# In vitro models | Neuronal cultures

2022-2023

# Introductory training for Cell Culture Basics

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# 1- Cell Culture Lab

A cell culture laboratory is restricted to cell culture work in order to maintain an aseptic work area.

- **Laboratory equipment**

Required for the sterile handling, incubation, and storage of cell cultures, reagents, and media

- **Material and Reagents**

Pipetting engines, plates and flasks, tubes….
Medium, enzymes, growth factors, antibiotics….

- **Getting the cells**

Primary cells or cell lines

- **Culture Workflow**

Protocols

# 1- Cell Culture Lab | Sterile handling

- To be successful in cell culture, it is essential to remain a contamination free environment (bacteria, fungi etc)
- Aseptic techniques ensure that no microorganisms enter the cell culture. Cell culture sterility is ensured by a set of procedures

| Handling | Reagents/Media | Workplace |
|---|---|---|
| Slow/careful handling.<br><br>Sterilization of all items before starting.<br><br>Sterile pipettes<br><br>No touching of sterile items to non-sterilized surfaces | Pre-sterilation of all reagents/ equipment.<br><br>No contamination in reagents (expiration date, appearance normal). | Cell culture hood works properly<br><br>Frequent de-contamination (hood, fridge etc)<br><br>Work area: sterile and tidy |

Table 1. Aseptic techniques required while working with cell culture.

# 2- Laboratory equipment

Laminar flow hood

Incubator

Inverted Microscope

Autoclave


Liquid Nitrogen (cell storage)


Water bath

Centrifuge

Vortex and Mini-Centrifuge

# 2- Laboratory equipment |Laminar flow hood

## Safety

The most important element of safety in a cell culture laboratory is the strict adherence to standard microbiological practices and techniques.

## Biosafety Levels (BSL)

BSL-1 is the basic level of protection common to most research and clinical laboratories. Appropriate for agents that are not known to cause disease in normal, healthy humans.

BSL-2 is appropriate for moderate-risk agents known to cause human disease of varying severity by ingestion or through percutaneous or mucous membrane exposure. Most cell culture labs should be at least BSL-2, but the exact requirements depend upon the cell line used and the type of work conducted.

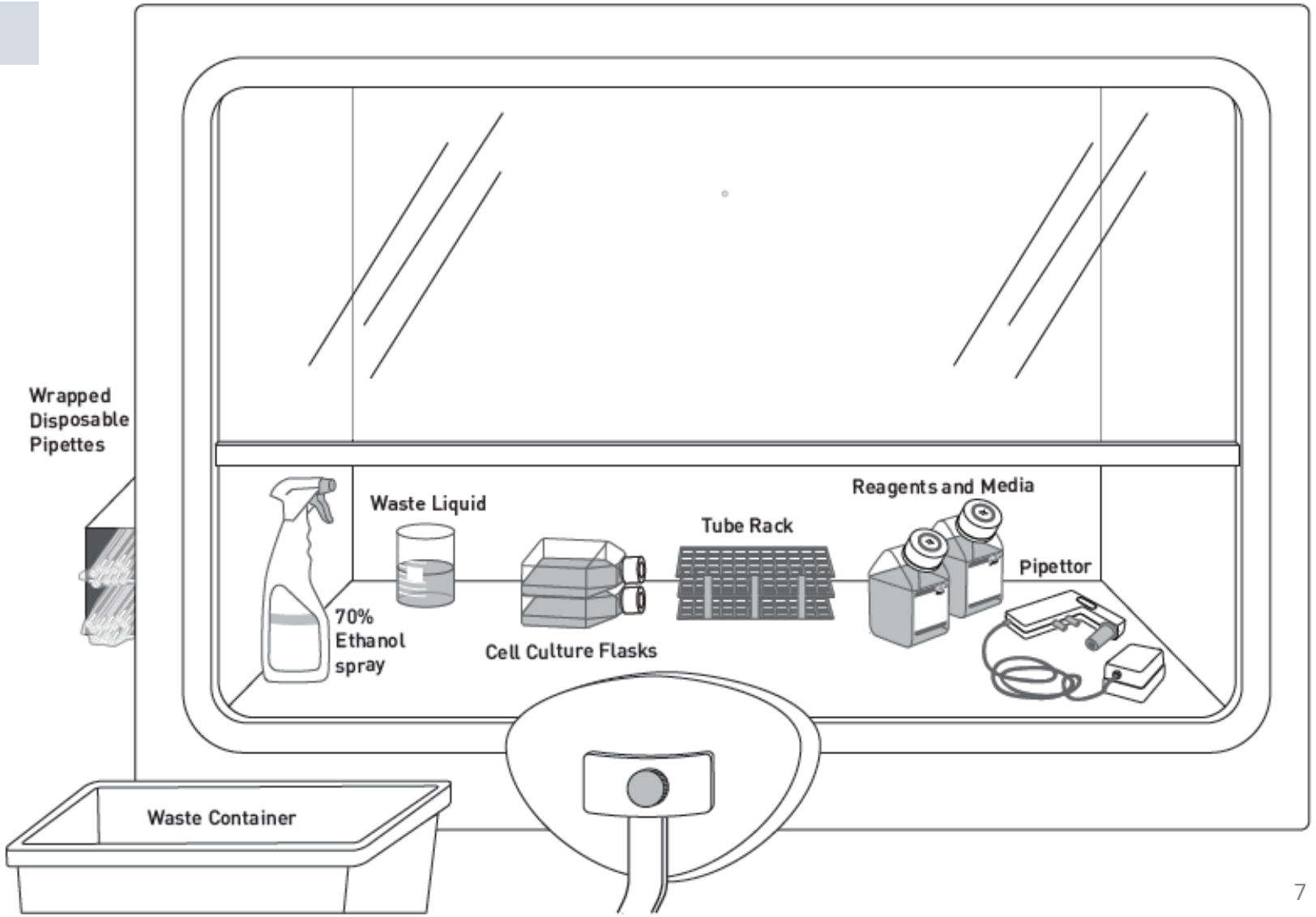
BSL-3 is appropriate for indigenous or exotic agents with a known potential for aerosol transmission, and for agents that may cause serious and potentially lethal infections.

BSL-4 is appropriate for exotic agents that pose a high individual risk of life-threatening disease by infectious aerosols and for which no treatment is available. These agents are restricted to high containment laboratories.

# 2- Laboratory equipment |Laminar flow hood

Basic cell culture equipment

The specific equipment of a cell culture laboratory depend on the type of research conducted, however, all cell culture laboratories have the same common equipment being free from pathogenic microorganisms



# 2- Laboratory equipment |Laminar flow hood

- Keep the work space in the cell culture hood clean…always….
- Disinfect hands, work area and all the containers, flasks, plates, and dishes with 70% ethanol before placing them in the cell culture hood
- Use autoclave to sterilize equipment, reagents and other supplies whenever possible
- Use ultraviolet light to sterilize the air and exposed work surfaces in the cell culture hood between uses.
- The work surface should contain only items required for a particular procedure; it should not be used as a storage area.

# 2- Laboratory equipment |Laminar flow hood

- Avoid pouring media and reagents directly from bottles or flasks.
- Always cap the bottles and flasks after use to prevent microorganisms and airborn contaminants. If you remove a cap or cover, and have to put it down on the work surface, place the cap with opening facing down
- Never uncover a sterile flask, bottle, petri dish, etc. until the instant you are ready to use it and never leave it open to the environment
- Perform your experiments as rapidly as possible to minimize contamination.

# 3- Cell culture environment

Ability to control and manipulate all physiochemical and physiological cell factors, such as, temperature, osmotic pressure, pH, gas, hormones, and nutrients.

| Media | pH | Temperature | $CO_2$ |
|---|---|---|---|
| Contains nutrients, growth factors, and hormones.<br><br>Sera source of growth, tipids,hormones. | Average pH for mammatian cells is pH 7.4. | Depends on body temperature of host.<br><br>Mammalian cell lines 36-37$^{0}$C.<br><br>Insert cell lines 27-30$^{0}$ C. | Controlled by media.<br><br>Organic or $CO_2$ bicarbonate buffer systems are popular.<br><br>Can impact pH.<br><br>4-10% $CO_2$ is most common. |

Table 1. Cell culture environment.

# 3- Cell culture environment

(1) Nature of the subtract
   (1) Solid – e.g. plastic
   (2) Semisolid (gel)– e.g. agar
   (3) Liquid – e.g. suspension culture in medium

(2) Medium constitution
   (1) Physico-chemical constitution
   (2) Physiological constitution

(3) Gas phase

(4) Temperature

# 3- Cell culture environment | Substract

## (1) Nature of the subtract

Cell culture ware

**Glass**

Cheap
Easily washed and Sterilized

**Disposable plastic**


Good optical quality
Flat grow surface

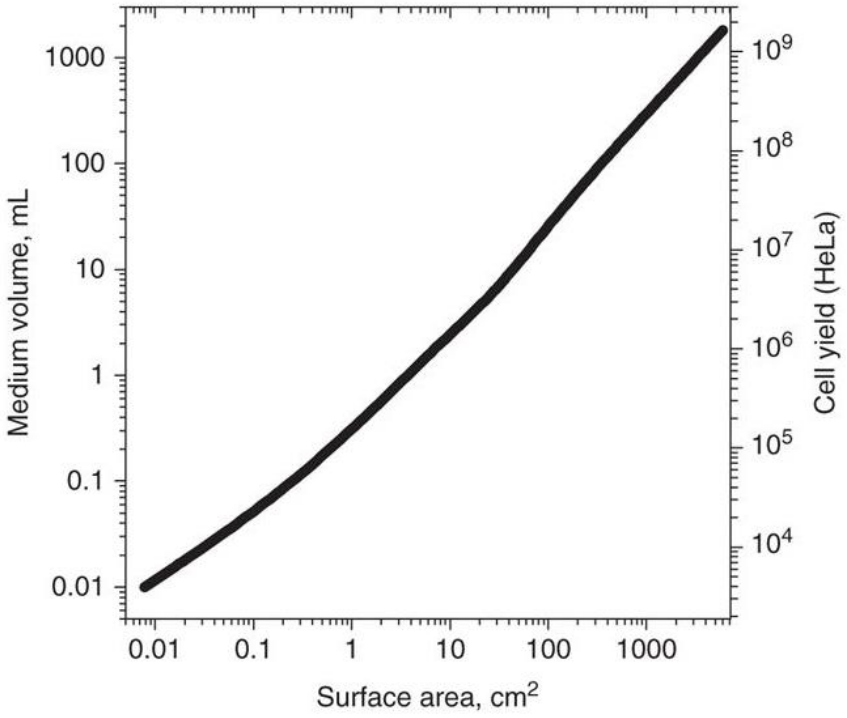
# 3- Cell culture environment | Substract

## (1) Nature of the subtract

Cell culture ware

Choose the best for appropriated cell proliferation and growth without compromising your experiments

- Cell mass/nº required
- Suspension/monolayer
- Vented or sealed atmosphere
- Frequency of sampling
- Type of analysis
- Cost



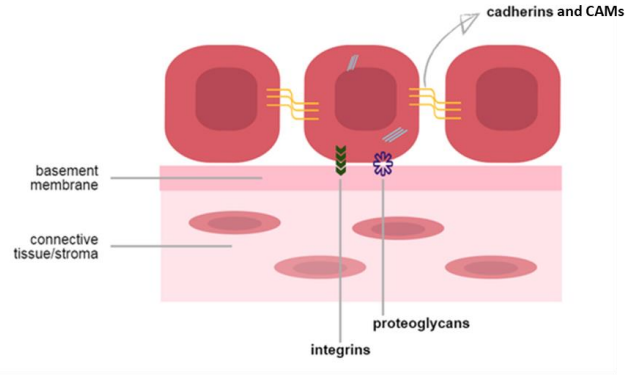
# 3- Cell culture environment | Substract

## (1) Nature of the subtract

**Matrix coating**

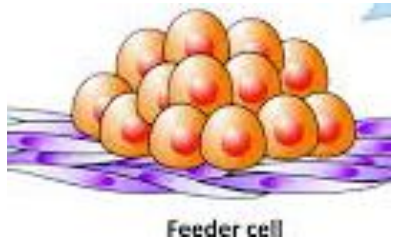
- Fibronectin
- Collagen
- Commercial available complex matrix
- …

Important for growth and may be important for differentiated functions




**Feeder layers**

- Support given by other cells
  - Supplementation of the medium
  - Metabolic leakage
  - Secretion of growth factors




**3D Matrices, Artificial substrates (microcarriers, …), Liquid-gel or Liquid-Liquid interface**

# 3- Cell culture environment | Medium

(1) Nature of the subtract
   (1) Solid – e.g. plastic
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   (1) Physico-chemical constitution
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(3) Gas phase

(4) Temperature

# 3- Cell culture environment | Medium

## (2) Medium constitution

Physicochemical properties:

pH
**Buffers**
$CO_2$ and $O_2$
Osmolality
T °C
Viscosity
Surface tension/foaming

"Complete" media:

Media +
Amino acids/Proteins
Vitamins
Salts
Glucose/Lipids
Organic supplements
Hormones and Growth factors
Antibiotics
**(Serum or not?)**

# 3- Cell culture environment | Medium

## (2) Medium constitution

**Growth Requirements**

- Salts and buffers
  - To simulate *in vivo* environment
- Serum
  - Portion of blood after the cells and fibers have clotted
  - From cow (bovine), horse, sheep
  - added to media as a nutrient source for growing cells
    - Lipids, proteins

# 3- Cell culture environment | Medium

## (2) Medium constitution

- Dulbecco' Modified Eagle's Media (DMEM)
  - Contains glucose, some proteins, and essential salts
  - Contains a pH indicator (**phenol red**) Media looks pink/red at pH 7.2
    - »**Acidic** -yellow or orange (cell growth, bacterial growth)
    - »**Basic** -purple (no cell growth, not enough $CO_2$)

# 3- Cell culture environment | Medium

**(2) Medium constitution**

- **Antibiotics** might be needed in the growth medium to inhibit contamination introduced from the host tissue.
- But what are the effects of these chosen antibiotics on the metabolism differentiation or gene expression of cultured neuronal cells?

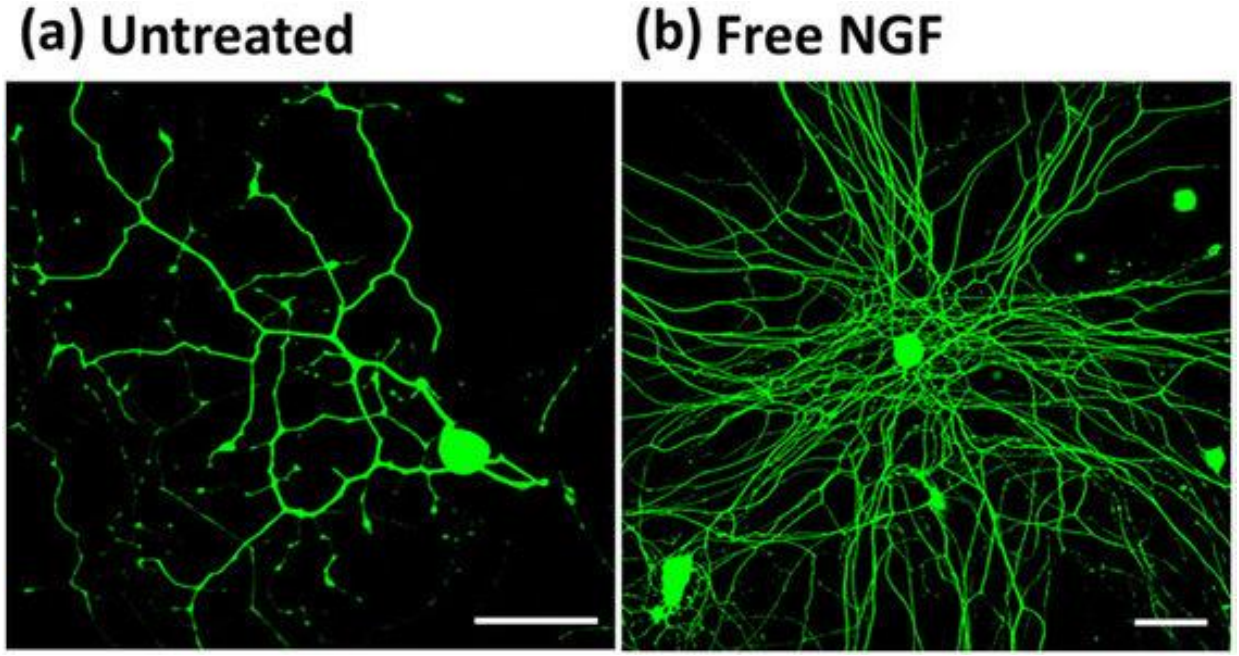
Do antibiotics indeed help to solve the contamination issue, or are they creating additional new problems?

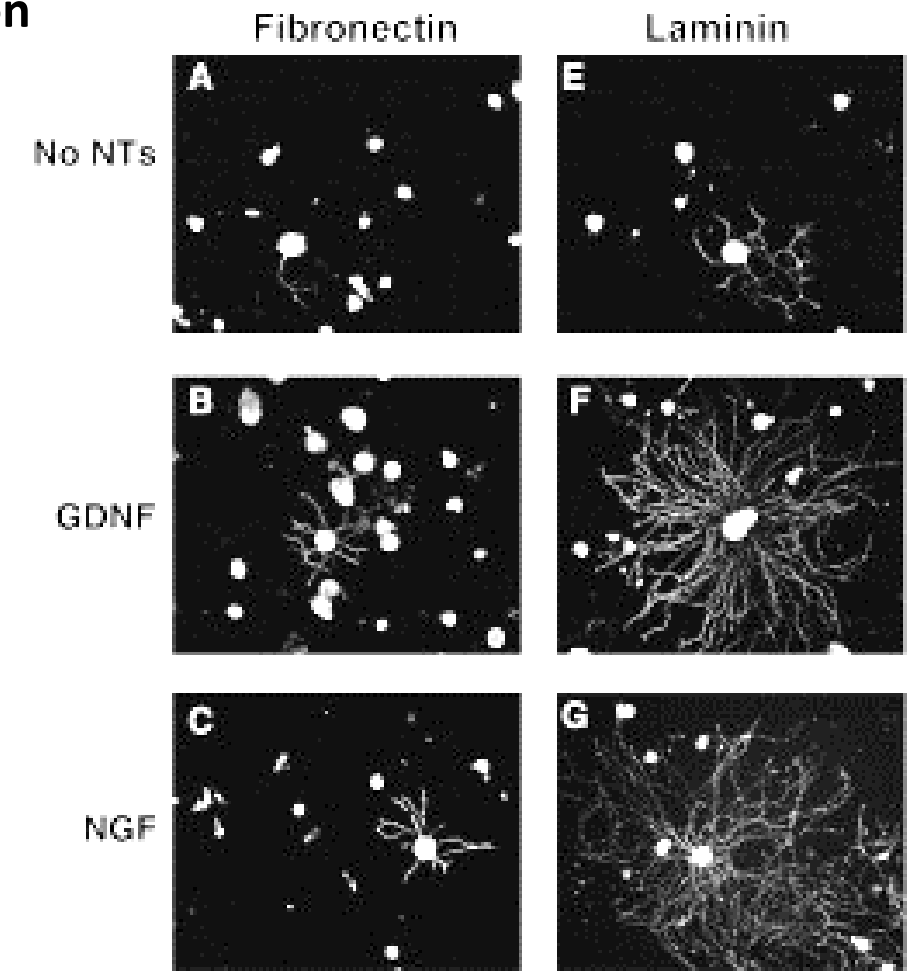
**Antibiotics deeply influence cell metabolism**

# 5- Maintenance of cell culture|Growth

Maintenance of neuronal cell culture

- **Special attention to subtract and media supplementation**



# 3- Cell culture environment | Cell incubator

(1) Nature of the subtract
   (1) Solid – e.g. plastic
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(2) Medium constitution
   (1) Physico-chemical constitution
   (2) Physiological constitution

(3) Gas phase

(4) Temperature

# 3- Cell culture environment | Cell incubator

**(3) Gas phase and (4) Temperature**

- Cells are grown/maintained at 37 °C, 5% $CO_2$, in a cell incubator.
  - contamination-free environment for cells while maintaining **temperature**, **humidity** and **O2/CO2 levels**.
    - door opening—even disrupts temperature and O2/CO2 levels and introduces potential contaminants.
  - High humidity (above 93%) prevents evaporation that can change the concentrations of important components in growth media.
  - $O_2$ levels is important for growing neurons (natural vs ischemic)

# 4- Cell culture workflow

## ISOLATE

- Separate fresh tissue of interest.
- Treat tissue with enzyme(s) (e.g., trypsin, collagenase, protease) and/or mechanically to isolate cells.
- Wash, count, and seed cells.

## VERIFY

- Examine cells under a brightfield microscope to assess their growth state, attachment to culture vessels/flasks, and to check for any signs of infection.
- Monitor cells for the following days until they reach confluence.
- Verify isolated cell types by their morphology and expressed biomarkers.

## CULTURE

- Passage cells to propagate the cell line.
- Make master and working cell banks.
- Immortalize cells if necessary.

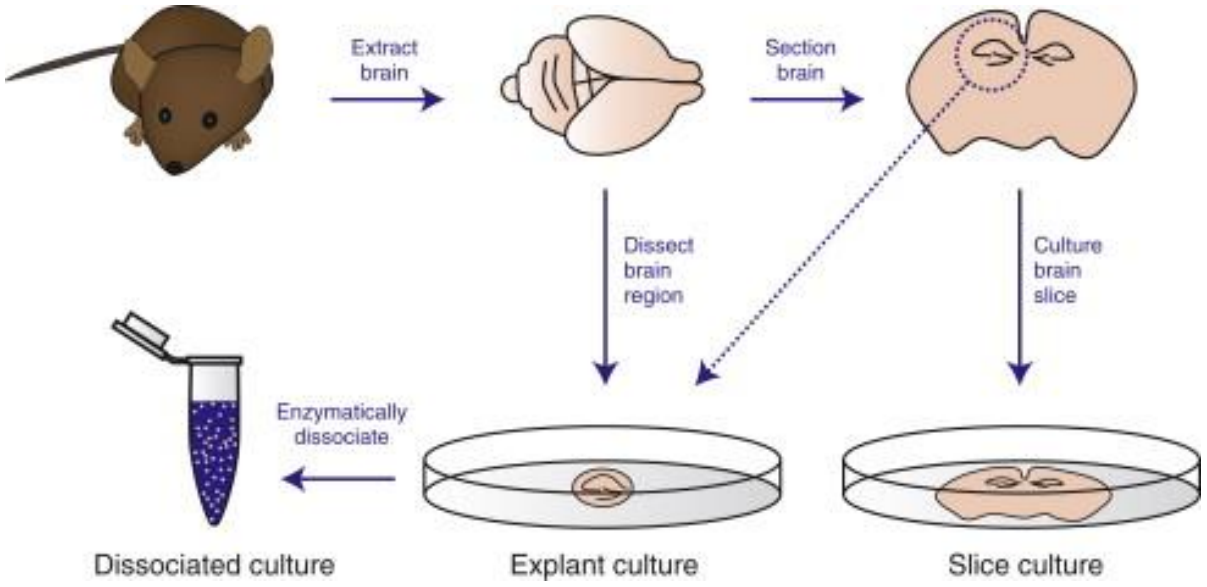
## INVESTIGATE

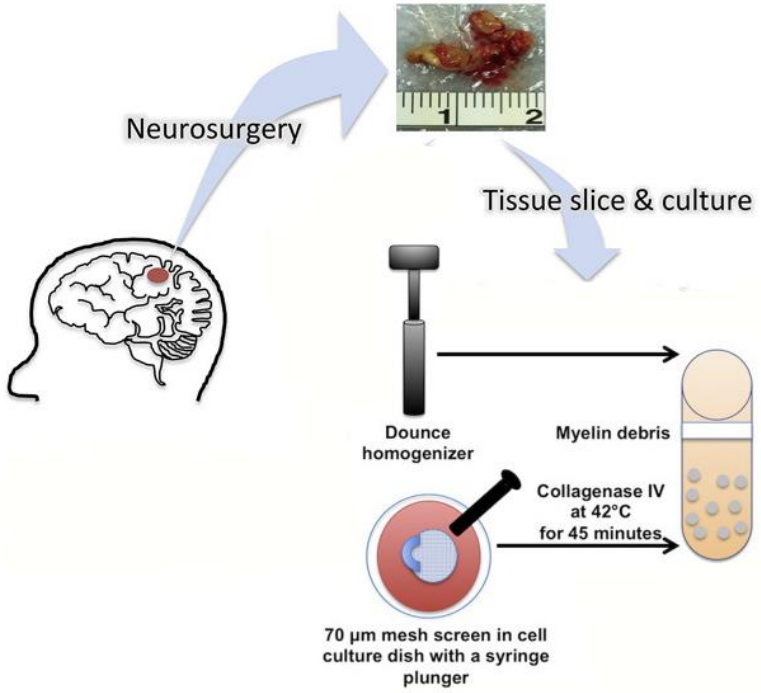
- Plan and execute experiments.
- Keep monitoring cell state and possible infections using a brightfield microscope.

# 4- Cell culture workflow | Getting the cells

**Obtaining primary cultures – from animals/human**

- Use the correct enzymes for disaggregation – depends on the tissue
- After obtaining the biopsy, the culture should be performed as soon as possible…



# 4- Cell culture workflow | Getting the cells

Enzymes commonly used for disaggregation

(A combination of more than one enzyme could be employed)

- **Trypsin** (protease)
- Elastase (protease)
- **Collagenase** (protease)
- Pronase (protease)
- Dispase (protease)
- Hyaluronidase (polysaccharidase)

# 4- Cell culture workflow | Getting the cells

**Obtaining primary cultures – buying**

**Receipt, storage and transport of primary cells**

- Do not store long-term in dry ice or -80 °C -> transfer to liquid $N_2$;
- During transport, keep cryovials submersed in dry ice;
- **Minimize** exposure to room temperature;

# 4- Cell culture workflow | Getting the cells

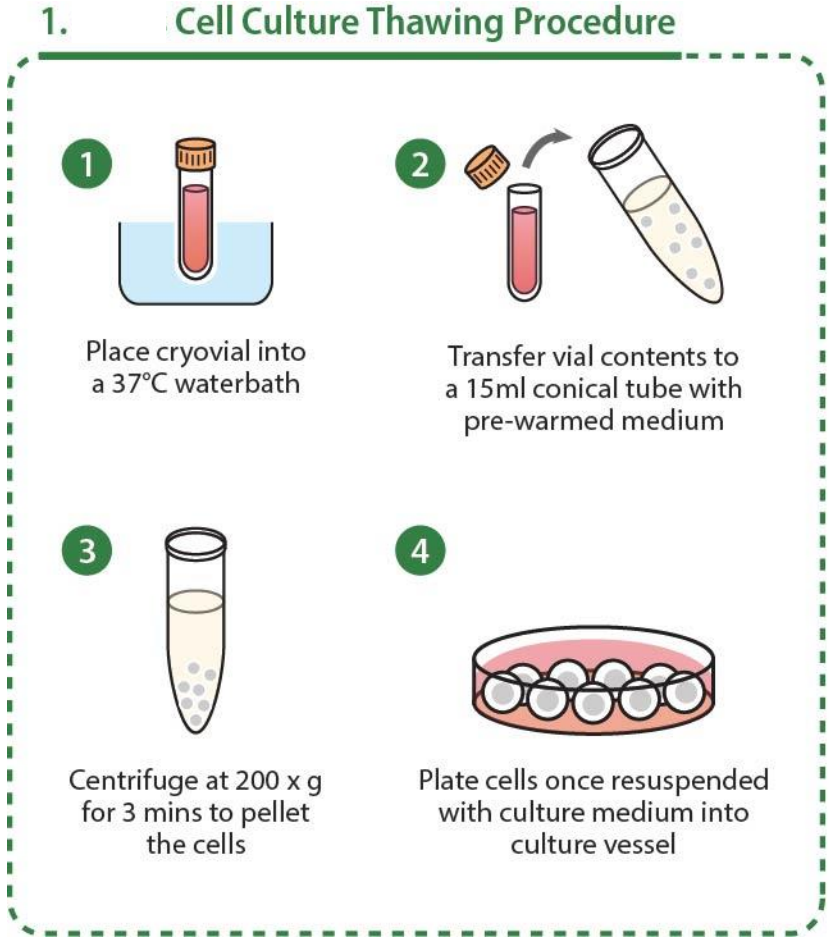
**Neuronal cell lines recovery**

- Thawing cryopreserved cells is a rapid process accomplished by immersing frozen cells in a 37°C water bath for about 1 to 2 minutes.

  •Do NOT over-thaw the cryovials (move from $N_2$ to dry ice and then thaw in 37 °C bath)

- Do not to centrifuge primary cells upon thaw as they are extremely sensitive to damage during recovery from cryopreservation.

- Plate cells directly upon thaw, and allows cultures to attach for the first 24 hours. Then remove media to clear remaining DMSO (harmful to primary cells and may cause a drop in post-thaw viability).





# 4- Cell culture workflow | Getting the cells

**Working with liquid nitrogen ($N_2$):**


- Always use the correct $N_2$ dewars;
  - Inadequate venting can result in excessive gas pressure which could damage or burst the container.

- $N_2$ potential hazards:
  - Extremely cold
    - Contact with the skin or eyes may cause serious freezing (frostbite) injury.
    - Wear protective clothing. Objects cooled by N2 will stick to you and tear the flesh when you attempt to free yourself!

  - Very small amounts of liquid vaporize into large amounts of gas
    - Nitrogen gas can cause suffocation without warning. Store and use liquid nitrogen only in a well ventilated place.
    - The cloudy vapor that appears when N2 is exposed to the air is condensed moisture, not the gas itself.

# 4- Cell culture workflow | Getting the cells

**Working with liquid nitrogen ($N_2$):**


- Know how to clean the dewar

- Know what do when:
  - a person is dizzy or loses consciousness
    - move to a well-ventilated area
    - If breathing has stopped, apply artificial respiration.
    - If breathing is difficult, give oxygen. Call a physician. Keep warm and at rest.

  - If exposed to liquid or cold gas, restore tissue to normal body temperature as rapidly as possible
    - Remove or loosen clothing that may constrict blood circulation to the frozen area. Call a physician.
    - Rapid warming of the affected part is best achieved by using water at 42°C (never over 44°C).
    - Do not rub the frozen part. The patient should neither smoke, nor drink alcohol.

# 4- Cell culture workflow | Getting the cells

**Working with dry ice (solid $CO_2$):**

- Dry ice potential hazards:
  - Very cold
    - skin contact can lead to severe frostbite; skin cells freeze and become damaged very quickly.
    - **Wear goggles or face shield, lab coat, and loose-fitting thermally-insulated gloves.**
  - Dry ice will sublime at any temperature above -78 °C.
    - Release of potentially substantial volumes of CO2, which can displace oxygen quickly causing difficulty breathing, loss of consciousness and death. This is especially of concern in nonventilated or confined spaces.

# 4- Cell culture workflow | Getting the cells

**Working with dry ice (solid $CO_2$):**

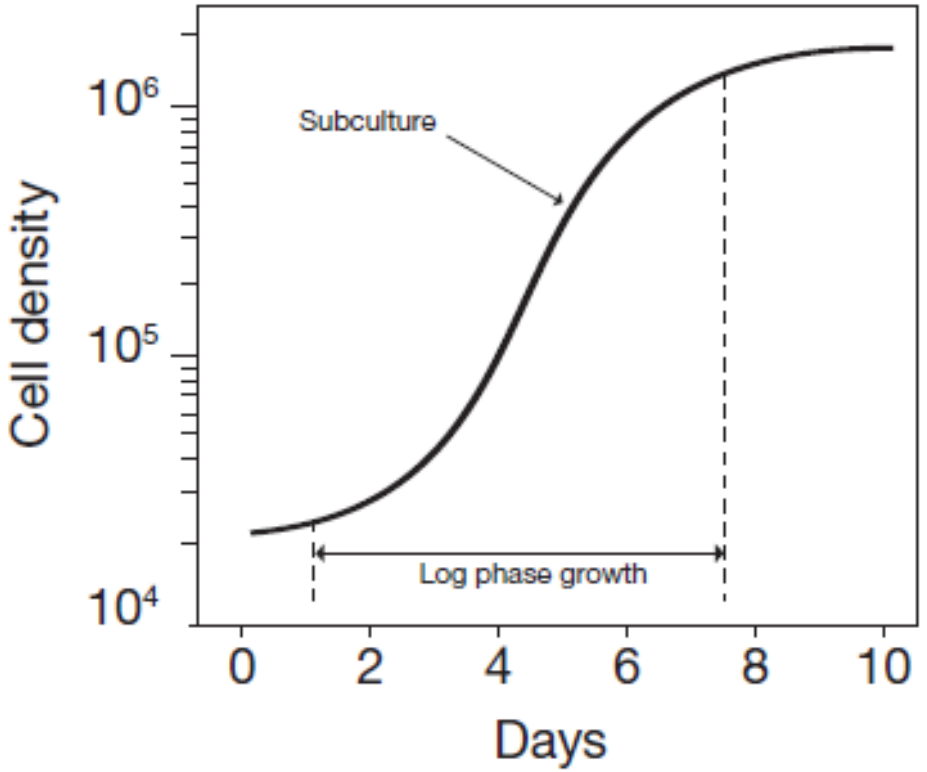
- Dry ice potential hazards:
  - Explosion Hazard
    - Due to the rapid emission of CO2 gas, if stored in a closed container can pressurize the container and explosion may occur, if the gas is not able to escape.
    - **If you receive a container that is swollen, bulging, or that you believe may be improperly packaged dry ice, secure the area and call for help. Do not try to release pressure in the container.**
  - Occupational Exposure Limits
    - 8h to 5000 ppm
    - 15’ to 30000 ppm

# 5- Maintenance of cell culture| Subculturing

**Subculturing**, or **passaging**, is the removal of the medium and transfer of cells from a previous culture into fresh growth medium, a procedure that enables the further propagation of the cell line or cell strain.

The growth of cells in culture proceeds from the **lag phase** following seeding to the **log phase**, where the cells proliferate exponentially occupying all the available substrate **(confluency)**

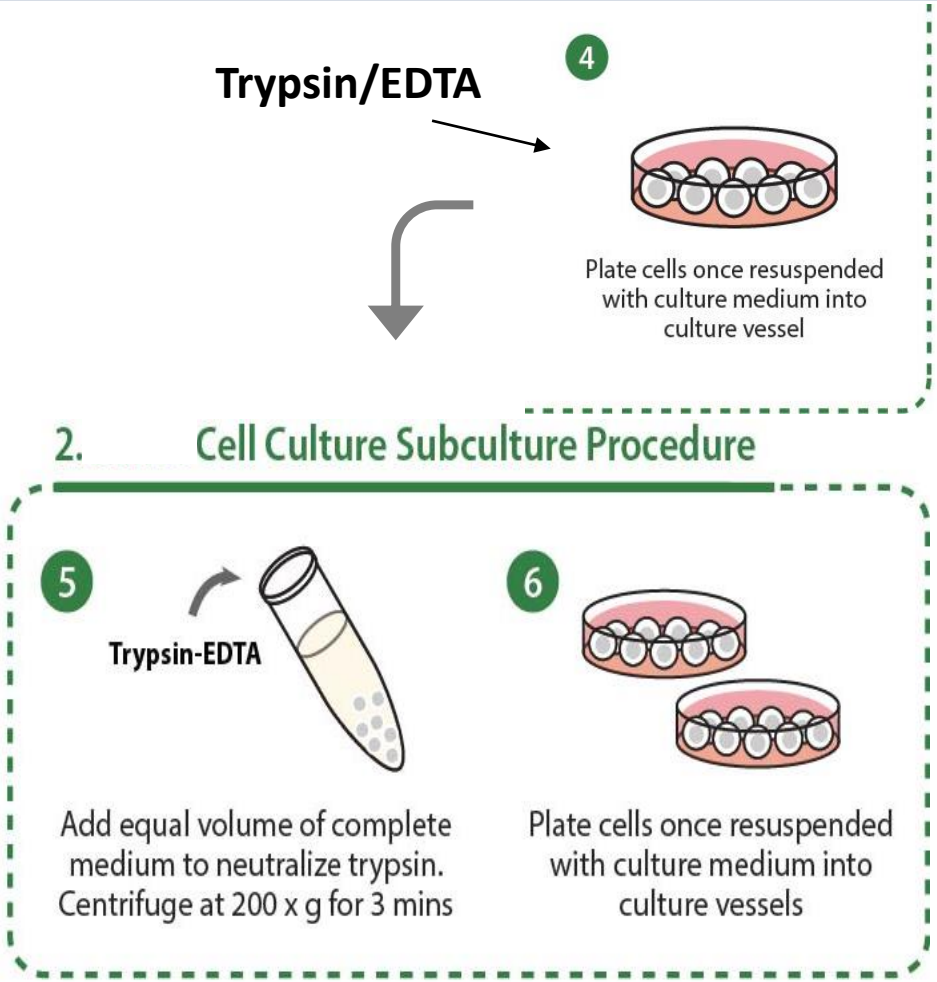
- **Cellular confluence**
  - Percentage of the culture vessel inhabited by attached cells.
    - Confluence end points - point cells need to be sub-cultured



# 5- Maintenance of cell culture| Subculturing

Maintenance of neuronal cell cultures

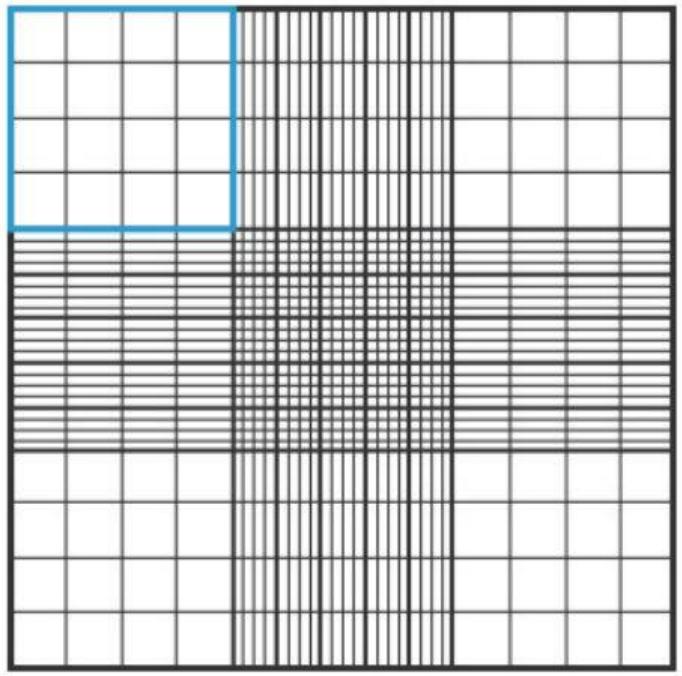
- **Maintenance and Subculture**
  - Attachment takes about 24 h.
  - Sub-cultivation -breakage of both inter- and intracellular cell-to-surface bonds.
    - Digestion of protein attachment bonds or separation from the monolayer or relevant tissue with a low concentration of a proteolytic enzyme such as **trypsin/EDTA**.
    - After the cell dissociation and dispersion into a single-cell suspension, they are counted and diluted to the appropriate concentration and transferred to fresh culture vessels.




https://www.youtube.com/watch?v=CMRKKl9XSDU

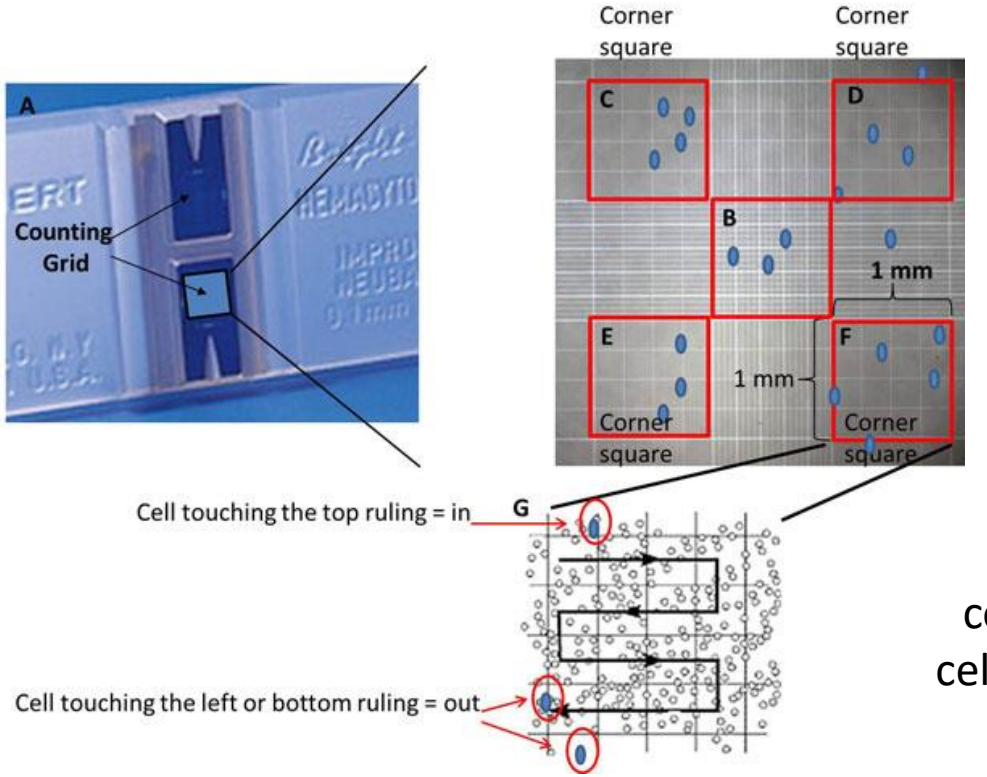
# 5- Maintenance of cell culture| Cell counting

- **Haemocytometers** + **Trypan Blue** (exclusion dye to access cell viability)



100 µl

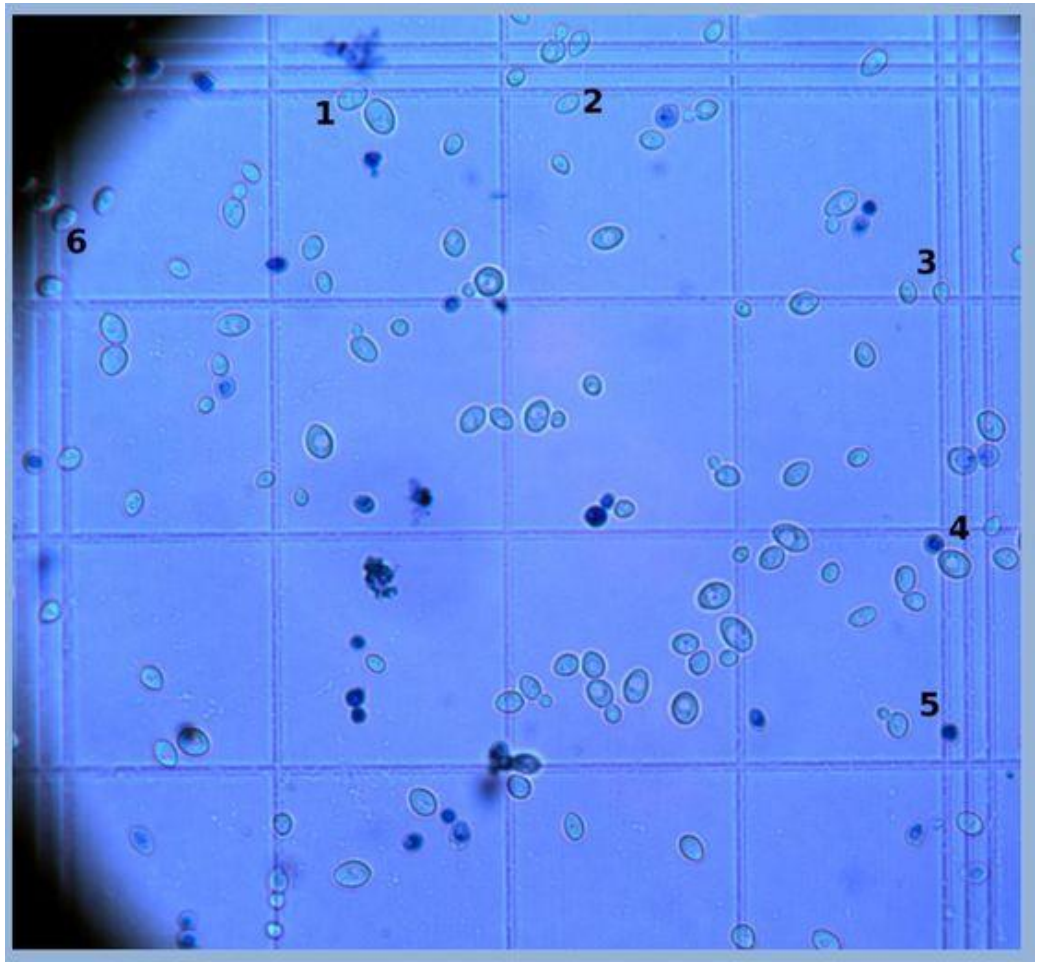
100 µL of “cells” + 400 µL 0.4% Trypan Blue (Cf = 0.32%)




Only compromised cells take Trypan blue

# 5- Maintenance of cell culture| Cell counting

(video)



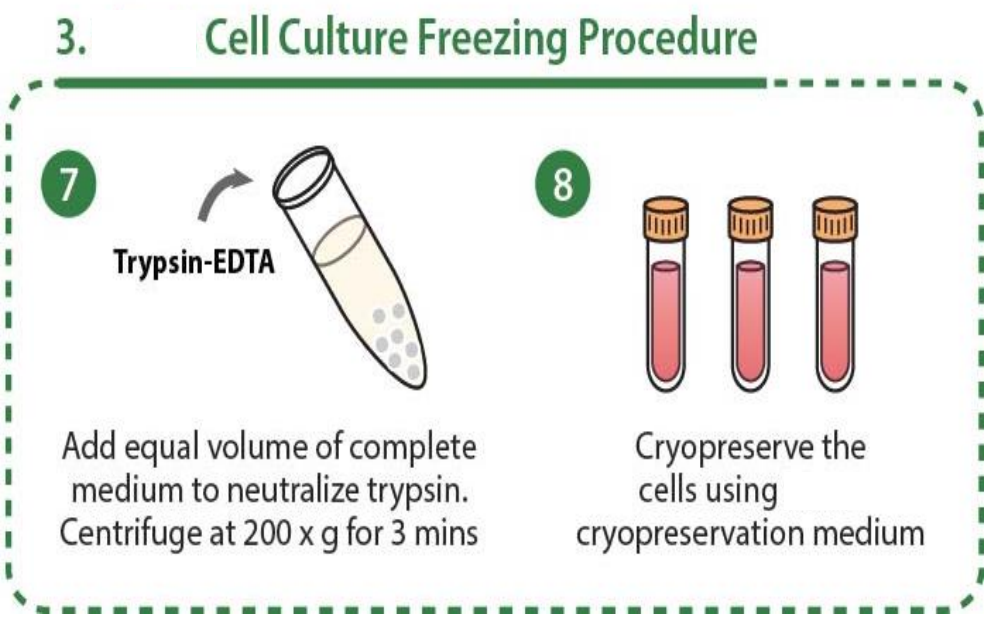

https://www.youtube.com/watch?v=pP0xERLUhyc

# 5- Maintenance of cell culture| Cell counting

Cell counting (automatic cell counter)

# 6- Cryopreservation

- Cryopreservation is the process to preserve structurally intact living cells using low temperatures.
- The freezing process needs to be slow, at a rate of -1°C per minute, to minimize the formation of ice crystals within the cells (use cryopreserved boxes and store at -80ºC for a couple hours)
- The frozen culture needs to be stored in the **vapour phase** of liquid nitrogen
- Use cryoprotectant, such as **DMSO** or **glycerol**
  - Commercial available
  - Homemade (Medium, 20%Serum, 10%DMSO)



# 7- Troubleshooting

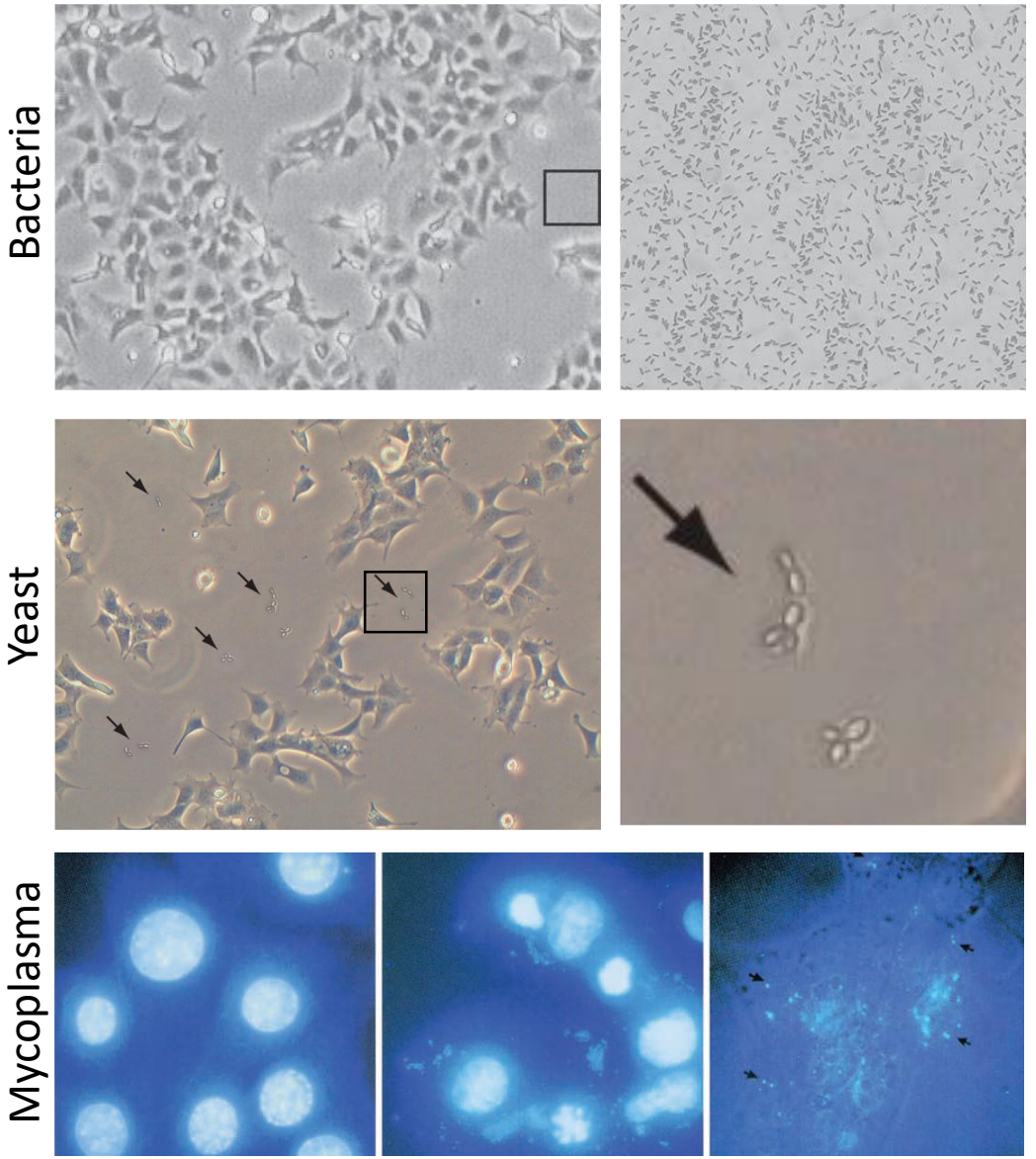
- Contamination

(Contamination of primary tissue when carried over to culture)

Contamination of cell cultures is easily the most common problem encountered in cell culture laboratories

**chemical contaminants -** impurities in media, sera, and water, endotoxins, plasticizers, and detergents;

**biological contaminants -** bacteria, molds, yeasts, viruses, mycoplasma, as well as cross contamination by other cell lines.



# 7- Troubleshooting

- Shifts in pH (Incorrect salt in the culture medium / **bacterial or fungal contamination** / insufficient bicarbonate buffering / incorrect carbon dioxide tension / ...)
- Detached cells (Insufficient or absence of attachment factors / contamination / overly trypsinized cells)
- Slow growth (Change in pH / depletion of nutrients or growth factors/ low contamination/ improper storage of reagents/…)

# 7- Troubleshooting

- Cell death (Temperature fluctuation/ absence of $CO_2$/ cell damage during thawing or cryopreservation/ increase concentration of toxic metabolite/ imbalanced osmotic pressure)

- Precipitation (no change in pH) (Use of frozen medium/ residual phosphate leftover while washing with detergent)

- Induced variability (Use of a variety of reagents and media induces variability in data acquired using primary cells. The handling methodology between the users may also contribute to the variability)

# 7- Troubleshooting

- Data reproducibility

Scientific progress demands reliable and reproducible data

- *"More than 70% of researchers have tried and failed to reproduce another scientist's experiments, and more than half have failed to reproduce their own experiments"*

Baker M., "1,500 scientists lift the lid on reproducibility" Nature, Volume:533, Pages:452–454 doi:10.1038/533452a 2016

https://www.tecan.com/watch-our-free-webinar-about-spark-cyto?utm_source=researchgate&utm_medium=native&utm_campaign=webinar

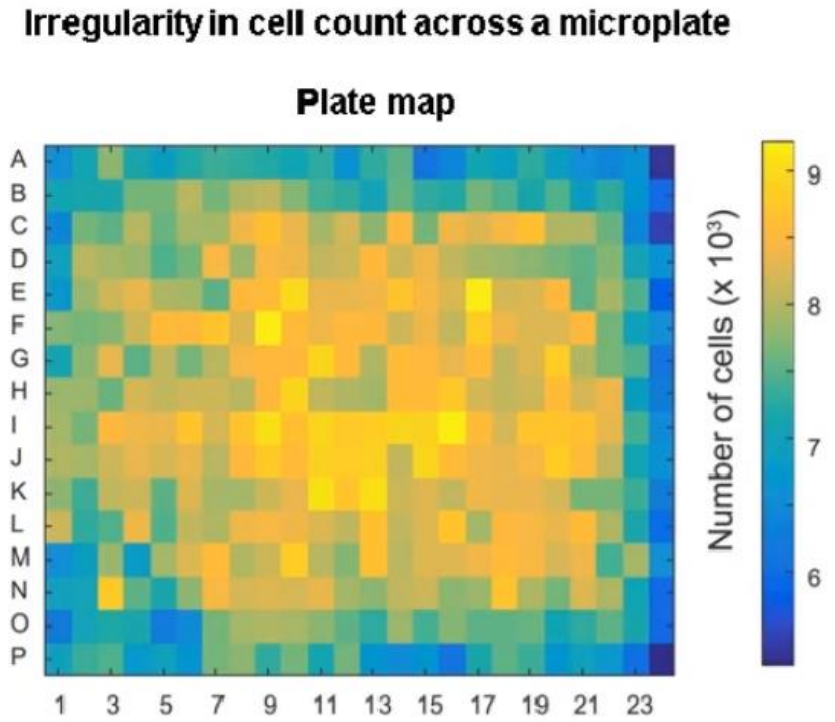
# 7- Troubleshooting

- Data reproducibility

- "Edge effects" in a microtiter plate led to variation in results between labs.

  - These so-called edge effects come from uneven evaporation of culture media and temperature gradients.

- Consequence: significant errors in dose response data




M. Niepel et al., "A multi-center study on the reproducibility of drug-response assays in mammalian cell lines," *Cell Systems*, doi:10.1016/j.cels.2019.06.005, 2019.

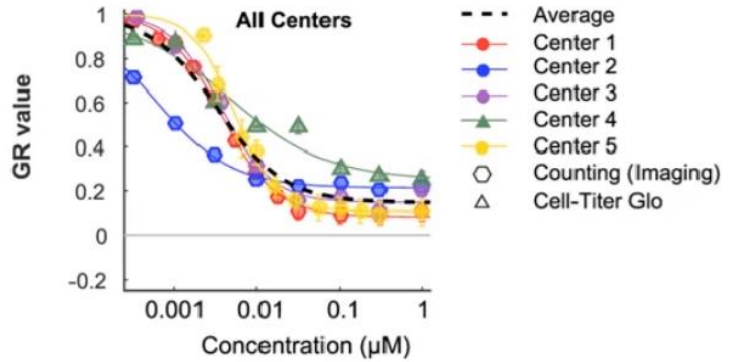
# 7- Troubleshooting

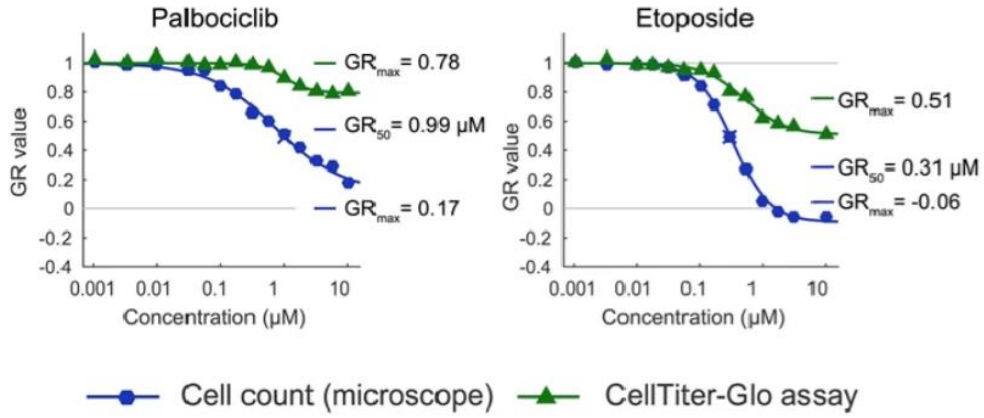
- Data reproducibility

- Observed centre-to-centre variation in drug potency of up to 200-fold
- **Why?**

  - Method for counting cells differed

  - Different image-processing algorithms to count live cells

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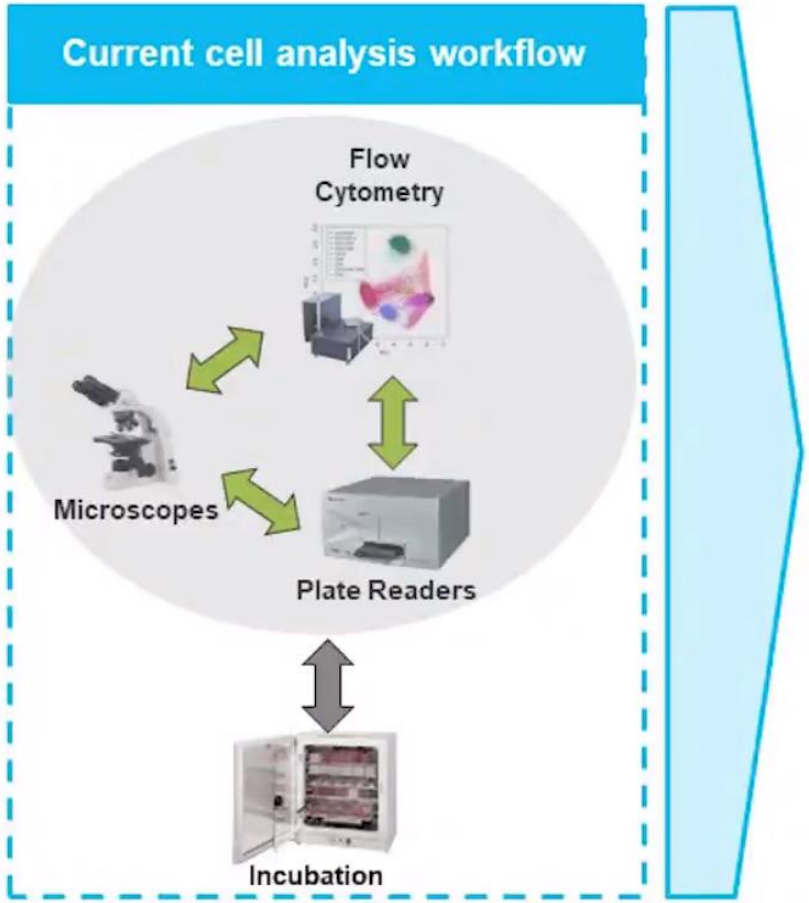
# 7- Troubleshooting

- Data reproducibility




**Data reproducibility is critical…**

- Multiple instruments required → increased handling of samples and expertise in each instrument necessary
- Parallel samples and sample vessels often required for various time points or instrument types
- Inconsistent environmental conditions
- Microscopes enable visual inspection, but no quantitative information – subjective assessment
- Long-term studies with multiple time points to investigate biological processes very laborious and often limited by user availability
- 'wellular' signals vs. cellular signals

**Consequences**

Pursuit of false leads

Failure in Pre-clinical trials

Wasted time and money

**Solutions**

Automation

Standardisation of protocols

Best practices in handling

**Result**

*High quality robust and reproducible data in a timely manner*

# 7- Troubleshooting

- Do NOT heat subculture reagents to 37 °C (use at RT or below)
- Do NOT over-trypsinize the cells (use correct enzyme concentration; follow protocol carefully)
- DO use the right kind of cell culture ware


about students project thesis employing cell culture models.