Clelland Lab

iPSC Cell Culture Protocol

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Healthy iPSC Maintenance

1. Coat four wells of a six-well plate with Matrigel solution and incubate at 37°C for at least 30 minutes.

   To make Matrigel solution: resuspend Matrigel 1:100 in KnockOut DMEM.

2. Make thawing medium.

   Add ROCK inhibitor (RI) to mTeSR Plus medium at 1:1000 (e.g., 20 mL medium with 20 µL RI). Warm the medium in a bead bath.

3. Thaw the frozen cells.

   Keep frozen cells on ice until ready to thaw. Thaw rapidly in a water bath or by holding the tube in your hand. Transfer the thawed cells (still in freezing media) into a 15 mL Falcon tube containing 2–5 mL of dPBS.

4. Centrifuge the cells at 150–300 rcf for 3 minutes.

   While cells are spinning, replace the Matrigel on the six-well plate with 2 mL of warm thawing medium per well.

5. Carefully aspirate the supernatant from the centrifuged cells.

   Tilt the tube to the side and avoid close contact with the cell pellet at the bottom.

6. Gently resuspend the cell pellet in 1 mL of thawing medium.

7. Transfer the cell solution into the prepared six-well plate.

   Optionally, create a concentration gradient across wells — for example, add 400 µL, 300 µL, 200 µL, and 100 µL of resuspended cell solution to consecutive wells.

8. Once colonies reach 4–6 cells each, switch to RI-free medium.

   Replace the thawing medium with mTeSR Plus (without RI), ideally around 24 hours after plating and no later than 48 hours.

9. Feed the cells daily until they reach 50–70% confluency.

   If cells are single cells or in very small colonies, you may feed every other day instead, until they reach 10–20% confluency or form large colonies.

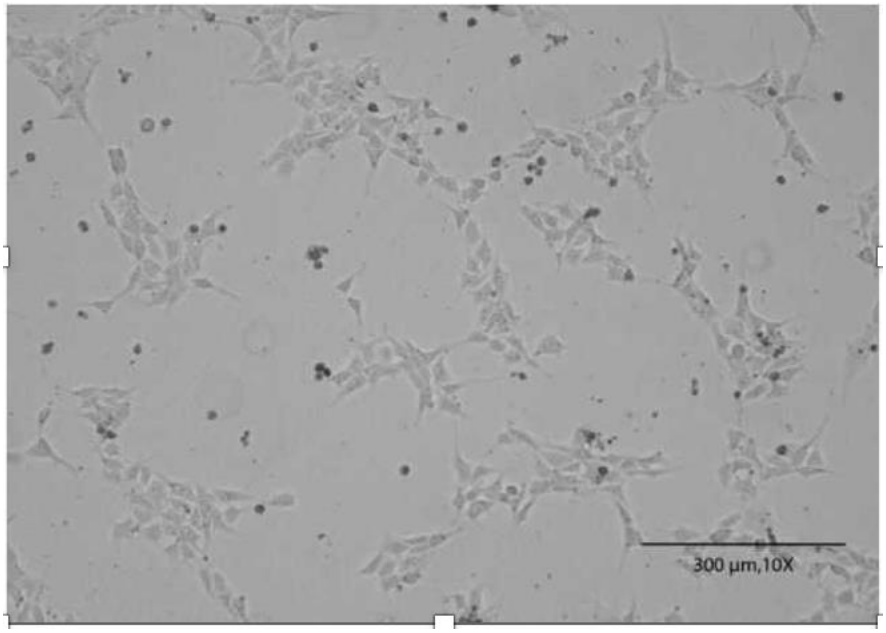

Fig. 1 — Cells on ROCK inhibitor, in colonies of 10–25 cells/colony. The spikey appearance is caused by RI. Dense, dark cells are debris or dead cells and will clear over time. (10x magnification)

Passaging iPSCs

10. Passage cells once they reach 50–70% confluency.

    Tip: *We split, or passage, cells before their density outstrips the nutrient capacity of the media and while they are still in log-phase growth. Near 100% confluency, cells enter a plateau (stationary) phase where fewer than 10% are actively dividing. This low division rate makes the line harder to maintain and increases the chance of unwanted mutations accumulating over time.*

11. Coat a six-well plate with Matrigel solution and incubate at 37°C for at least 30 minutes.

    To make Matrigel solution: resuspend Matrigel 1:100 in KnockOut DMEM.

12. Aspirate medium from a well of healthy iPSCs at the appropriate confluency.
13. Wash once with dPBS.
14. Add 1 mL of ReLeSR and incubate at room temperature in the hood for 30–40 seconds.
15. Aspirate the ReLeSR.
16. Incubate the dry well at 37°C for 3–9 minutes.

    While incubating, replace the Matrigel on the new plate with warm mTeSR Plus (optionally with RI added at 1:1000).

17. Resuspend the cells in 1 mL of mTeSR Plus.

    Pipette mTeSR Plus over the well to lift the cells, going over the well only 1–2 times. Cells that don't lift without mechanical force are the unhealthy ones — leave them behind.

18. Distribute the resuspended cells across 1–6 wells of the new plate.
19. Gently rock the plate back and forth, then side to side, to evenly distribute the cells.
20. Label the plate with cell line, passage number, initials, and date.
21. Check the cells under the microscope to confirm even distribution and assess quantity.
22. Return the plate to the incubator.
23. After 48 hours, switch to RI-free medium.

Replace the medium with fresh mTeSR Plus (without RI). Continue feeding every 24 hours. Once cells reach 50–70% confluency — typically after about three days — passage them into a new plate.

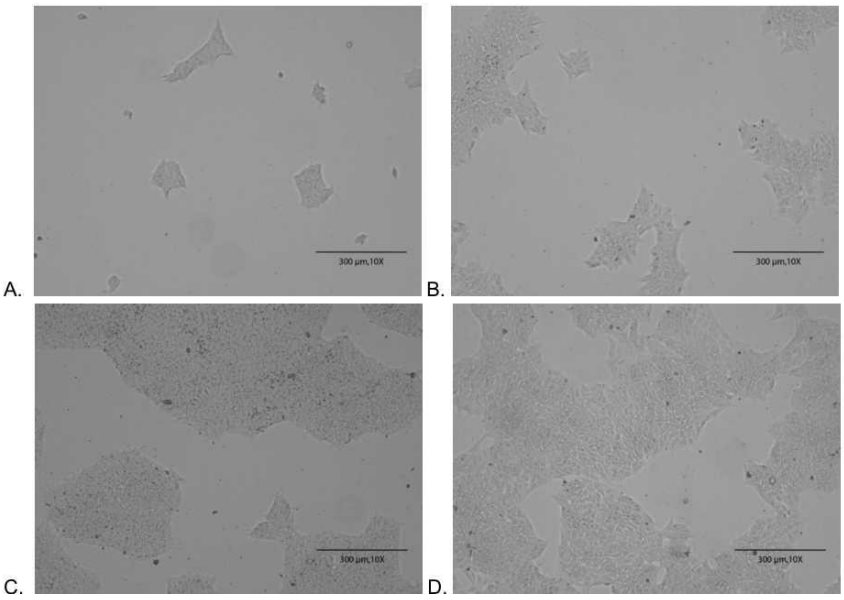

Fig. 2 — Cell confluency guide (10x magnification). A. 10% confluency B. 20% confluency C. 60% confluency D. 70% confluency

Freezing Cells

24. Freeze cells once the well reaches 70% confluency.
25. Make freezing media.

    Combine 90% mTeSR Plus with 10% DMSO, then add ROCK inhibitor at 1:1000 (e.g., 9 mL mTeSR Plus, 1 mL DMSO, 10 µL RI).
26. Label cryovials with cell line, passage number, initials, and date.

    Freezing counts as a passage, so increment the passage number accordingly.
27. Aspirate medium from a well of healthy iPSCs at 70% confluency.
28. Wash once with dPBS.
29. Add 1 mL of ReLeSR and incubate in the hood for 40 seconds.
30. Aspirate the ReLeSR.
31. Incubate the dry well at 37°C for 3 minutes.
32. Resuspend the cells in 1 mL of freezing medium or CryoStor.

    Pipette the medium over the well to lift the cells, going over the well only 1–2 times. Cells that remain stuck down are the unhealthy ones — leave them behind.
33. Transfer 1 mL of the resuspended cell solution into a labeled cryovial.
34. Place the cryovials in a Corning CoolCell and store at −80°C for 24–72 hours.
35. Transfer the vials to the liquid nitrogen tank.

    Place them in the appropriate box and update the liquid nitrogen tracking spreadsheet.

Troubleshooting

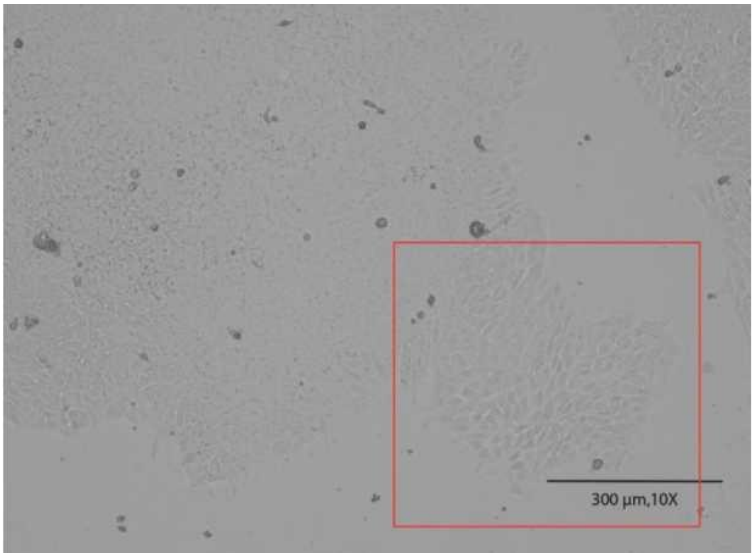

Fig. 3 — Example of spontaneous differentiation, showing spikey cells at the colony edges and a lacunar (cobblestone) appearance within the colony. (10x magnification)

36. Identify spontaneous differentiation.

    Signs include: cells on the edge of colonies appearing "spikey," and slower-than-normal growth. The first line of treatment is to passage very lightly, taking care to pick up only healthy cells.

Infection

37. Recognize the signs of bacterial infection.

    Watch for: yellow, cloudy medium; moving debris under the microscope beyond normal Brownian motion; and increased cell death.

38. If the plate is not precious, discard it.

    Spray all wells in the infected plate with 10% bleach, leave in the sink for at least 30 minutes, and dispose of in the biohazard bin. Sterilize the incubator and BSC hood, and notify the lab via email.

39. If the line is precious, you may be able to save unaffected wells with antibiotic treatment.

    Any severely infected well should still be bleached, since infection is likely to spread to other wells and the incubator. For example, add antibiotic-antimycotic at 1:100 to the media and feed the plate. Effective antibiotics may vary.