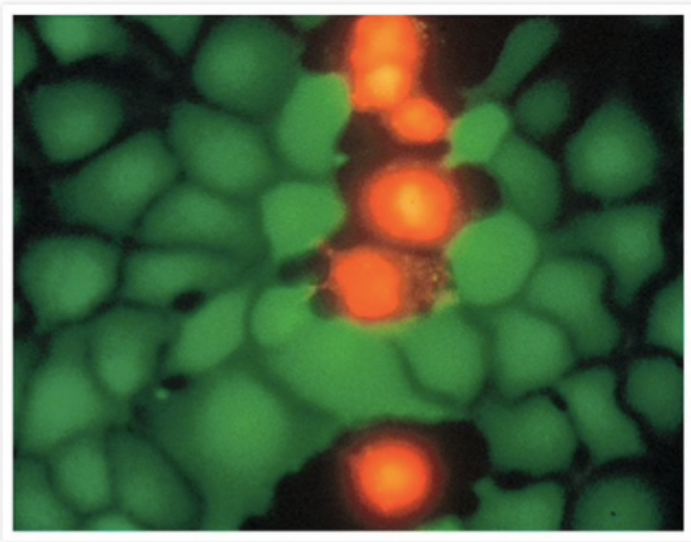
Module 5: Cell Counting and Viability Assays

BMES Cell Team

Fall 2020

Outline

- Cell Counting
    - Manual and Automated Methods
    - How to use a Hemocytometer
- Viability
    - MTT Assay
    - MTS Assay
    - Live-Dead Assay
    - Luminescent Assay: Cell Titer Glo
- Protocol Overview



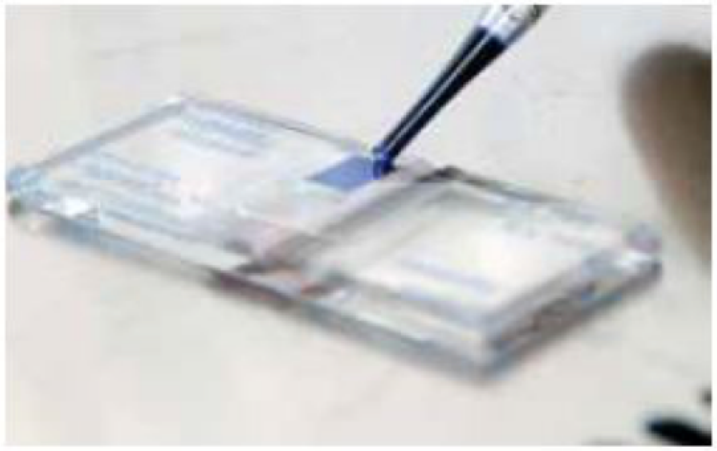
https://www.thermofisher.com/order/catalog/product/L3224#/L3224

Introduction to Cell Counting

- **Definition:** Cell counting determines the number of cells in a sample to estimate the total number of cells in a flask.

- Cell counting is embedded in almost every cell culture procedure

- Result is expressed as cell concentration ($\frac{number\ of\ cells}{mL\ solution}$)



https://www.sigmaaldrich.com/technical-documents/protocols/biology/cell-quantification.html

Why do we need to count cells?

- **Seeding Purposes**
  - **Knowing cell concentration increases seeding accuracy**
    - Avoids underseeding and overseeding
    - Improves confluency estimates

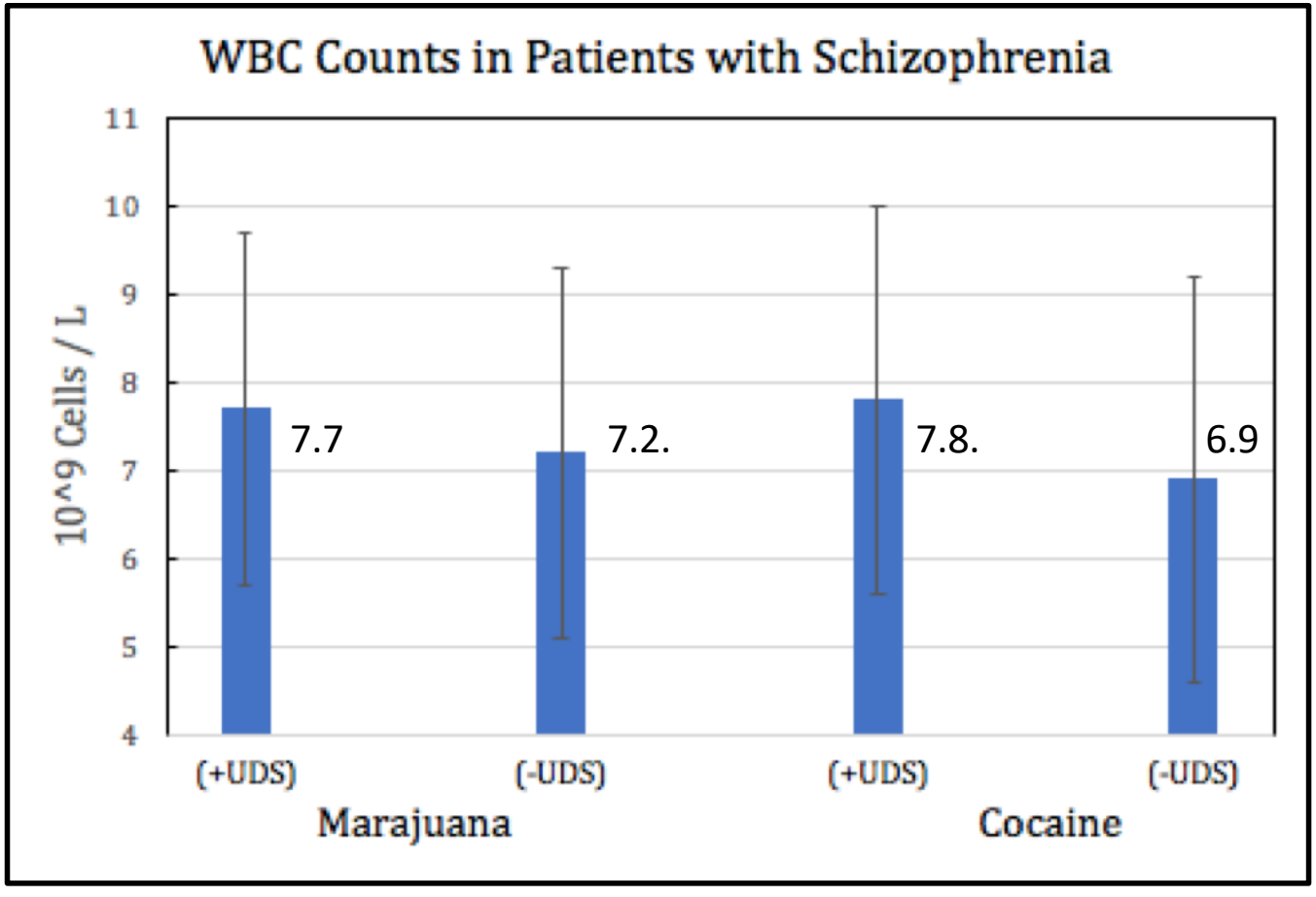
- **Experimental Purposes**
  - **An accurate cell count ensures reproducibility**
    - Transfections
    - Drug treatment studies
      - Drugs that depleted blood cells
    - CRISPR

- **Diagnostic Purposes**
  - **The diagnosis of some diseases is based on cell count**
    - HIV → AIDS
      - [CD4 T Cells] < 200 cells / uL blood

https://journals.lww.com/jonmd/FullText/2019/08000/Total_and_Differential_White_Blood_Cell_Counts..4.aspx?casa_token=H99TN9FOttIAAAAA:BgYXkgcUng8uZiCDl4BfOmGCxRNqltkBu6NBzrmSGucemqG0KMWWkcP80tVeHH7V7arGJDFIfHGNhS-negRGJis https://www.clinicalleader.com/doc/are-absolute-cell-counts-essential-in-drug-discovery-0001

Example: Why do we need to count cells?

Impact of Marijuana and Cocaine Use on White Blood Cell Counts of Patients with Schizophrenia



Data from the August 2019 Journal of Nervous and Mental Disease

https://journals.lww.com/jonmd/FullText/2019/08000/Total_and_Differential_White_Blood_Cell_Counts..4.aspx?casa_token=H99TN9FOttIAAAAA:BgYXkgcUng8uZiCDl4BfOmGCxRNqltkBu6NBzrmSGucemqG0KMWWkcP80tVeHH7V7arGJDFIfHGNhS-negRGJis https://www.clinicalleader.com/doc/are-absolute-cell-counts-essential-in-drug-discovery-0001

Methods of Cell Counting

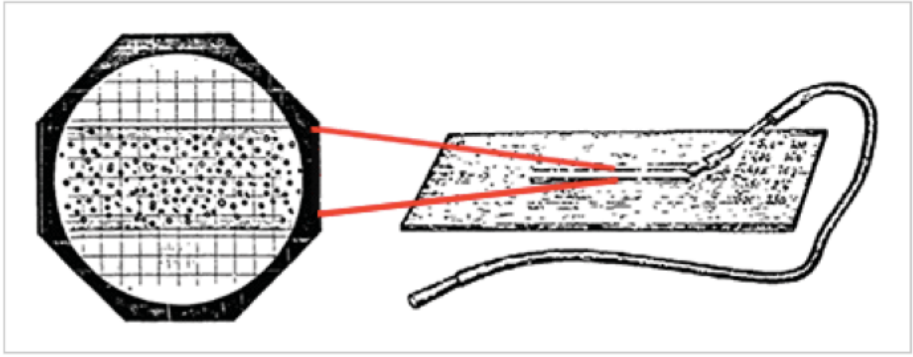
- **Manual**
    - **Hemocytometer**
        - Insert sample and count cells under a microscope
- **Automated**
    - **Flow Cytometry**
        - Optical system that counts and sorts marked cells
    - **Coulter Counter**
        - Two chamber system detects cell type and cell number
    - **Image Analysis**
        - Software counts cells from microscope images

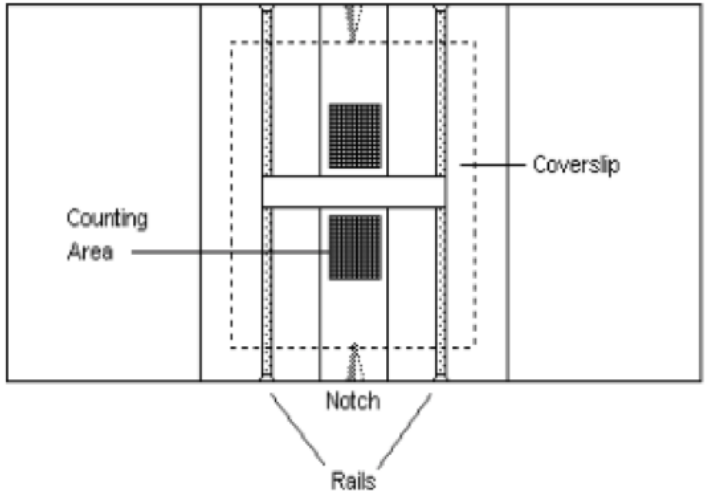
https://docs.google.com/document/d/1yx3zMS82iSiy2l7tmFbCObKcMOQAaBox_iB1NGTwaco/edit

Hemocytometers

- **Definition:** A hemocytometer is an instrument that allows for the visual counting of cells in a fluid sample.

- Originally created to count blood cells
- Sample loaded into the hemocytometer notch
- Travels to grid via capillary action

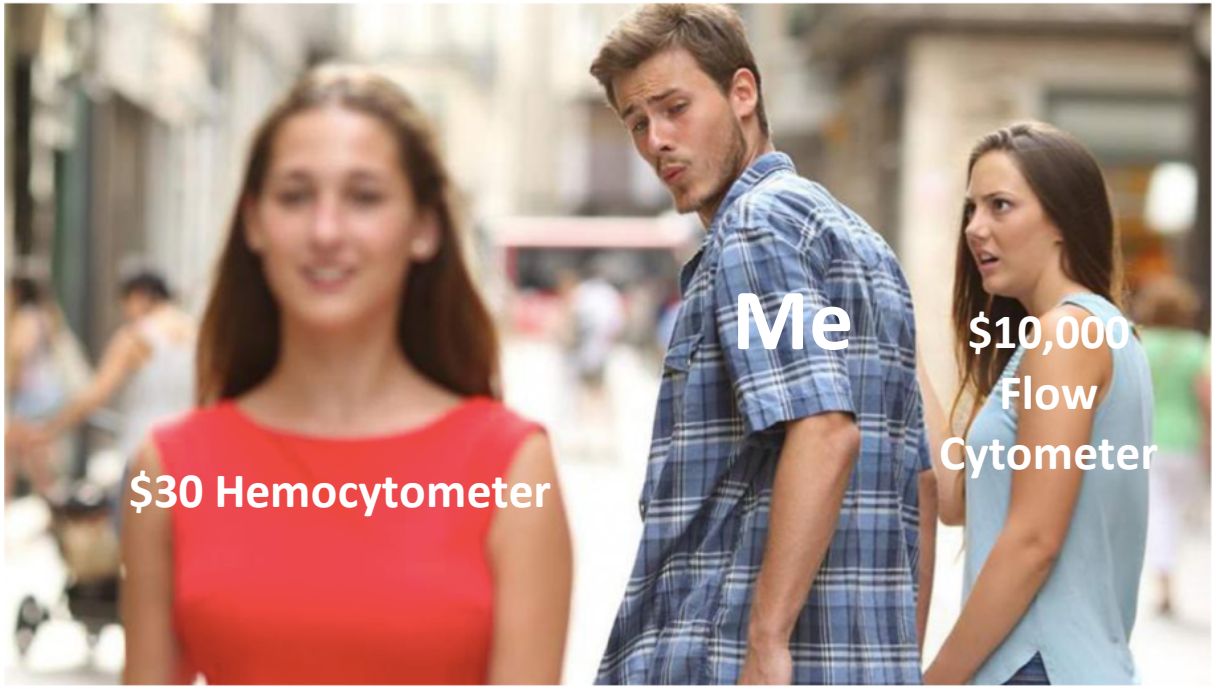




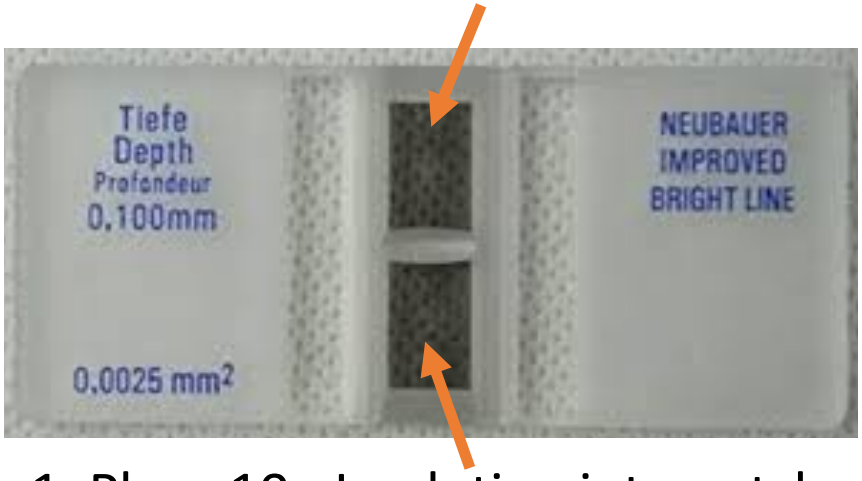
https://www.nexcelom.com/applications/cellometer/cell-counting/history-of-the-hemacytometer/ https://en.wikipedia.org/wiki/Hemocytometer https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3335093/

Why do we use Hemocytometers?

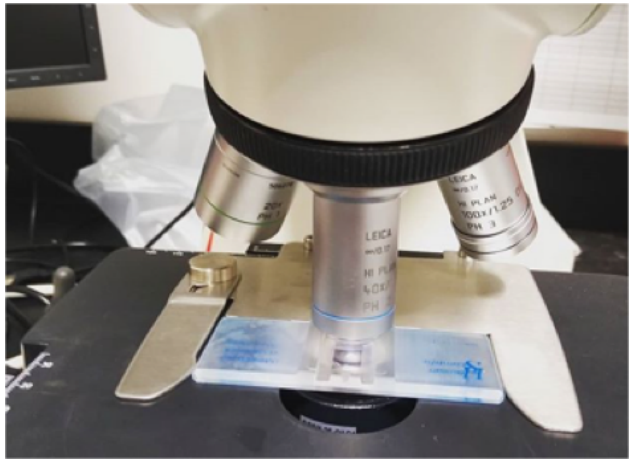
- Cheap
- Fast
- Compact
- Reusable
- Relatively simple
- 71% of the 400 researchers examined use hemocytometers in their research (Millipore)



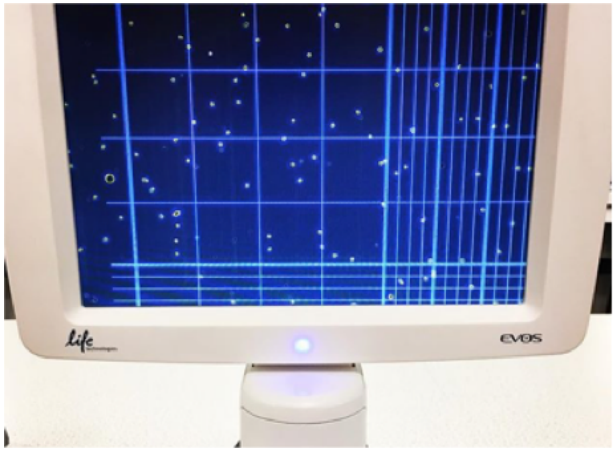
How to use a Hemocytometer



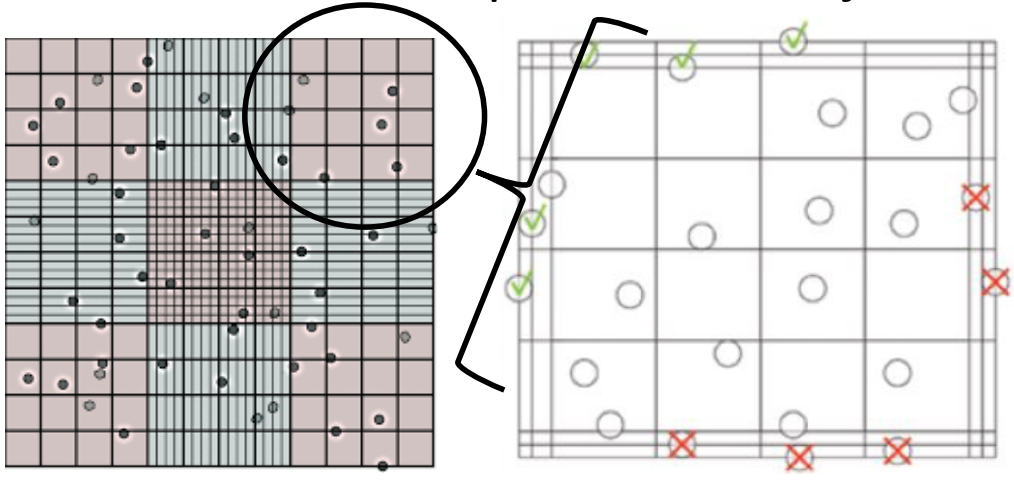
1. Place 10 uL solution into notch (between hemocytometer and coverslip)



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

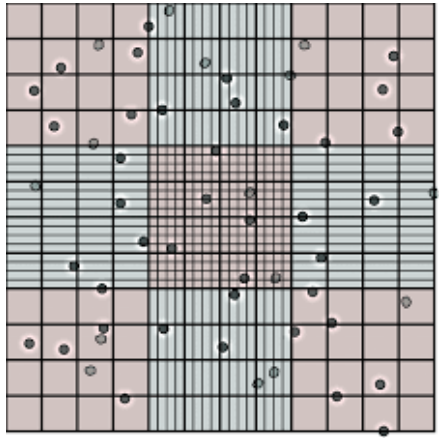
Hemocytometer Calculations

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

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

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

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



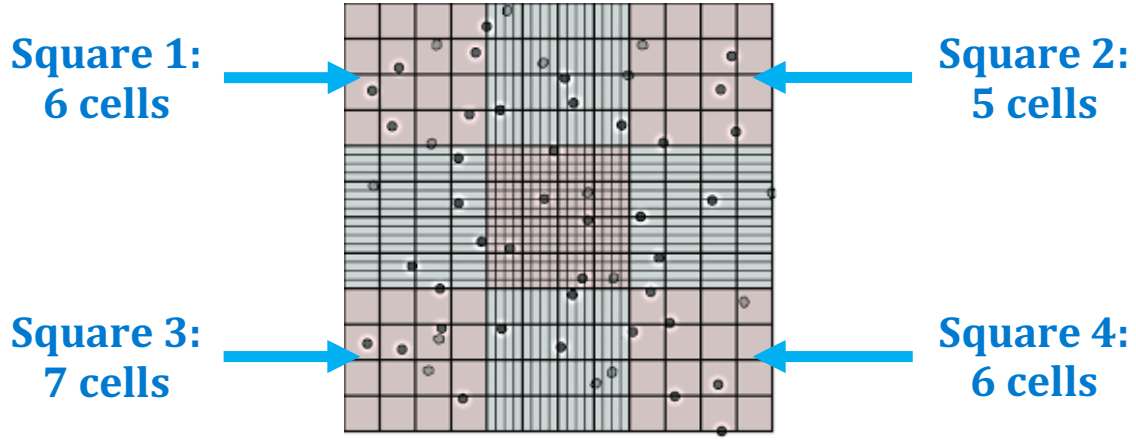
Hemocytometer Calculations Example

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

$$\left(\frac{average\ \#\ cells}{counting\ square}\right) = \frac{6+5+7+6}{4\ counting\ squares} = 6\ \frac{cells}{counting\ square}$$

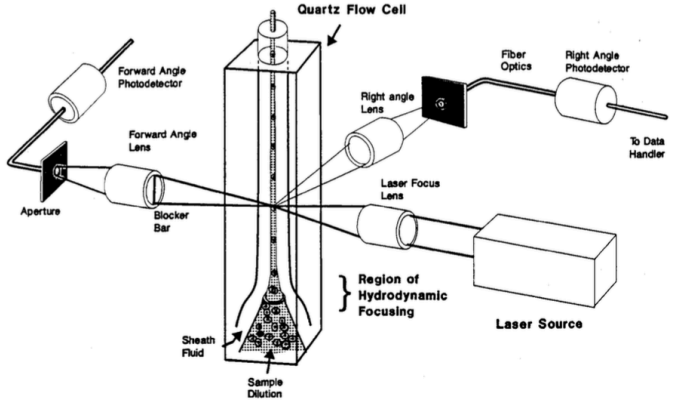
- **Cell Concentration ($\frac{\#\ cells}{mL}$)**

$$\left(\frac{\#\ cells}{mL}\right) = \left(\frac{6\ cells}{counting\ square}\right)*\left(\frac{counting\ square}{0.1mm^3}\right)*\left(\frac{1mm^3}{10^{-3}cm^3}\right)*\left(\frac{1cm^3}{1\ mL}\right) = 6 \cdot 10^4\ \frac{cells}{mL}$$



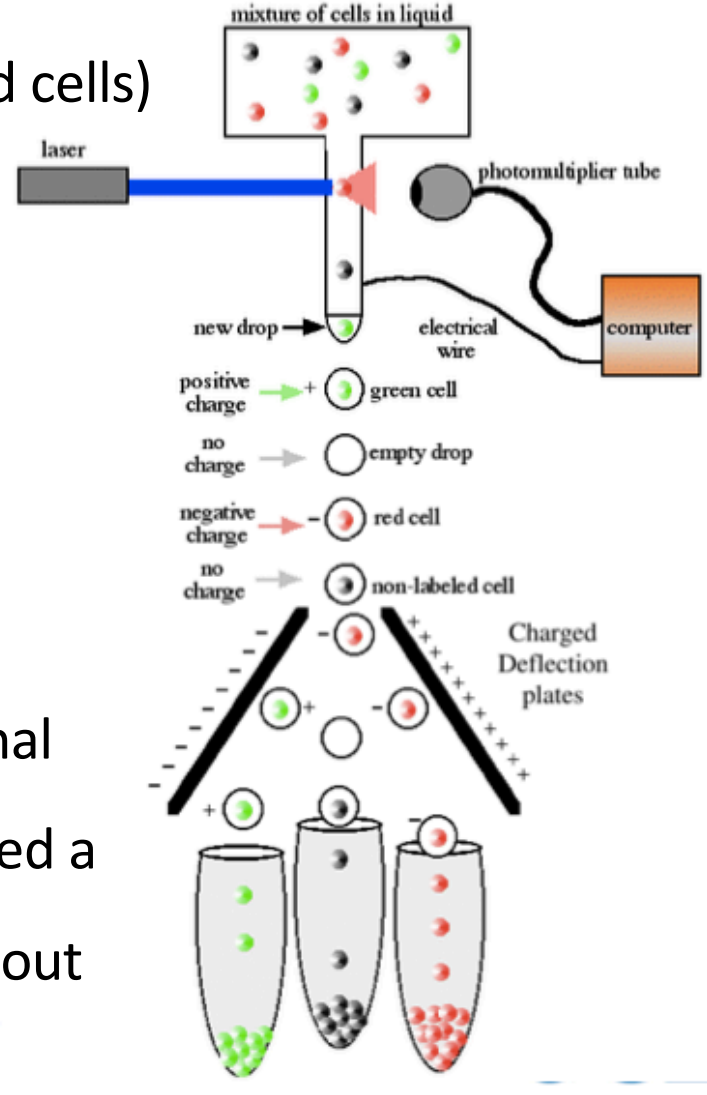
Flow Cytometry

- **Utility**
  - Counts, sorts, and detects marked cells
- **Mechanism**
  - Sheath fluid linearizes cells
  - Focused laser beam hits the cell
    - Creates forward scattered light (FSC) at low angle and side scattered light (SSC) at high angle
  - Photodetectors pick up FSC and SSC
    - FSC correlates to cell volume (cell size)
    - SSC correlates to internal complexity (cell type)

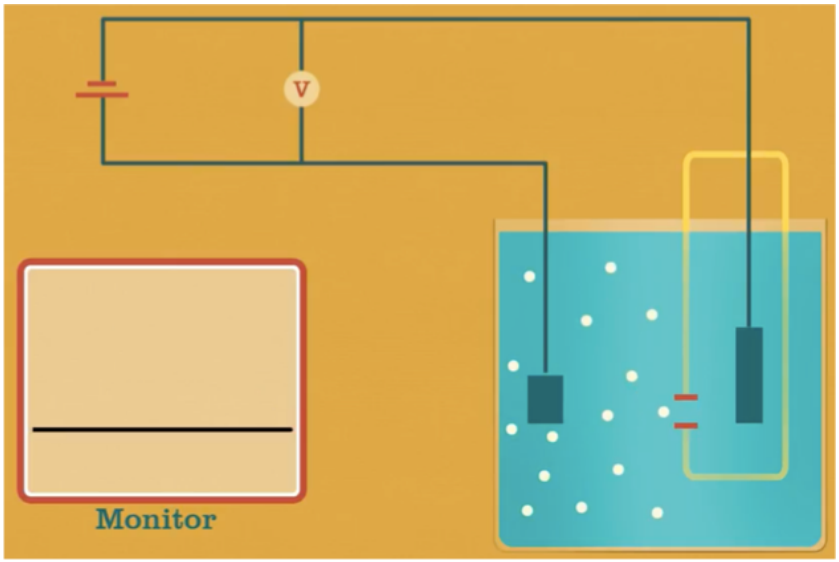


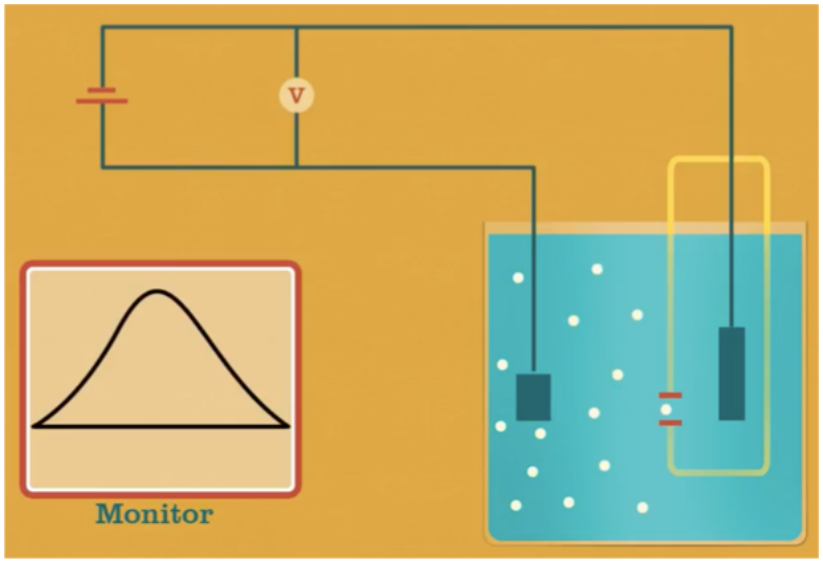
Fluorescence Activated Cell Sorting (FACS)

- Specialized flow cytometry that uses fluorescence for cell sorting
- **Utility**
  - Separates mixture of cell types (ex: blood cells)
- **Mechanism**
  - Fluorescently-tagged antibodies added
  - Sheath fluid linearizes cells
  - Focused laser beam hits the cell
    - Tagged cells fluorescence
  - Photodetectors pick up fluorescence signal
    - If they fluoresce, cells are assigned a charge that is used to sort them out



Coulter Counter



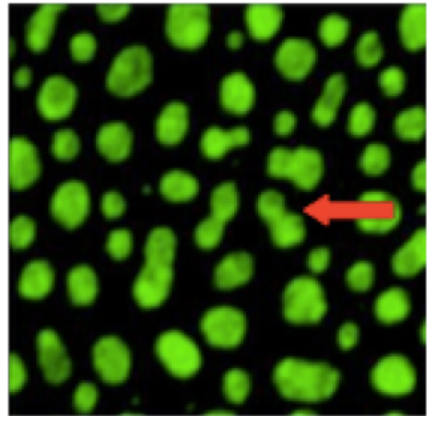


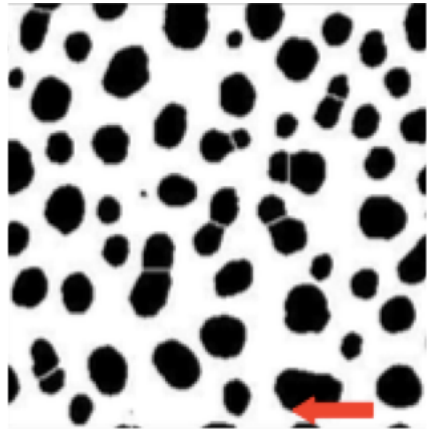
- **Utility**
  - Counts, sorts, and detects marked cells
- **Mechanism**
  - Device contains two chambers filled with electrolytic solution with microchannel(s) between the chambers
  - A particle entering a microchannel changes the liquid's electrical resistance
  - Counter records resistance change (ΔR)
    - $\Delta R \propto$ cell volume

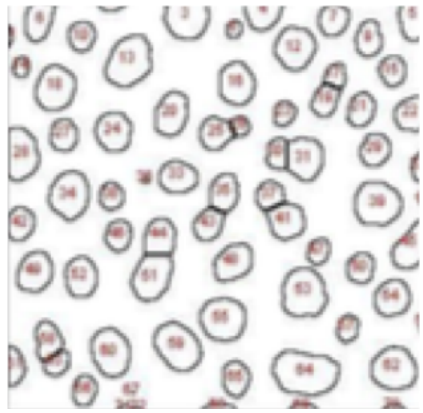
https://www.youtube.com/watch?v=rsPQ8G9-GgI

Image Analysis

- **Utility**
  - Counts cells in uploaded images
  - Saves time when processing large image sets
- **Mechanism**
  - Greyscale image
  - Remove background
  - Divide touching particles
  - Analyze Particles to obtain cell count
- **Frequently Used Software**
  - ImageJ
  - CellProfiler







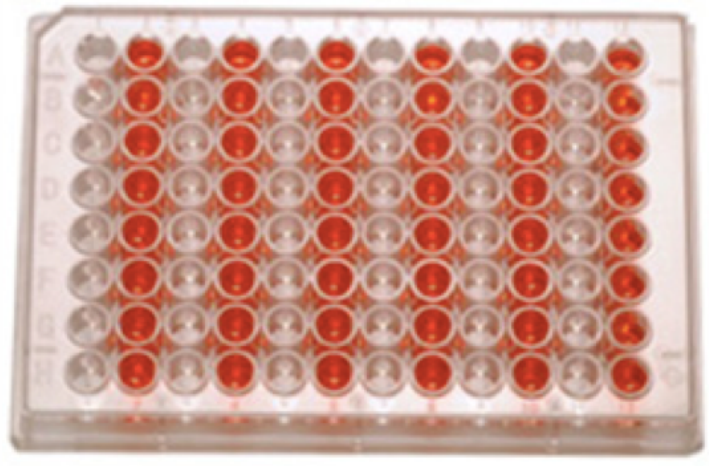
Count	Total Area	Average Size	Area Fraction
72	21286.00	295.64	32.7

https://www.unige.ch/medecine/bioimaging/files/3714/1208/5964/CellCounting.pdf

Introduction to Viability Assays

- **Definition:** An assay is a laboratory procedure that quantifies target presence. A cell viability assay quantifies the living cells in a sample.

- Typically conducted in a 96 well plate
- Use linear relationship between living cell count and absorbance/luminescence



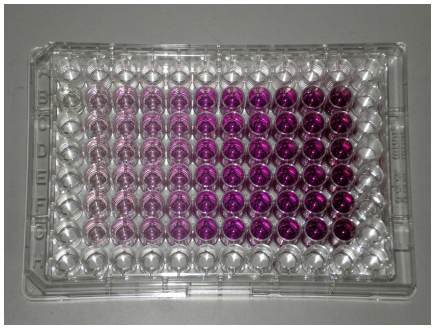
https://www.rpicorp.com/products/laboratory-equipment/cell-culture/cell-culture-plates-96-well.html

Why do we need Viability Assays?

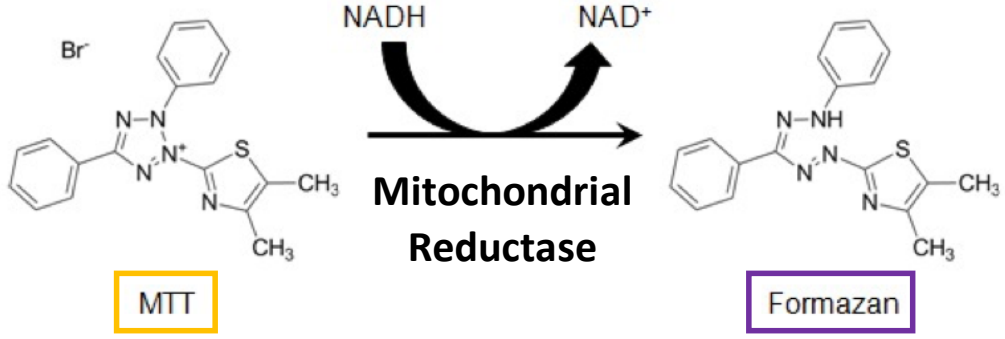
- **To Quantify Cells**
    - **Number of live and dead cells helps access treatment results**
        - Compare control cell count vs. drug-treated cell count
        - Examine the impart of growth factors on cell behavior
- **To Visualize Cells**
    - **Experiment success is dependent on the survival of seeded cells**
        - Cells seeded into scaffolds
        - Cells deposited into hydrogels
        - Cells encapsulated in hydrogels

MTT Assay

- **Utility:**
  - Estimates cell viability using mitochondrial activity
  - The first modern viability assay



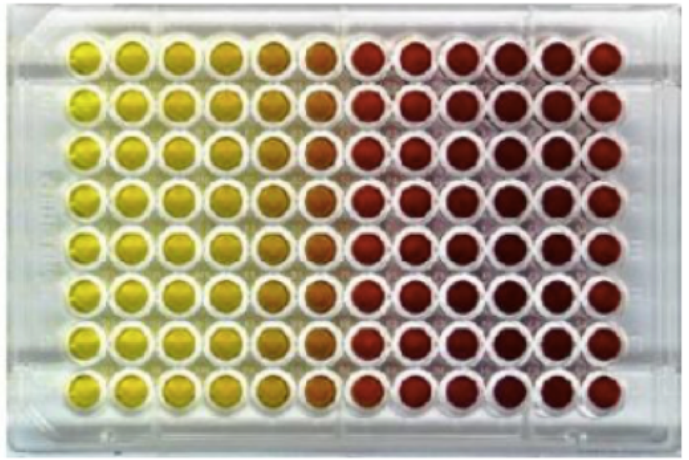
- **Mechanism:**
  - **Yellow MTT** (a tetrazole) is added to cells in culture
  - Mitochondrial enzymes reduce MTT to **purple Formazan** precipitate
  - The insoluble precipitate accumulates in the well
    - Absorbs maximally at 570 nm
  - Absorbance reading at 570 nm ∝ # metabolically active cells



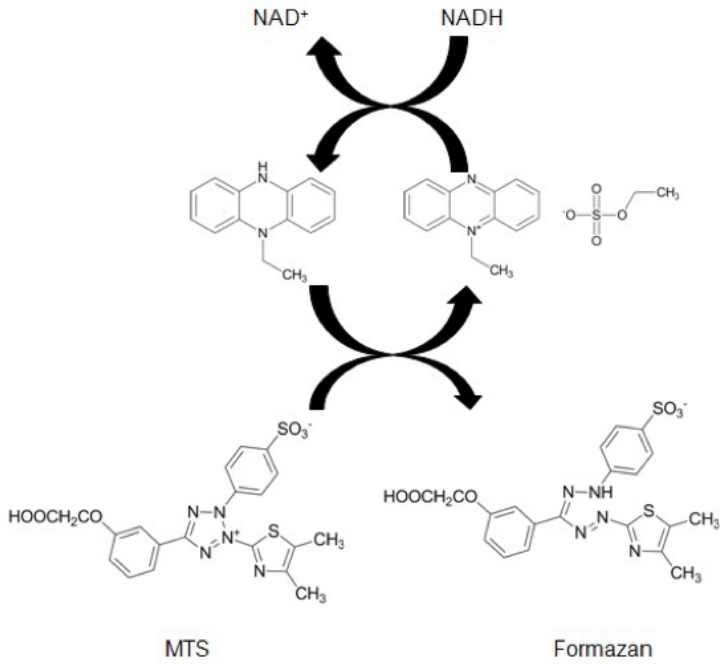
https://www.ncbi.nlm.nih.gov/books/NBK144065/

MTS Assay

- **Utility:**
  - Estimates metabolic activity of cells
  - "One step" MTT Assay
    - Yields product that is soluble in cell culture media
    - Nontoxic, so cells can return to culture after assay



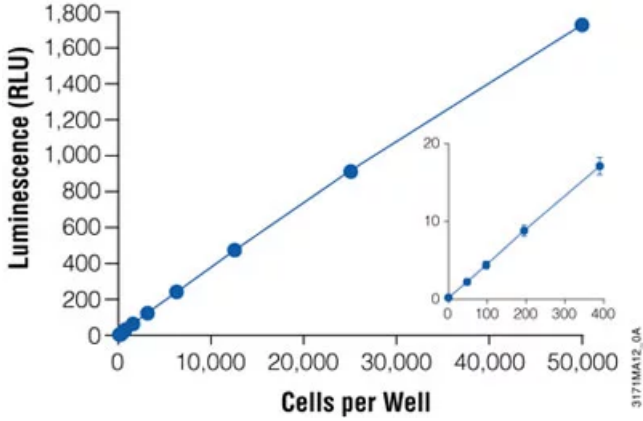
- **Mechanism:**
  - **Yellow MTS** is added to cells in culture with **PMS**
  - PMS enters cells, gets electron from NADH, and exits cells
  - Electron rich PMS reduces MTS to soluble **purple Formazan**
    - Absorbs maximally at 490 nm
  - Absorbance reading at 490 nm ∝ # metabolically active cells

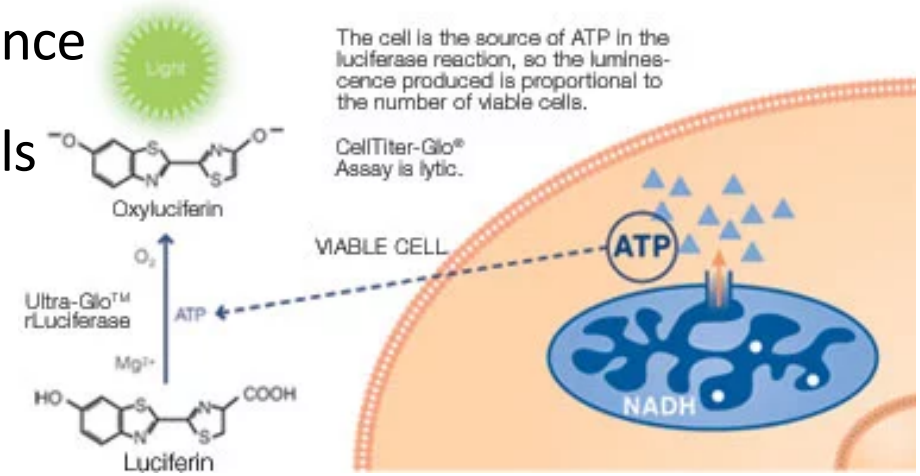


https://www.sciencedirect.com/topics/medicine-and-dentistry/mts-assay https://mappingignorance.org/2017/04/12/drug-discovery-works-finding-new-strategies-kaposis-sarcoma/
https://www.ncbi.nlm.nih.gov/books/NBK144065/

Cell Titer Glo

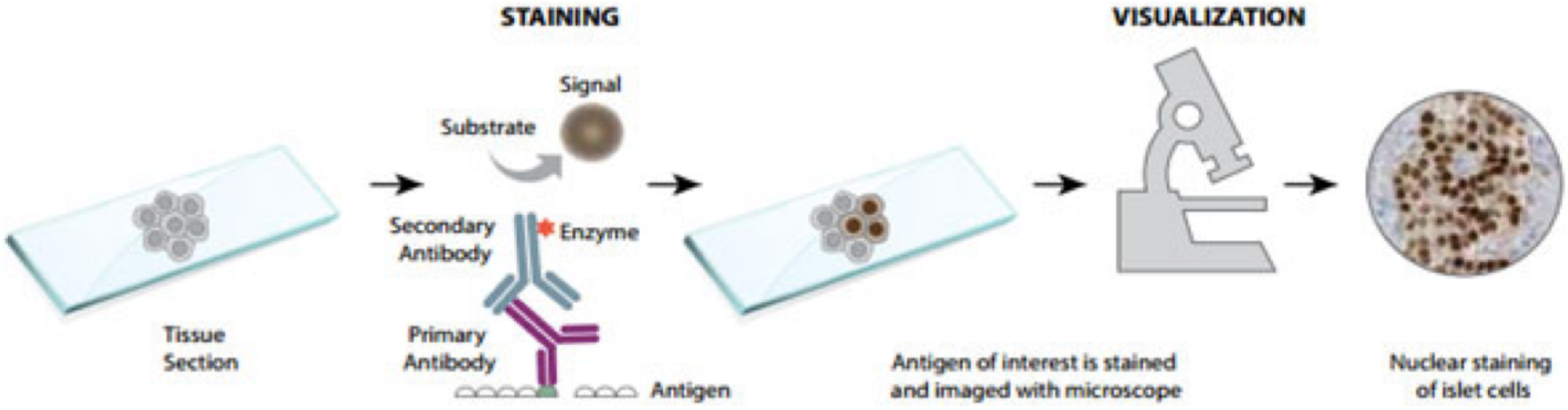
- **Utility:**
  - Estimates metabolic activity of cells
    - Quantifies ATP using luminescence
  - Requires only one reagent
- **Mechanism:**
  - The **CellTiter-Glo Reagent** is added to cells in media
  - The reagent lyses cells and causes a **luciferase reaction**
    - Produces bioluminescence
  - Luminescence ∝ # ATP ∝ # cells



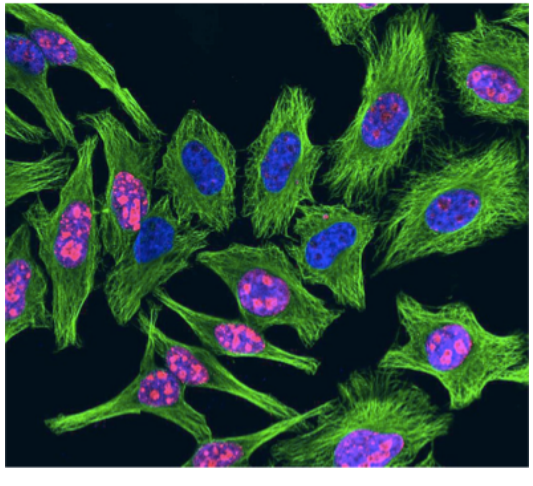


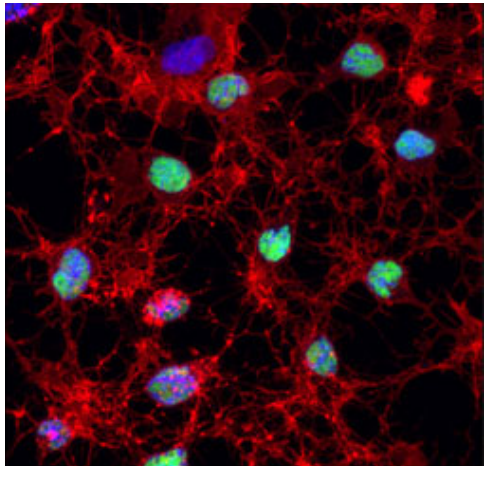
https://docs.google.com/presentation/d/1ZU2UWFywjxa6lQ3uVwyWfeI8GsWtQMkphH0QmrLb6Sc/edit#slide=id.g7cf3b4a8cb_0_137https://www.biocompare.com/9956-Assay-Kit/90549-CellTiterGloreg-Luminescent-Cell-Viability-Assay/

Immunostaining



- **Utility:**
  - Detects protein markers associated with cell viability
  - Visualizes proliferating and dying cells
- **Cell Proliferation Markers:**
  - Ki-67
- **Cell Death Markers:**
  - PARP-1

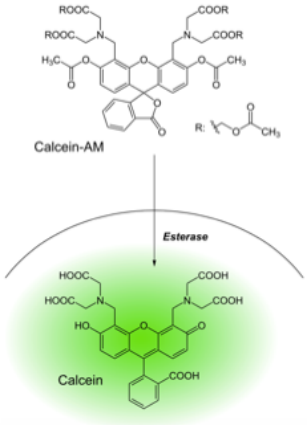


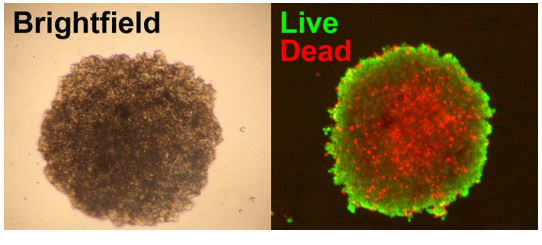


https://www.cellsignal.com/products/primary-antibodies/cleaved-parp-asp214-d64e10-xp-rabbit-mab/5625?site-search-type=Productshttps://en.wikipedia.org/wiki/PARP1 https://en.wikipedia.org/wiki/Ki-67_(protein) https://www.novusbio.com/application/ihc

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

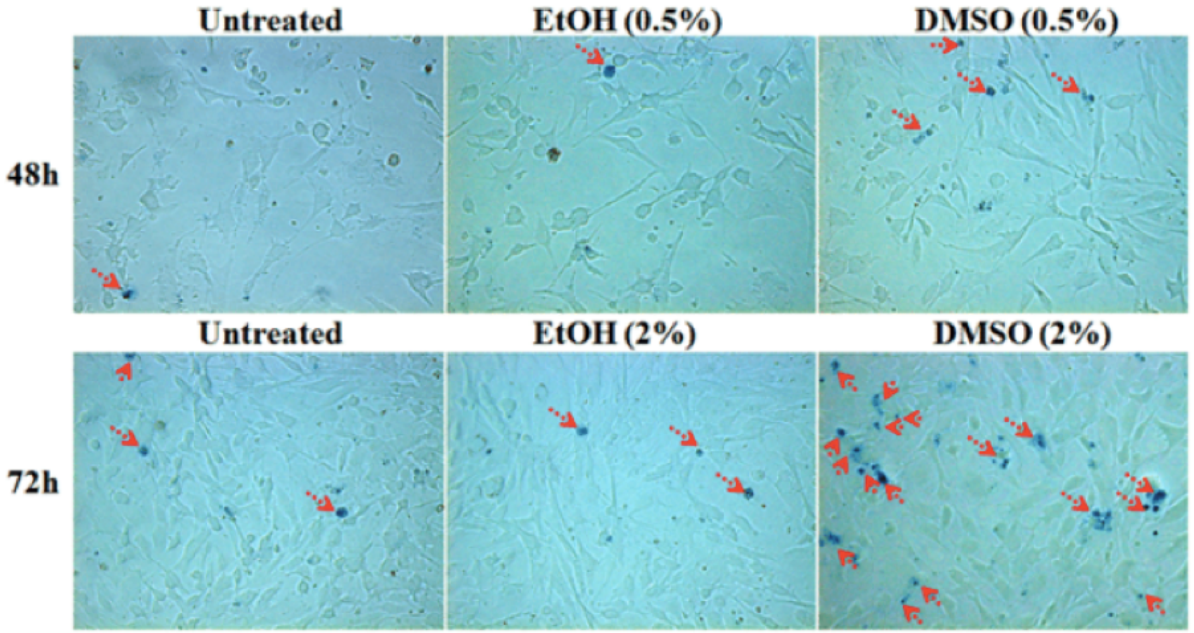




https://quizlet.com/341886021/be-167l-lab-practical-questions-flash-cards/ https://en.wikipedia.org/wiki/Calcein

Killing Cells: Dimethyl Sulfoxide

- DMSO creates pores in the cell membrane → apoptosis (cell death)
- If we are able to do in person experiments this year, we will:
    - Seed cells with increasing concentrations of DMSO
    - Use a Live/Dead Assay to determine cell viability as a function of DMSO concentration



Effect of DMSO (visualized through Trypan Blue Exclusion)

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