	200 mM	2 mM	1 mL
MEM Non-Essential Amino Acids Solution (Cat. no. 11140-050)	10 mM	0.1 mM	1 mL
bFGF (Cat. no. PHG0024)	10 µg/mL	4 ng/mL	40 µL
β-mercaptoethanol (Cat. no. 21985-023)	1000X	1X	100 µL

ii. KnockOut™ DMEM (Cat. no. 10829-018)

To prepare 100 mL of complete PSC Culture Medium using KnockOut™ DMEM, aseptically combine the components listed in the table below.

Component	Stock Concentration	Final Concentration	Volume
KnockOut™ DMEM (Cat. no. 10829-018)	—	1X	78 mL
KnockOut™ SR (Cat. no. 10828-028)	—	20%	20 mL
GlutaMAX™ -I (Cat. no. 35050-061)	200 mM	2 mM	1 mL
MEM Non-Essential Amino Acids Solution (Cat. no. 11140-050)	10 mM	0.1mM	1 mL
bFGF (Cat. no. PHG0264)	10 µg/mL	4 ng/mL	40 µL
β-mercaptoethanol (Cat. no. 21985-023)	1000X	1X	100 µL

B. Alternative bFGF pack sizes

Product Name	Cat. no.	Product Size
FGF-basic (AA 1-155) Recombinant Human	PHG0264	10 µg
FGF-basic (AA 1-155) Recombinant Human	PHG0266	25 µg
FGF-basic (AA 1-155) Recombinant Human	PHG0261	100 µg
FGF-basic (AA 1-155) Recombinant Human	PHG0263	1 mg

C. Dissociation Enzymes/Tools for Harvesting iPSC

Dissociation Enzyme /Tools	Application	Suggested concentration
StemPro® EZPassage™ tool (Cat. no. 23181-010)	Manual passaging	Sterile, disposable tool
StemPro® Accutase® (Cat. no. A11105-01)	Monolayer of cells post passage, Dissociation into single cells	1X ready to use (1–2 minutes incubation at 37°C)
Dispase (Cat. no. 17105-041)	Colony-like morphology post passage	2 mg/mL (2–3 minutes incubation at 37°C)
TrypLE™ Express (Cat. no. 12604-021)	Dissociation to single cells	1X ready to use

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