Cell Passaging & Subculturing

General Notes

- Passaging (also known as subculturing or splitting) refers to the removal of the medium and transfer of the cells from a previous culture into a new culture in fresh growth medium.
- Cells should be passaged at ~80% confluence (the percentage of the culture vessel surface area that appears covered by a layer of cells).
- The passage number will increase whenever the cells touch new plastic. Thus, during cell passaging, the passage number will increase by one.

Materials

- Mack cells, in culture
- Incubator, set to 27C without CO2
    - We use the heratherm style of incubator: https://www.thermofisher.com/order/catalog/product/50129111
- Micropipettes and autoclaved tips
- KimWipes
- Ethanol (140 proof)
- Centrifuge with attachments for 15 and 50 mL tubes
- Counterbalancing tube(s) for centrifuge
- Chemical-resistant laboratory marker
- Pipette controller and serological pipettes
- Conical tubes and tube racks
- Biosafety cabinet
- Light and phase contrast microscope
- Mack-GM
    - L-15 with 20% FBS, 1X anti-anti, 1 ng/mL recombinant human FGF2, and 20 mM HEPES pH 7.4
- iMatrix recombinant laminin-511-E8
    - Iwai North America #N892021
    - 0.5 mg/mL
- Trypsin-EDTA (0.05 or 0.25%, 1X)
- Gibco DPBS, no calcium, no magnesium (STERILE)
- Microcentrifuge tube, 1.5 mL, autoclaved
- Tissue culture flasks or well plates (vented)

Method

1. Warm growth media to room temperature.
2. View cells under light microscope and note observations.
3. Thaw trypsin from the -20°C to room temperature.

    a. The volume will depend on the surface area of plates/flasks from which you are passaging. Recommended volumes are shown the reference volumes table at the bottom of this protocol.
4. Once the media and trypsin are warm, carefully aspirate the spent media (i.e., media that remains after the cells have utilized the components) from the flask using an aspirating pipette connected to the vacuum inside the biosafety cabinet.
    a. NOTE: For greater precision, a 200 µL micropipette tip can be placed on the tip of the aspiration pipette.
5. Add the appropriate volume of sterile DPBS (see reference volumes table) using a serological pipette.
    a. This step washes off residual growth media, as FBS can inhibit trypsin.
6. Carefully aspirate DPBS using an aspirating pipetted connected to vacuum in the biosafety cabinet.
7. Add the appropriate volume of warmed trypsin (see reference volumes table) using a serological pipette. Tilt the culture vessel gently to ensure even distribution.
8. Incubate for 5 minutes at room temperature
9. After 5 minutes, check cell attachment on the culture vessel using the microscope.
    a. The cells should appear circular and detached (Fig. 1). If cells are circular but not detached, firmly tap (~5 times) each side of flask to help cells detach. Wait (~30 sec) and repeat as necessary until no/very few cells are visibly attached under microscope.
    b. NOTE: Once detached, cells are in suspension and frequently will be moving quickly across field of view. To test if cells detached, gently agitate flask and observe if cells remain in place (i.e., do not move with flow of liquid movement).

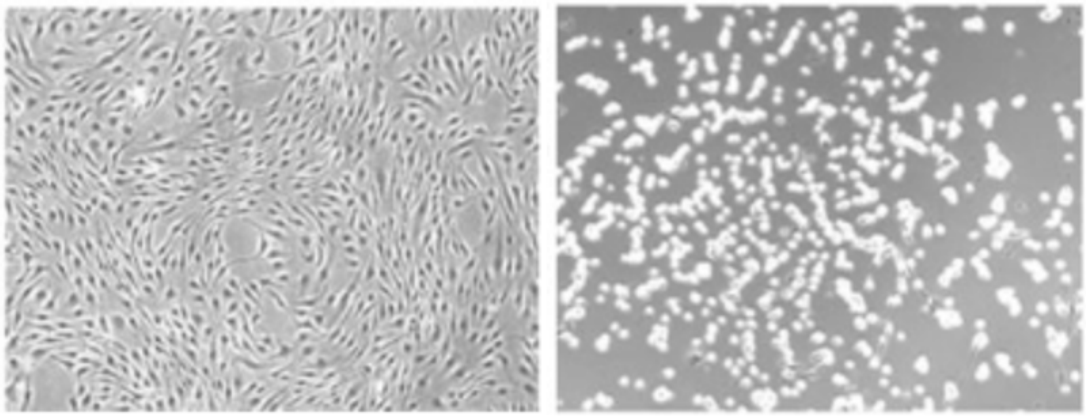

Fig. 1: Example images of adherent cell culture before (left) and after (right) cell detachment with trypsin[1]

10. Immediately after observing no/very few visibly attached cells, bring flask back to biosafety cabinet and add an equal volume (in comparison to the volume of trypsin) of growth media using a pipette. Using the same pipette, rinse the flask surface by pipetting up and down (~5 times), dispensing directly onto entire bottom of flask to help cells detach. Transfer cell suspension to labeled (species/cell type, passage number, initials, date) tube.
    a. NOTE: Be quick about adding GM as soon as you observe no/very few visibly attached cells; the longer the trypsin is left with the cells, the more cells can be damaged by excessive exposure to protease. Adding media (which includes FBS) neutralizes trypsin and inactivates its proteolytic activity.

b. NOTE: Since adding the growth media neutralizes the trypsin, it is also important to move the cells out of the culture vessel, as they could re-adhere to the tissue culture treated plastic (but will not adhere to the conical tube's plastic).

11. Spin the sterile cell suspension in the centrifuge at 300 RCF for 5 minutes.
12. Bring the centrifuged cells into the biosafety cabinet.
13. Carefully aspirate off supernatant without disturbing cell pellet using aspirating pipette connected to vacuum in biosafety cabinet.
14. Resuspend the cell pellet in 2-10 mL of growth media. Mix well by pipetting up and down.
    a. Note: The volume of media will depend on the expected cell number. Typically, aim for between 200k – 1 million cells/mL
    b. Note: You should see the cell pellet at the bottom of the tube. When you add the media, be sure to pipette rather vigorously up and down to first dislodge the pellet and then suspend the cells evenly throughout the media. When you pipette, you want to be forceful but try to avoid introducing bubbles.
15. Count the cells on your cell counter.
16. Generally, we recommend seeding 3,000-8,000 cells/cm^2. The exact cell density will depend on your schedule and the cell growth rate.
    a. The Mack1 doubling time is roughly 24-30 hours.
    b. We typically passage 3-4 days after seeding cells at 4,000 cells/cm^2.
17. While optional, cell attachment is improved if the cells are seeded with laminin. With the laminin-511 E8 fragment, 0.25 μg/cm^2 is recommended.
18. Calculate the volume of cell suspension needed for your seeding. Mix the cells with the appropriate amount of media and optionally laminin for your culture vessel (see Reference Volumes at the bottom), either directly in the flask or in a conical tube, after which you will add cell suspension to the culture vessel.
19. Mix the cells by tilting the vessel to all 4 sides slowly.
20. Place cells in the 27C incubator.

References

1. https://sciencellonline.com/blog/13-technical-tips-for-successful-primary-cell-culture/
2. https://www.stemcell.com/how-to-count-cells-with-a-hemocytometer.html

Reference volumes

Vessel	**Surface (cm2)**	**~PBS volume**	**~Trypsin volume**	**Laminin-511 E8 (0.5 mg/ml) volume**	**~Media volume**
6-well plate	9.6	1.5 mL	750 µL	4.8 µL	2 mL
12-well plate	3.5	1 mL	500 µL	1.75 µL	1 mL
24-well plate	1.9	800 uL	400 µL	0.95 µL	1 mL
48-well plate	1.1	400 uL	200 µL	0.55 µL	500 uL
96-well plate	0.32	200 uL	100 µL	0.16 µL	200 uL
T-25	25	3 mL	1.5 mL	12.5 µL	5 mL
T-75	75	9 mL	4.5 mL	37.5 µL	12 mL
T-175	175	20 mL	10 mL	87.5 µL	30 mL