Cell Team Cell Passaging and Live-Dead Staining Protocol for Detecting the Effect of DMSO Treatment on Cell Viability

The following two protocols were followed by last year's Cell Team during their Cell Passaging and Cell Viability labs.

Background Information:

These experiments allowed students to practice their cell passaging and manual cell counting techniques and examine the impact of dimethyl sulfoxide (DMSO) treatment through a Live-Dead Stain.

The protocol was completed using the **3T3** cell line, a fibroblast cell line often used in elementary passaging procedures. This cell line was derived from **embryonic mouse fibroblasts** in the 1960s and has historically been used to study the effect of genetic modifications on cell morphology and behavior. 3T3 cells grow on standard tissue culture plastic in an adherent monolayer, allowing for accurate confluency measurements from a single view microscope. 3T3 cells reach a cell density of 40,000 $\frac{cells}{cm^2}$ at confluence, with a confluent T25 flask containing approximately one million cells. 3T3 cells can be passaged for twenty to thirty generations before losing functionality, lending to their utility in experiments with students learning how to passage.

The viability assay will examine the effect of DMSO treatment on 3T3 cell behavior. **Dimethyl sulfoxide (DMSO)** is an organosulfur that functions as a weak acid and mild oxidant in chemical reactions. High concentrations of DMSO generate pores in cell membranes, disrupting the electrochemical gradient between the inside of the cell and the outside of the cell. This results in apoptosis, killing the cell.

This protocol employed a **LIve-Dead Stain** to assess cell viability. A traditional Live-Dead stain uses green and red fluorescent dyes to visualize live and dead cells under a fluorescent microscope. When the fluorescent dye is delivered to the experimental well, nonpolar **Calcein-AM** readily enters the living cell. Esterases inside of the cell convert nonpolar Calcein-AM into polar, fluorescent **Calcein**. This polar Calcein cannot readily pass through the cell membrane and is therefore stuck inside of the cell, marking the live cell by its green fluorescence. When a cell undergoes apoptosis, the plasma membrane is disrupted, allowing polar, fluorescent Calcein to leave the cell. These membrane disruptions create binding sides for fluorescent **Ethidium homodimer-1**, with the red fluorescence of Ethidium homodimer thereby marking dead cells.

Protocol for T25 Flask Cell Passaging:

Some General Lab Reminders:

- Spray everything you place in the BSC or incubator (including your hands) with ethanol EVERY TIME the items enter the BSC
- Make sure to cap containers immediately after use to prevent contamination
- Try not to disrupt the airflow in the BSC by moving your hands around or obstructing the vents
- Bring extra pipettes into the BSC for each step of the procedure in case you make a mistake and need another ethanol-sprayed pipette
- Anything that touched cells should be discarded into biohazardous waste
- Make sure to take notes as you work. Record notes on your protocol, cell numbers, and information about the reagents you used (type, brand, lot number, expiration date)

Set-Up:

1. Turn on the BSC and raise the shield to the shield line (about one foot)
2. Spray down BSC with 70% ethanol and wipe with Kimwipes
3. Remove starting T-25 flask from the incubator
4. Look at the flask under the microscope to gauge confluency
    - orange or yellow media signifies that it is time to passage/change media
5. Place media aliquot in the water bath to warm the media up

Removing cells:

6. Attach an aspirating pipette to the vacuum and aspirate the old media from the flask, being careful not to disturb the adhered cells
7. Use a 5 mL pipette to add 5 mL of PBS and gently shake flask to ensure the entire surface is covered
8. Use an aspirating pipette to aspirate the PBS
9. Use a 5 mL pipette to add 3 mL of trypsin and gently shake flask to ensure the entire surface is covered
10. Place the flask in the incubator and ley incubate for 8 minutes
    - remember to wipe the flask down with ethanol before placing it in the incubator
11. Remove the flask from the incubator and look at it under the microscope; tap the sides with the side of your hand to ensure cell removal
    - cells should look like small, floating beads not adhered fibroblasts

Centrifuging:

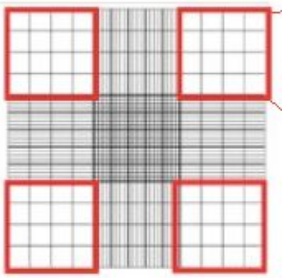
12. Return flask to BSC (wipe the flask down with ethanol before placing inside!)
13. Use a 5 mL pipette to add 5 mL of media to the flask, pipette up and down gently to collect all the cells
    - The fetal bovine serum in the media deactivates the trypsin
14. Use the 5 mL pipette to transfer all the liquid from the flask into a 50 mL conical tube
15. Label the tube with cell type (3T3), the new passage number (P2), your initials, and the date
16. Place the conical tube in the centrifuge, making sure to balance the centrifuge with equal volumes
17. Set the centrifuge for 5 minutes at 1000 rpm and press start
18. While the cells are being centrifuged, clean up BSC for next step
    - throw away trash in biohazard or sharps bin
    - return trypsin and PBS to fridge

Moving Cells to New Flasks:

19. Use an aspirating pipette to aspirate the supernatant (liquid and cell debris) from the conical tube.
    - Be careful not to touch the pellet, as you may lose viable cells!
    - Go slow, and when in doubt, it is better to leave a tiny bit of supernatant than to lose your cell pellet
20. Use a 1000 uL pipette to resuspend the cell pellet in 1 mL of media. Gently pipette up and down to resuspend the cell pellet in the new media
    - The cells should no longer be visible after resuspension
21. Use a 5 mL pipette to add 5 mL of media to each new flask
22. Add the resuspended cells to the new flask at a volume according to the split ratio (1:4)
    - For this experiment, add 250 uL of cell suspension to the flask
23. Aspirate any remaining volume of the cell suspension (or place in other flasks!)
24. Label the new flask with the cell type (3T3), passage number (P2), your initials, and the date in one corner
    - This allows you to still view your cells under the microscope
25. Place the flask in the incubator
    - remember to wipe the T25 flask down with ethanol before placing it in the incubator!
26. Clean up BSC: throw away trash in biohazard or sharps bin, return media to fridge, wipe down the BSC with ethanol, and spray surface with ethanol
27. Close the BSC shield and turn the BSC off.

Protocol for Counting 3T3 Cells with a Hemocytometer:

1. Passage cells according to previous protocol. Stop after aspirating supernatant (Step 19)
2. Resuspend cell pellet in 4mL of media.
3. Transfer 20uL of cell solution into an eppendorf tube.
4. Clean the hemocytometer and glass slide with ethanol and a kimwipe.
5. Place the glass slide onto the hemocytometer and carefully pipette 10uL into one side of the hemocytometer.
6. Place the hemocytometer under a microscope set at 10x objective.
7. Count the total number of cells in four corner quadrants.



In your cell count, only include cells touching two of the four sides of the quadrant corners. For example, if you choose to count cells touching the top and left sides, only count cells touching the top and left sides on all four quadrants.

8. Calculate cell concentration (cells/mL):

$$\_\_\_\_\_ \text{ cells counted} \times \frac{1}{4 \text{ quadrants}} \times \frac{1 \text{ quadrant}}{10^{-4} \text{ mL}} = \_\_\_\_\_ \text{ cells/mL}$$

9. Calculate total number of cells in solution:

$$\frac{\text{cells}}{\text{mL}} \times \underset{\text{(total volume of cell solution)}}{\_\_\_\_\_ \text{ mL}} = \_\_\_\_\_ \text{ cells}$$

10. Add 500uL of media to each well of a 24 well plate
11. Seed 50,000 cells/well
    a. Determine the volume of cell solution you need to transfer using the cell concentration (cells/mL)
        - 50,000 cells / cell concentration = volume in mL
    b. Add that cell solution volume to each well
12. Put 24 well plates back in incubator, spraying with ethanol before putting them in the incubator
13. Clean up the lab bench by spraying the hemocytometer with ethanol and putting the media away.

Protocol for Cell Viability Staining:

1. Wrap a conical tube in aluminum foil to prevent photobleaching.

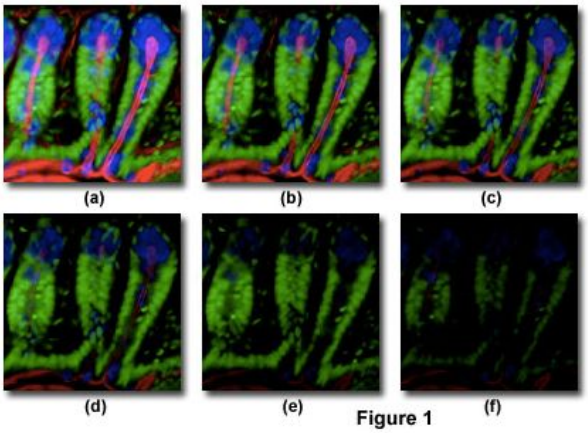


Figure 1

Photobleaching occurs when a fluorophore permanently loses its ability to fluoresce. This happens when a fluorophore undergoes photon-induced chemical damage and covalent modification. Wrapping the conical tube containing the fluorophores in aluminum foil prevents photon damage.

2. Aliquot 8mL PBS + 1uL calcein + 2uL ethidium homodimer into conical tube to make the working solution.
3. Observe 24 well plate under microscope. Place the 24 well plate under the fluorescence shield to prevent photobleaching.
4. Write down your observations.
5. Aspirate media from the 24 well plate, being careful not to touch the tissue culture plastic and aspirate off the cells.
6. Add 500uL of working solution to each well.
7. Add specified DMSO volume to one well.
    a. 2% DMSO: 500 uL solution + 10 uL DMSO
    b. 5% DMSO: 500 uL solution + 25 uL DMSO
    c. 10% DMSO: 500 uL solution + 50 uL DMSO
    d. 25% DMSO: 500 uL solution + 125 uL DMSO
8. Incubate at 37^{o}C for 30 minutes.
9. Visualize under fluorescent microscopy, and take pictures.
    a. Brightfield to see all cells
    b. GFP to see calcein marking live cells
    c. RFP to see ethidium homodimer marking dead cells
10. Write down your observations.