iPSC Cell Culture Protocol

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This protocol describes regular maintenance of induced pluripotent stem cell (iPSC) cultures.

Healthy iPSC Maintenance

Thawing iPSCs

1. Coat four wells of a six-well plate with matrigel solution and incubate at 37°C for at least 30 minutes.
    a. To make matrigel solution, resuspend matrigel 1:100 in KnockOut DMEM.
2. Make thawing medium:
    a. Add ROCK inhibitor (RI) 1:1000 to mTeSR Plus media (ex: 20 mL medium with 20 µL RI). Warm medium in a bead bath.
3. Frozen cells should be kept on ice until ready to thaw. Thaw rapidly in a water bath or by holding tube in hand. Transfer cells suspended in freezing media to a 15 ml falcon tube with 2-5 ml of dPBS.
4. Centrifuge cells at 150-300 rcf for 3 minutes.
    a. While cells are spinning, replace matrigel on six well plate with 2 mL warm thawing medium per well.
5. Carefully aspirate supernatant from centrifuged cells by tilting the tube to the side, avoiding close contact with the pellet at the bottom of the tube.
6. Gently resuspend the cell pellet in 1 mL thawing medium.
7. Transfer cell solution into the six well plate with media. Optionally, make a concentration gradient between wells, for example:
    a. Add 400 µL, 300 µL, 200 µL, 100 µL of resuspended cell solution into each well, respectively.
8. Once cells are in colonies of at least 4-6 cells per colony, replace thawing media with mTeSR Plus without RI, ideally after about 24 hours and up to 48 hours.
9. Continue to feed every day, until 50-70% confluent.
    a. If cells are single cell or very small colonies, may opt to feed every other day until 10-20% confluency or large colonies.

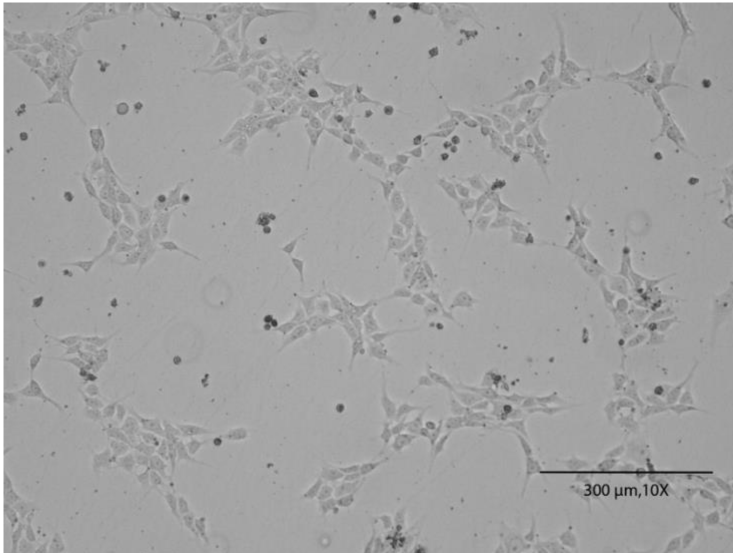

Fig. 1. Cells on ROCK Inhibitor in colonies of 10-25 cells/colony. The spikey appearance of cells is due to RI. Dense/dark cells are cell debris/cell death and will clear over time. Imaged at 10x magnification.

<u>Passaging iPSCs</u>

1. When cells are 50-70% confluent, they should be passaged
    a. tip: we 'split' or passage cells before their density outstrips the nutrient capacity of the culture media and while they are still in log growth phase. Once cells are approaching 100% confluency, they reach the plateau (stationary) phase where fewer than 10% of cells are actively dividing, and the cells are more susceptible to stress. This low rate of division makes it hard to maintain the line and increases the chances of unwanted mutations accumulating in the line over time.
2. Coat a six-well plate with matrigel solution and incubate at 37°C for at least 30 minutes.
    a. To make matrigel solution, resuspend matrigel 1:100 in KnockOut DMEM.
3. Aspirate medium from a well with healthy iPSCs at appropriate confluency.
4. Wash once with dPBS.
5. Add one mL of ReLeSR and incubate at room temperature in the hood for 30-40 seconds.
6. Aspirate ReLeSR.
7. Incubate dry well at 37°C for 3-9 minutes.
    a. While incubating, replace matrigel with warm mTeSR Plus on the new plate (optionally add RI at 1:1000).
8. Resuspend cells with one mL mTeSR Plus.
    a. To resuspend, pipette mTeSR Plus over the well to lift cells. Only go over the well 1-2 times, leaving behind cells that do not lift without mechanical force (the cells that do not lift are the unhealthy cells).
9. Distribute resuspended cells across 1-6 wells of your new plate.
10. Gently rock plate back and forth and side to side to evenly distribute cells across the wells.

11. Label plate with cell line, passage number, initials, and date.
12. Look at your cells under the microscope to confirm even distribution and check the quantity of cells passaged.
13. Leave plate in incubator.
14. After 48 hours, replace media with fresh mTeSR Plus without RI.
    a. Continue to feed cells every 24 hours.
    b. When the cells are 50-70% confluent (typically after about three days), passage into a new plate.

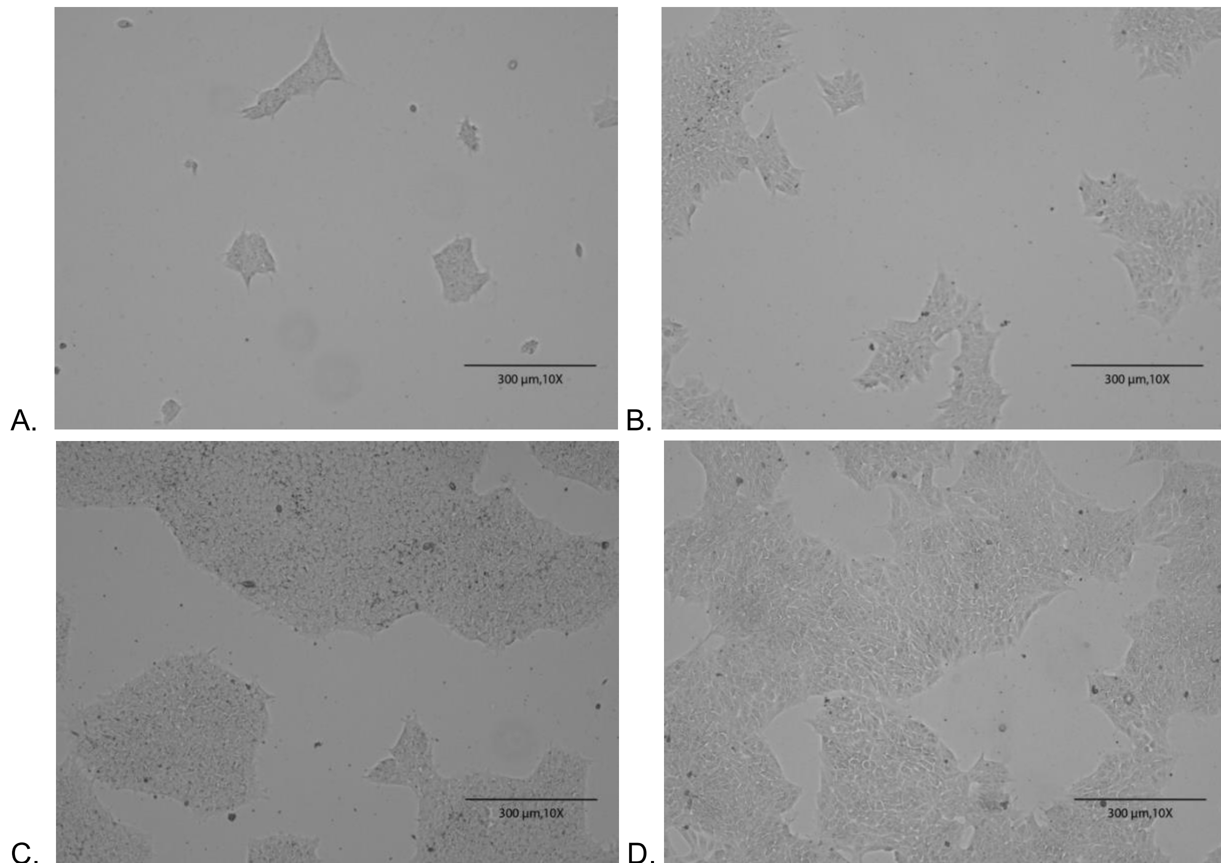

Fig. 2. Cell Confluency Guide, Imaged at 10x magnification. A. 10% confluency B. 20% confluency C. 60% confluency D. 70% confluency

<u>Freezing Cells</u>

1. Freeze cells when the well is at 70% confluency.
2. Make freezing media:
    a. Make a solution of 90% mTeSR Plus and 10% DMSO
    b. Add rock inhibitor 1:1000
    c. Ex: 9 mL mTeSR Plus, 1 mL DMSO, 10 μL RI
3. Label cryovials with cell line, passage number (freezing counts as a passage), initials, and date.
4. Aspirate medium from a well with healthy iPSCs at 70% confluency.

5. Wash once with dPBS.
6. Add one mL of ReLeSR and incubate in the hood for 40 seconds.
7. Aspirate ReLeSR.
8. Incubate dry well at 37°C for 3 minutes.
9. Resuspend cells with one mL freezing medium or Cryostor.
    a. To resuspend, pipette medium over the well to lift cells. Only go over the well 1-2 times, leaving behind cells that do not lift without mechanical force (cells that remain stuck down are the unhealthy cells).
10. Transfer one mL of resuspended cell solution into a labeled cryovial.
11. Add cryovials to a Corning CoolCell and leave in the -80°C freezer for a minimum of 24 hours and a maximum of 72 hours.
12. Transfer vials to a box in the liquid nitrogen tank and update the liquid nitrogen spreadsheet.

Troubleshooting

Spontaneous Differentiation

1. Identify spontaneous differentiation
    a. Signs of spontaneous differentiation
        i. Cells on the edge of the colonies look "spikey"
        ii. Cells are growing slowly
2. The first line of treatment is to passage very lightly, being careful to only pick up healthy cells.

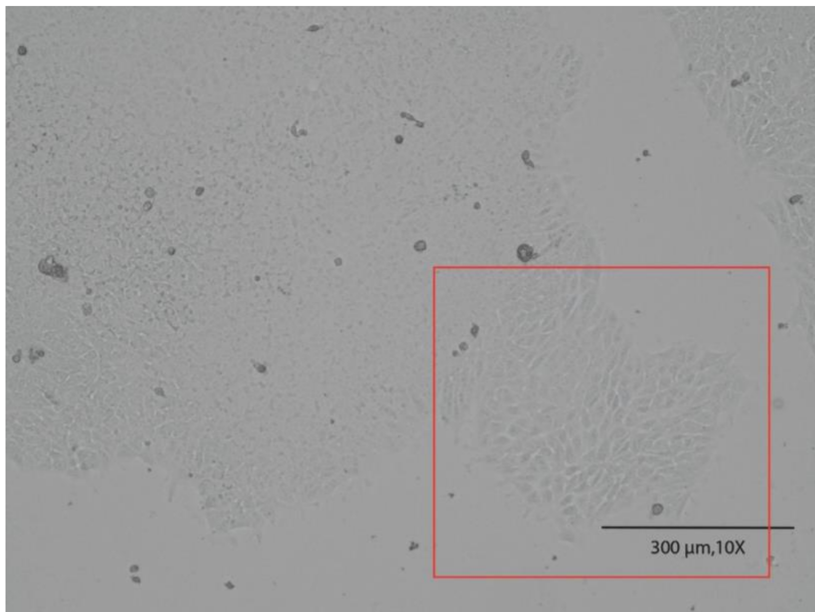

Fig 3. Example of spontaneous differentiation with spikey cells at edges of colony and lacunar or cobblestone appearance of cells within the colony. Imaged at 10x magnification.

Infection

1. Signs of bacterial infection:
    a. Yellow, cloudy medium
    b. Moving debris visible under the microscope, beyond Brownian motion
    c. Increased cell death
2. Unless the plate is precious, spray all of the wells in the infected plate with 10% bleach, leave in the sink for at least 30 minutes, and throw away in the biohazard bin.
3. Sterilize the incubator and BSC hood. Notify the lab via email.
4. If the line is precious, it is sometimes possible to save other wells in the plate by treating with antibiotics (any severely infected well should be bleached as this is likely to spread to other wells and the incubator)
    a. For example, make media with antibiotic-antimycotic 1:100, and feed the plate.
    b. Effective antibiotics may vary.

Reagents:

Reagent	**Manufacturer**	**Catalog Number**
Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix, Phenol Red-free, LDEVfree	Corning	356231
KnockOut DMEM	ThermoFisher	10829018
mTeSR Plus	STEMCELL Technologies	100-0276
ROCK Inhibitor (Y-27632 2HCl)	Selleckchem	S1049
PBS, pH 7.4	ThermoFisher	10010023
ReLeSR	STEMCELL Technologies	05872
CryoStor CS10	STEMCELL Technologies	7930
DMSO	Sigma-Aldrich	D8418
Antibiotic-Antimycotic (100X)	ThermoFisher	15240062

Equipment:

Equipment	**Manufacturer**	**Catalog Number**
Thermo Scientific™ Nunc™ Cell-Culture Treated Multidishes	Thermo Scientific	140685
Falcon™ 15 mL Conical Centrifuge Tubes	Falon	352196
CoolCell Containers	Corning	432002