Welcome to Protocol Walkthrough 3!

- **Today's Pre-Module Playlist**
  - Young – Vallis Alps
  - Confidence – Oscar Scheller
  - suburban wonderland– BETWEEN FRIENDS

- **Team Check Ins**
  - Checking in with Groups 2 & 3 this week

- **Cell Team Schedule for the Rest of Winter 2021**
  - Week 6
    - Protocol Walkthrough 3 (Tuesday)
    - Protocol Discussion 3 (Thursday)
  - Week 7
    - Module 8 (Tuesday)
    - Group 2 Presentation (Thursday)
  - Week 9
    - Group 3 Presentation (Thursday)

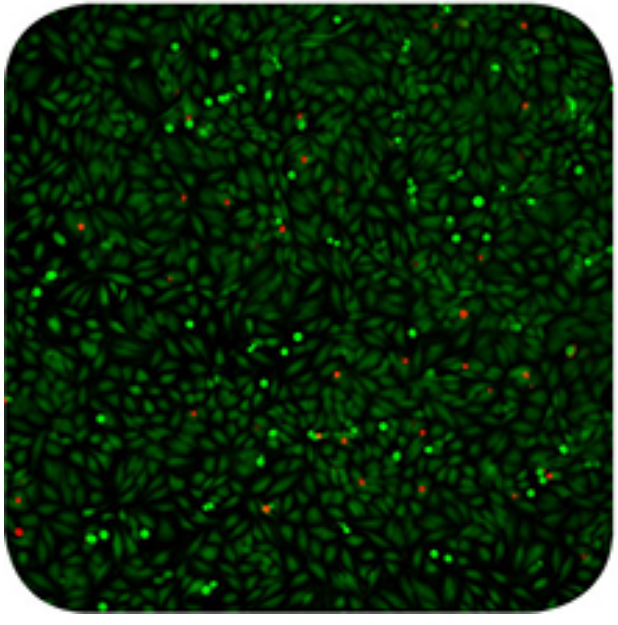
Protocol Walkthrough 3:
Cell Passing, Cell Counting and Viability Assays

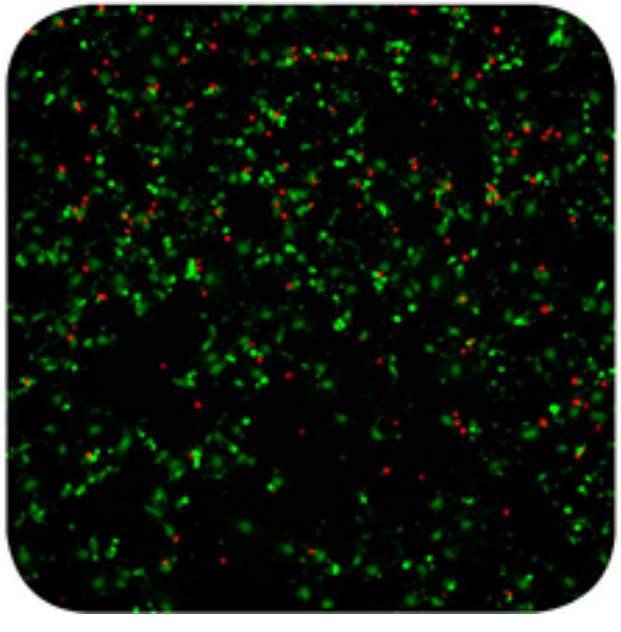
BMES Cell Team

Winter 2021

Outline

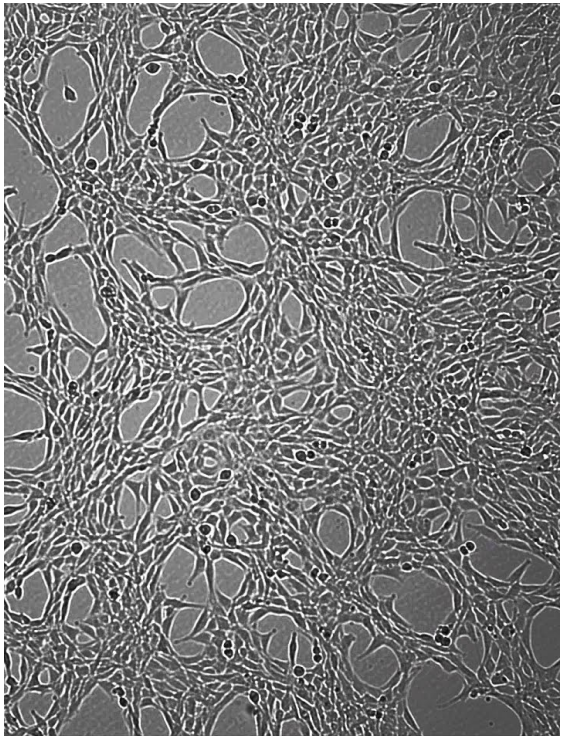
- Background
- Cell Passaging
    - 3T3 Cells in a T25 Flask
- Cell Counting
    - Hemocytometer Count
- Viability Assays
    - Live-Dead Stain





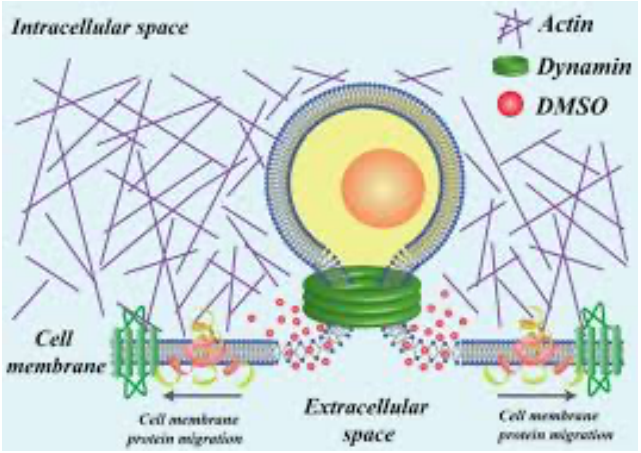
Background: 3T3 Cells

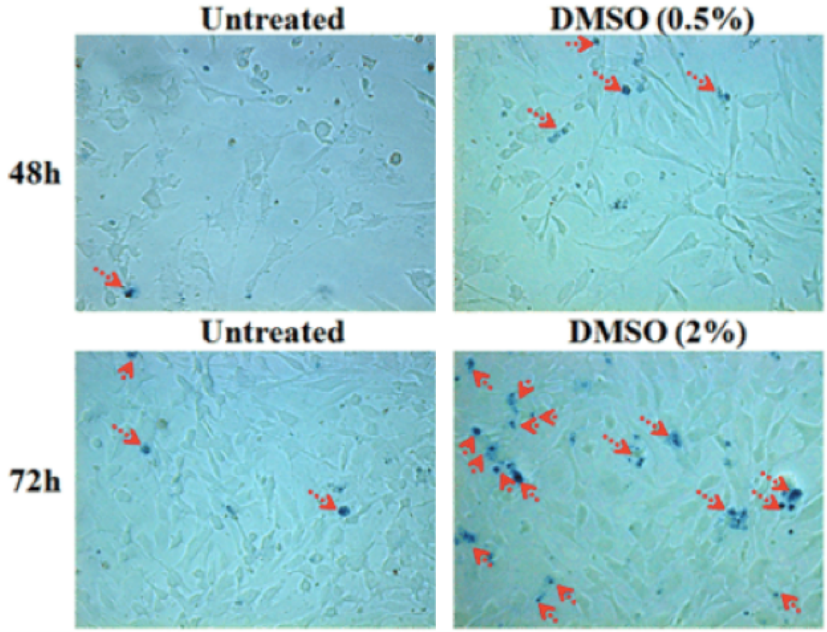
- To learn the cell passaging procedures, students often use 3T3 cells
- 3T3 cells come from a mouse fibroblast line
- Why they are used:
    - They grow in a monolayer
    - They are functional for many generations (20 - 30)
    - They are receptive to genetic modification



Background: Dimethyl Sulfoxide (DMSO)

- DMSO is an organosulfur that is capable of serving as a mild oxidant
- DMSO is involved in chemical reactions that create pores in the plasma membrane, disrupting the electrochemical gradient
- High concentrations of DMSO can lead to apoptosis and the resulting cell death

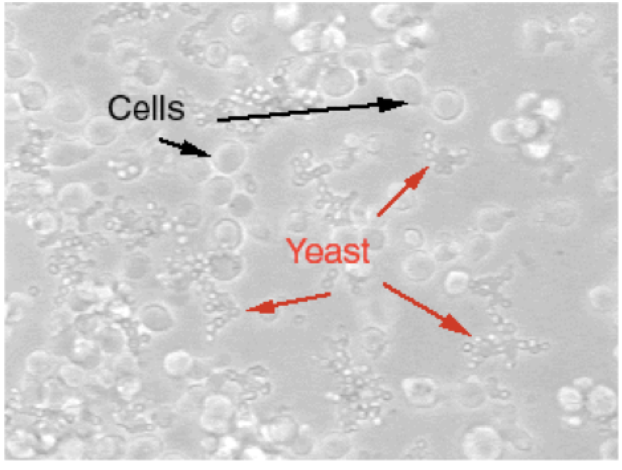


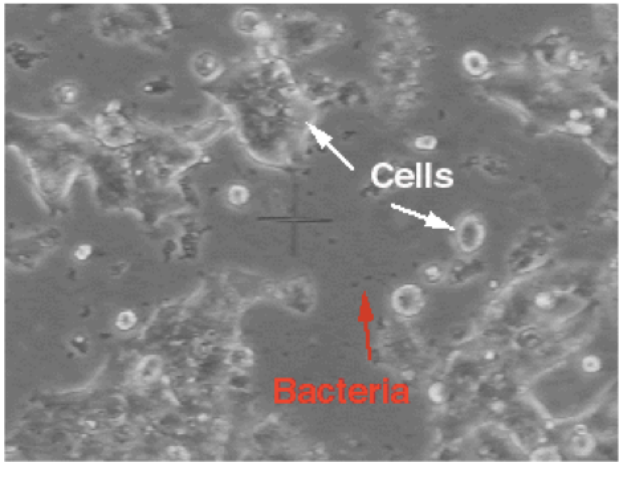


https://www.researchgate.net/figure/Representative-microscope-images-of-live-and-dead-cells-after-solvent-treatments-Cells_fig5_324113812

Background: Some General Lab Reminders

- You are constantly trying to avoid contamination, so follow good laboratory practices, like:
  - Spraying EVERYTHING with ethanol
  - Cap containers immediately after use
  - Don’t move quickly inside the BSC
  - Bring extra pipettes into the BSC
  - Dispose of anything that touched cells in biohazardous waste
  - Take notes as you work





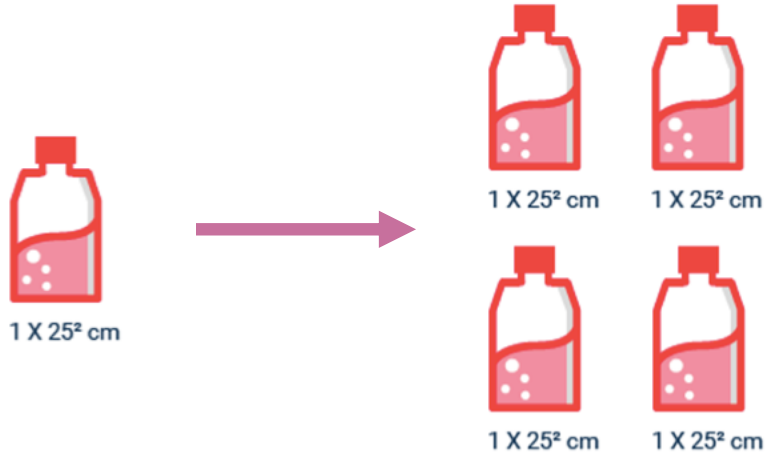
Background: Setting Up for the Experiment

- Turn on the BSC and place necessary equipment inside the work station

- Determine if your cells needs to be passaged

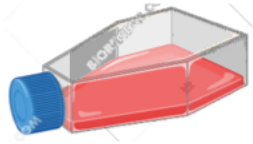
Part 1: Cell Passaging

- **Cell passaging** is the transfer of cells between culture containers
- Protocol for transferring cells from one confluent T25 flask to four new T25 flasks
- Performed inside of a Biological Safety Cabinet (BSC)

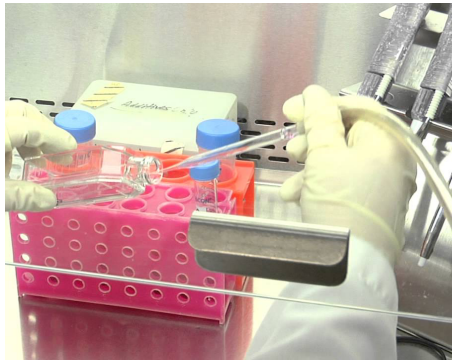


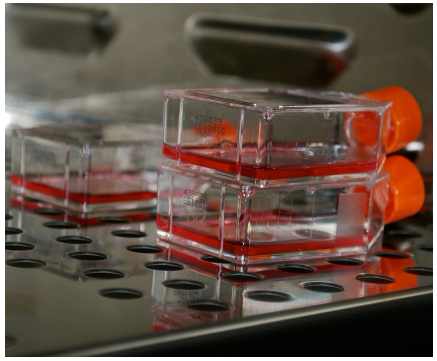
Cell Passaging: Removing Cells

Starting flask

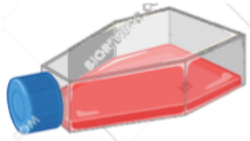


Aspirate off media

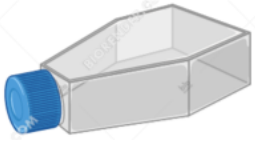


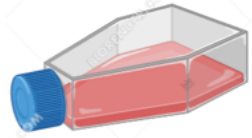


Ending flask



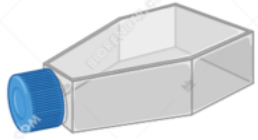
Add media

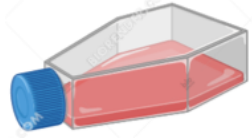




Add PBS

Incubate



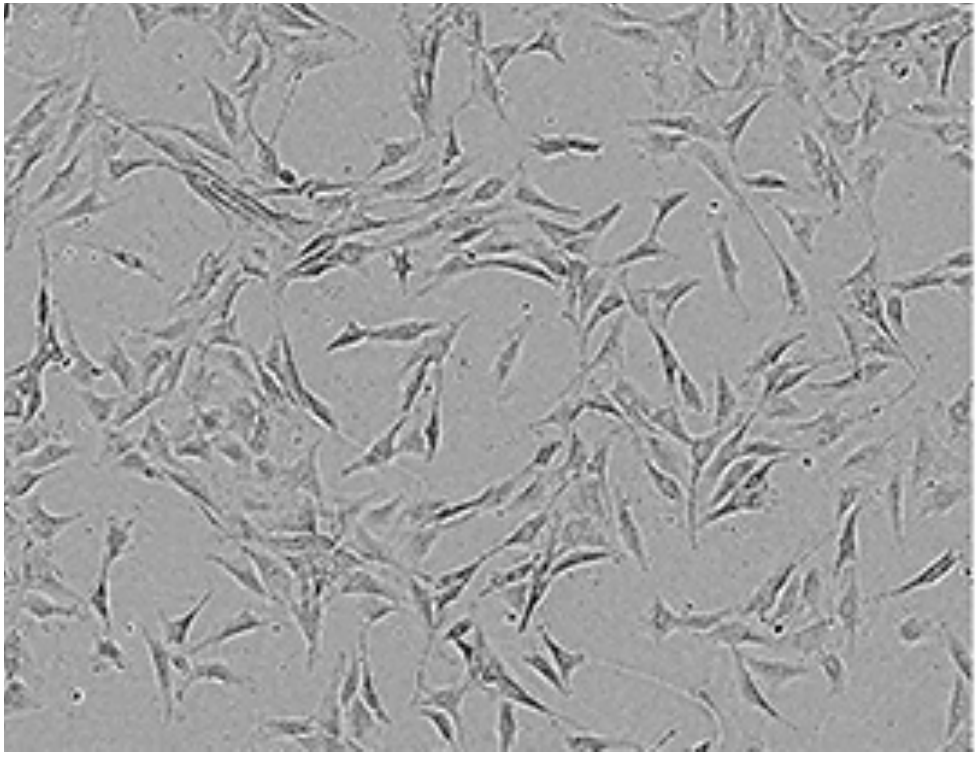


Aspirate off PBS

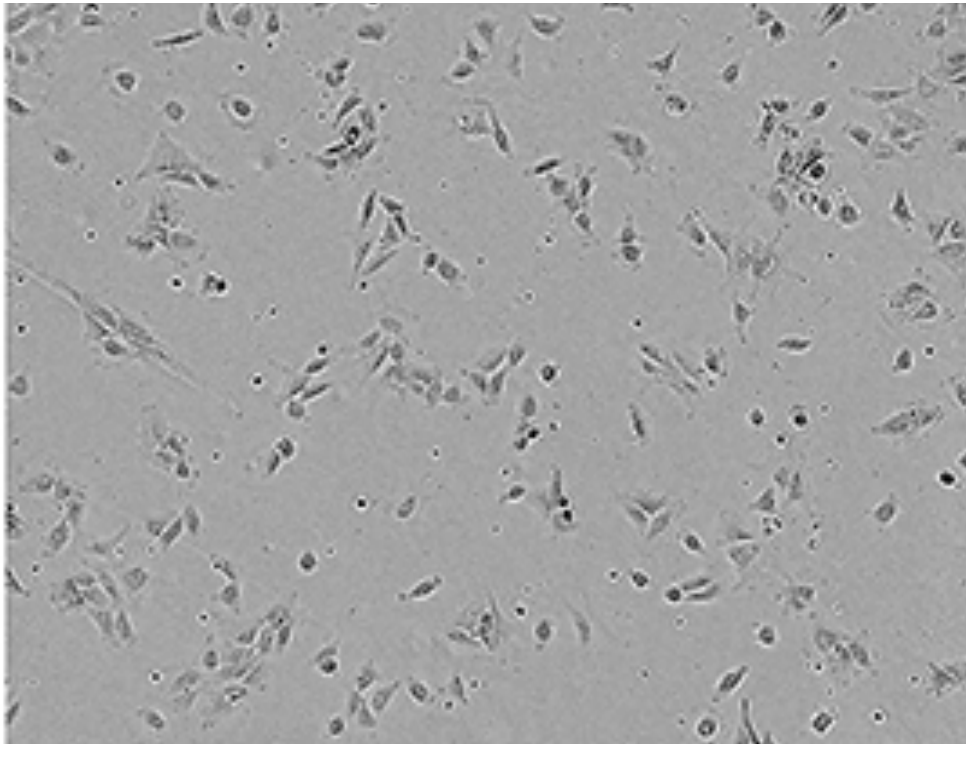
Add Trypsin

Cell Passaging: Removing Cells

Before trypsin

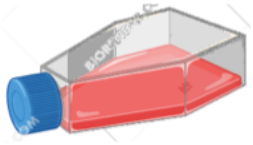


After trypsin



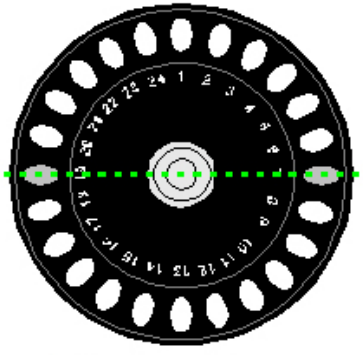
Cell Passaging: Centrifuging

Return flask to BSC

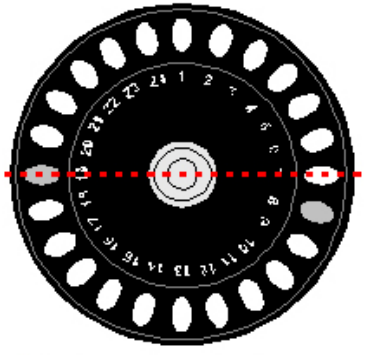


Transfer flask contents to a 50 mL conical tube

Make sure samples are balanced in the centrifuge



✔ Tubes are balanced



✘ Tubes are not balanced

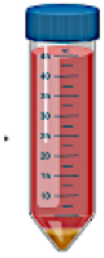
Place in centrifuge

Run at 1000 RPM for 5 minutes

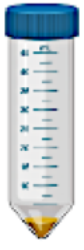
Yields cell pellet

Cell Passaging: Moving Cells to New Flasks

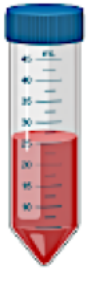
Centrifuged tube

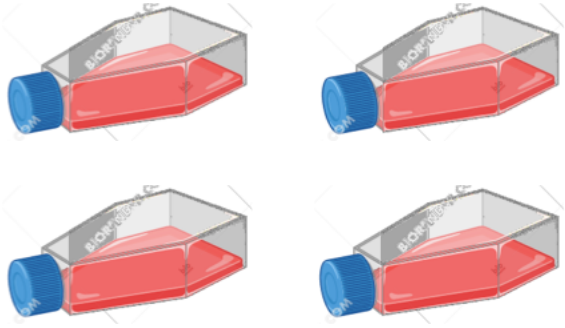


Aspirate off supernatant

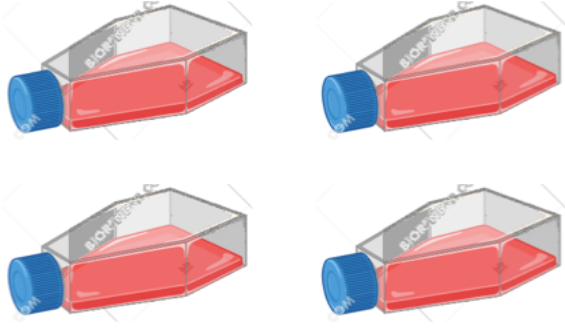


Resuspend in 1 mL media

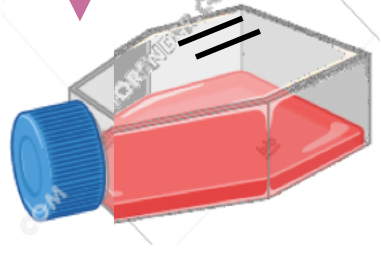




Add 5 mL media to 4 T25 flasks



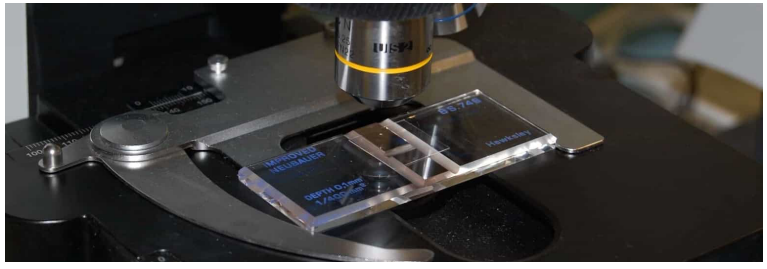
Add 250 uL cell solution to 4 T25 flasks



Label the flasks

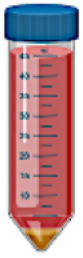
Part 2: Cell Counting

- **Cell counting** with a hemocytometer is conducted to yield an estimate of seeded cell concentration
- Protocol for obtaining a hemocytometer count while passaging cells to new T25 flasks
- Performed on the lab bench under a EVOS Fluorescence Microscope

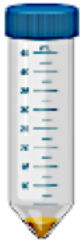


Cell Counting: Transferring Cells to the Hemocytometer

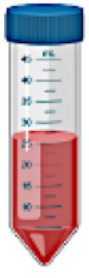
Centrifuged tube



Aspirate off supernatant

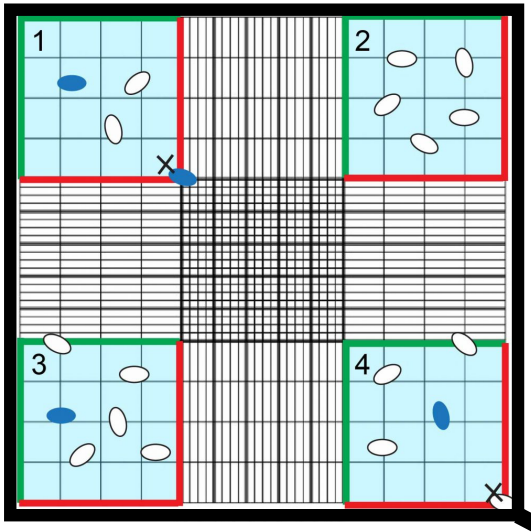


Resuspend in 4 mL media

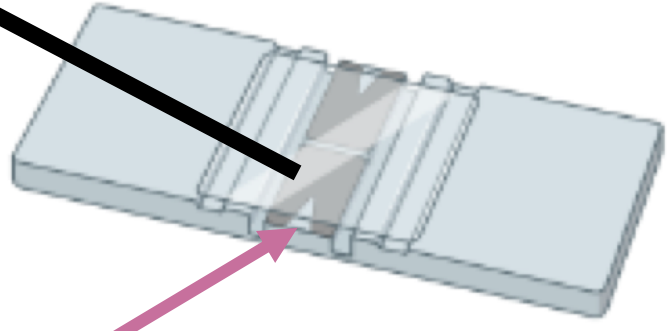


Transfer 20 uL to eppendorf tube

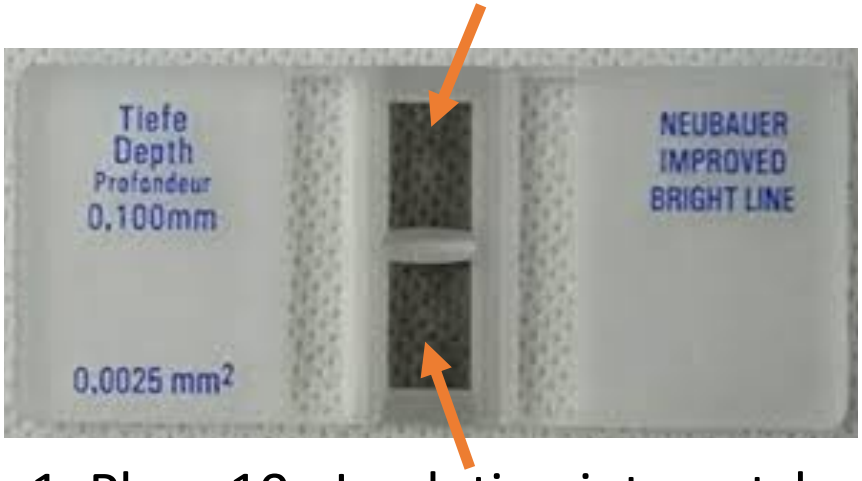
Pipette 10 uL cell solution into hemocytometer



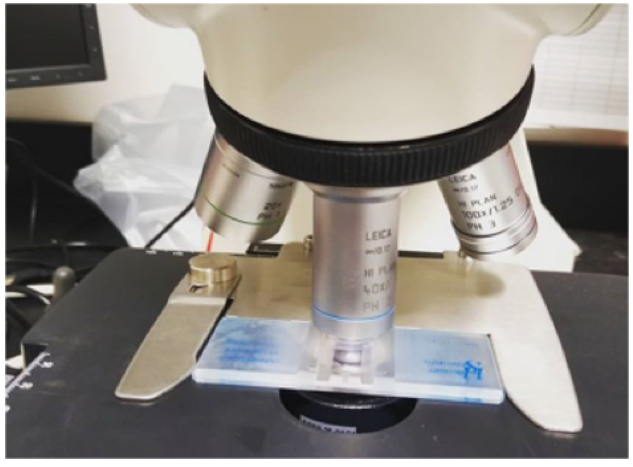
Count the cells in the four corner quadrants



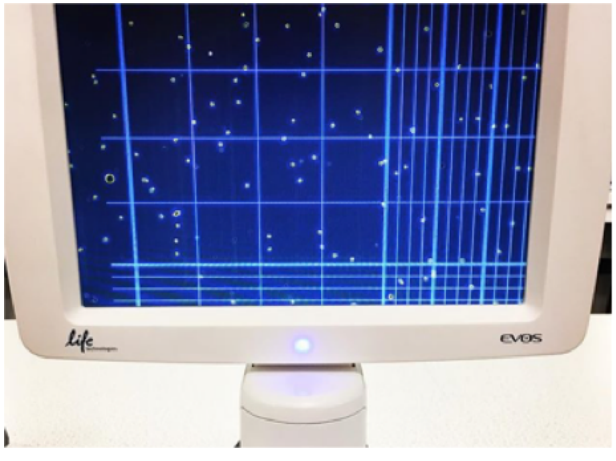
Cell Counting: How to use a Hemocytometer



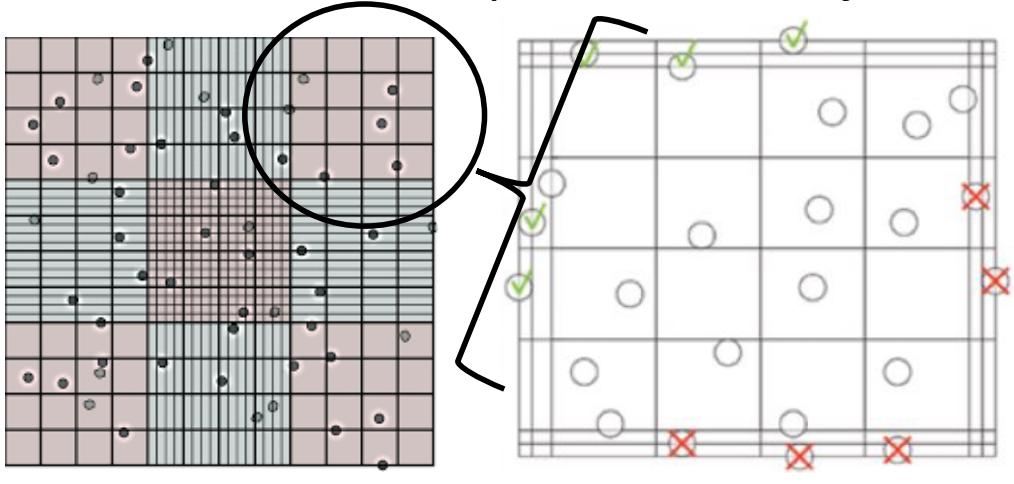
1. Place 10 uL solution into notch (between hemocytometer and coverslip)



2. Place hemocytometer under microscope with 10x objective



3. Bring grid into view and cells into focus



4. Count total number of cells in four corner quadrants

Cell Counting: Determining Seeding Volume

- **Average Number of Cells Per Counting Square ($\frac{\boldsymbol{average\ \#\ cells}}{\boldsymbol{counting\ square}}$)**

$$\left(\frac{average\ \#\ cells}{counting\ square}\right) = \frac{\#\ cells\ in\ Square\ 1 + \#cells\ in\ Square\ 2 + Square\ 3\ cells + Square\ 4\ cells}{4\ counting\ squares}$$

- **Cell Concentration ($\frac{\boldsymbol{\#\ cells}}{\boldsymbol{mL}}$)**

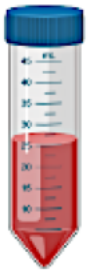
$$\left(\frac{\#\ cells}{mL}\right) = \left(\frac{average\ \#\ cells}{counting\ square}\right) * \left(\frac{counting\ square}{0.1mm^3}\right) * \left(\frac{1mm^3}{10^{-3}cm^3}\right) * \left(\frac{1cm^3}{1\ mL}\right)$$

- **Seeding Volume ($\boldsymbol{mL}$)**

$$mL = \frac{desired\ number\ of\ cells}{cell\ concentration} = \frac{desired\ number\ of\ cells}{\frac{\#\ cells}{mL}}$$

Cell Counting: Seeding Cells

Transfer desired volume of cell suspension into a 24 well plate

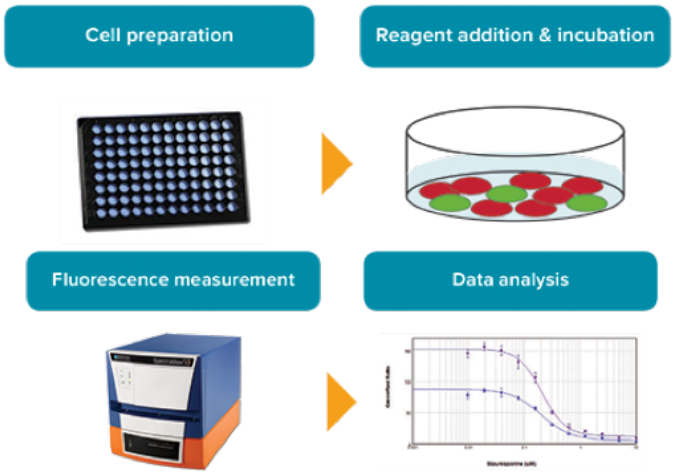


These seeded wells will be used in the viability assay in the next section

For this experiment, 50,000 cells were seeded in each well

Part 3: Cell Viability

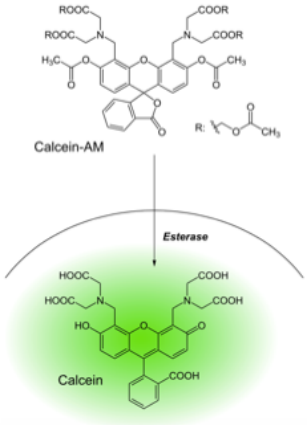
- **Cell viability** is assessed using colorimetric assays to determine the metabolic activity of the cell population
- Protocol for conducting a Live-Dead Stain on 3T3 cells treated with DMSO
- Performed on the lab bench under a EVOS Fluorescence Microscope

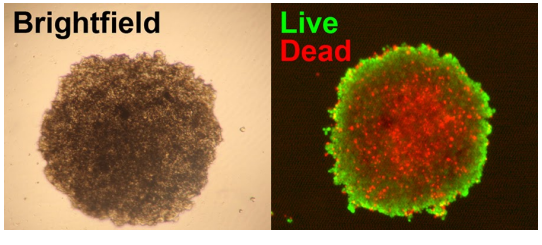


https://www.moleculardevices.com/en/assets/app-note/reagents/earlytox-live-dead-assay-kit-on-spectramax-fluorescence-readers

Live-Dead Assay

- **Utility:**
  - Visualizes live and dead cells using fluorescent dyes
- **Mechanism:**
  - Nonpolar **Calcein-AM** enters the living cells
  - Esterases convert Calcein-AM into polar, fluoresecent **Calcein**
  - The polar product cannot leave the cells → green marks live cells
  - When the cell dies, the plasma membrane is disrupted
    - Calcein can leave the cell
  - The fluorescent dye **Ethidium homodimer-1** tags broken membranes, binding to the dead cells → red marks dead cells





https://quizlet.com/341886021/be-167l-lab-practical-questions-flash-cards/ https://en.wikipedia.org/wiki/Calcein

Cell Viability: Transferring Cells to the Hemocytometer

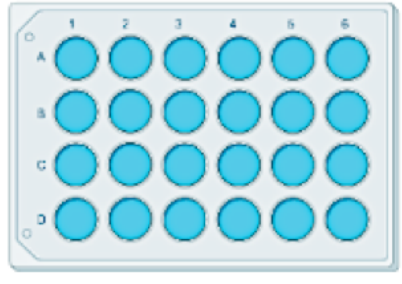
Starting with an empty conical tube

Note: conical tube is wrapped in aluminum foil to prevent photobleaching

Observe 24 well plate under a microscope

Add PBS, Calcein, and Ethidium Homodimer

Aspirate off media



Examine under microscope

GFP shows Calcein labeling
RFP shows Ethidium Homodimer labeling

Protocol Walkthrough WrapUp

- **For Thursday**
    - Come to Protocol Discussion with ImageJ installed
    - We will be analyzing images from a cell passaging procedure
- **Team Check Ins**
    - Group 2: stick around for check ins
- **Check out the Class Planning Workshop if you can!**