Cell Culture Systems
Course 3

Subculturing & Passaging

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What is Sub-culture?

- Subculturing, also referred to as 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.

Phases of Cell Growth

- It is important to know and record the growth characteristics of the cell line in use before starting any experiments.
- An alteration in cellular growth can indicate a significant problem within the cell line and if undetected can have detrimental effects on experimental results.

Phases of Cell Growth

1. **Log Phase**
2. **Lag Phase**
3. **Stationary Phase**
4. **Death (Decline) Phase**

1. Lag Phase

- **At this stage the cells do not divide.**
- During this period the **cells adapt to the culture conditions**
- The length of this phase will depend upon the growth phase of the cell line at the time of subculture and also the seeding density.

2. Logarithmic (Log) Growth Phase

- **Cells actively proliferate and an exponential increase in cell density arises.**
- The cell population is considered to be **the most viable** at this phase, therefore it is recommended to assess cellular function at this stage.

2. Logarithmic (Log) Growth Phase

- Each cell line will show different cell proliferation kinetics during the log phase
- It is therefore **the optimal phase for determining the population doubling time.**
- **Cells are also generally passaged at late log phase.**
- Passaging cells too late, can lead to overcrowding, apoptosis and senescence.

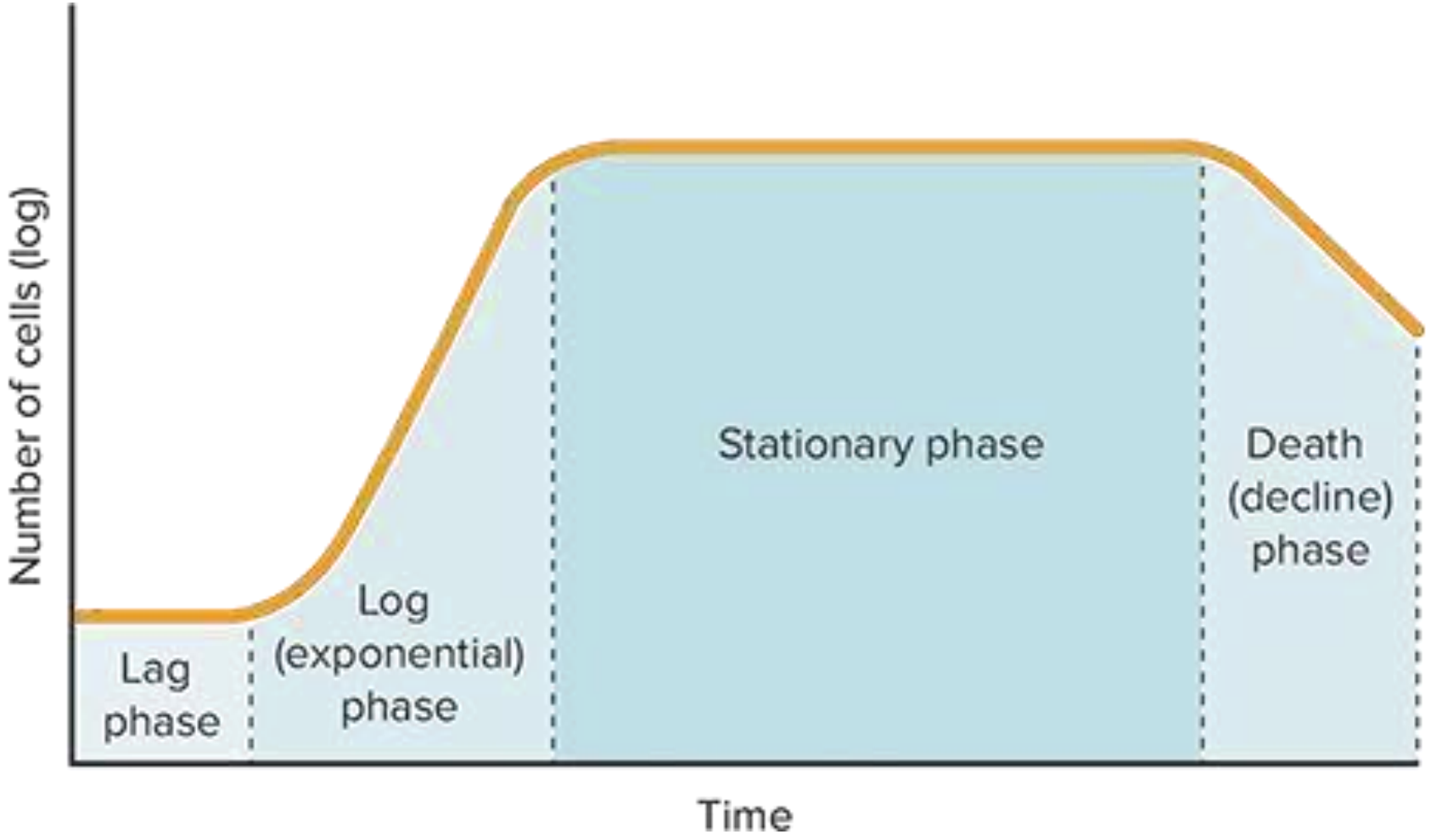
3. Plateau (or Stationary) Phase

- **Cellular proliferation slows down due to the cell population becoming confluent.**
- It is at this stage the number of cells in the active cell cycle drops to 0-10% and the cells are most susceptible to injury

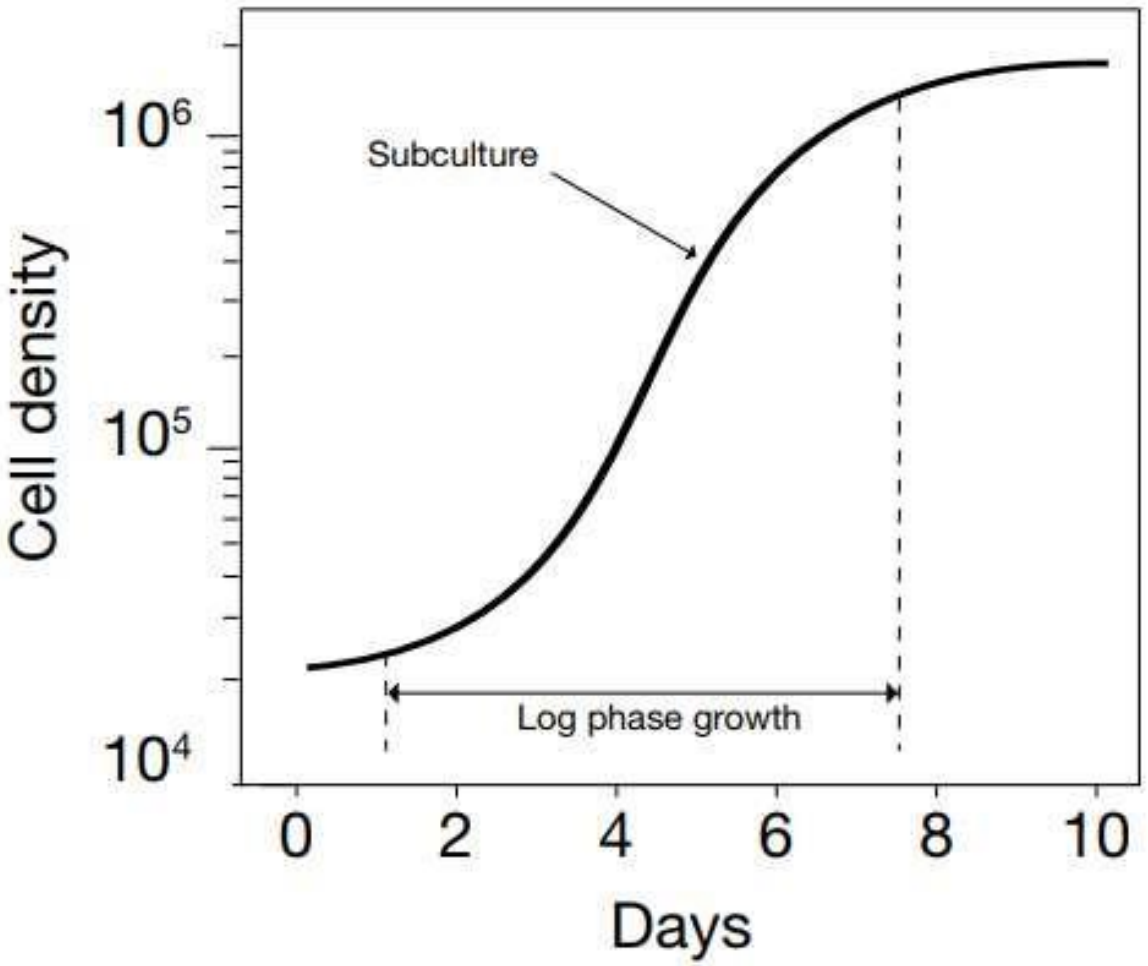
4. Decline Phase

- Cell death predominates in this phase and there is a reduction in the number of viable cells.
- Cell death is **not only due to the reduction in nutrient supplements but also the natural path of the cellular cycle.**

Phases of Cell Growth



Phases of Cell Growth



Factors affecting culture environment

a) Serum:

- Vitally important source of growth factors, adhesion factors, hormones, lipids and minerals

- **Regulates cell membrane permeability:**

- Serves as a carrier for lipids, enzymes, micronutrients, and trace elements into the cell.

Factors affecting culture environment

b) pH Level:

Cell line	Optimal pH
Mammalian cell lines	7.4
Transformed cell lines	7.0 – 7.4
Normal fibroblast cell lines	7.4 – 7.7
Insect cell lines	6.2

Factors affecting culture environment

c) CO_2 Level

- The growth medium controls the pH of the culture and buffers the cells in culture against changes in the pH.
- Buffering is achieved by an organic (e.g., HEPES) or CO_2-bicarbonate based buffer
- **4 – 10%** CO_2 is common for most cell culture experiments

Factors affecting culture environment

d) Temperature

- The optimal temperature for cell culture largely depends on the body temperature of the host from which the cells were isolated.

Cell line	Optimal Temperature
Human and mammals	36°C - 37°
Insect cells	27°C
Avian cell lines	38.5°C
Cold-blooded animals (e.g., amphibians, cold-water fish)	15°C - 26°C

Media

- The culture medium is **the most important component of the culture environment**, because it provides the **necessary nutrients, growth factors, and hormones for cell growth**, as well as regulating the pH and the osmotic pressure of the culture.

Basic Constituents of Media

- Inorganic salts
- Carbohydrates
- Amino Acids
- Vitamins
- Fatty acids and lipids
- Proteins and peptides
- Serum
- Trace Elements

Inorganic Salts

- The inclusion of inorganic salts in media performs several functions.
- Primarily they **help to retain the osmotic balance of the cells and help regulate membrane potential by provision of sodium, potassium and calcium ions.**
- All of these are required in the cell matrix for cell attachment and as enzyme cofactors.

Buffering Systems

- Most cells require pH conditions in the range 7.2-7.4 and close control of pH is essential for optimum culture conditions.
- There are major variations to this optimum.
- Fibroblasts prefer a higher pH (7.4-7.7) whereas, continuous transformed cell lines require more acid conditions pH (7.0-7.4).

- Regulation of pH is particularly important immediately following cell seeding
- It is usually achieved by one of two buffering systems;

(i) a “natural” buffering system where **gaseous CO2** balances with the CO3 /HCO3 content of the culture medium

(ii) (ii) chemical buffering using a zwitterion called **HEPES**

- Cultures using natural bicarbonate /CO2 buffering systems need to be maintained in an atmosphere of 5-10% CO2 in air usually supplied in a CO2 incubator.
- **Bicarbonate/CO2 is low cost, non-toxic and also provides other chemical benefits to the cells**

- HEPES has superior buffering capacity in the pH range **7.2-7.4** but is relatively expensive and can be toxic to some cell types at higher concentrations
- **HEPES buffered cultures do not require a controlled gaseous atmosphere.**

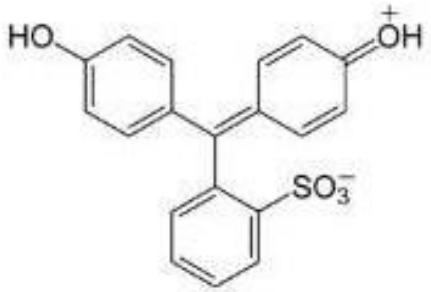
Phenol Red

- Most commercial culture media include phenol red as a **pH indicator** so that the pH status of the medium is constantly indicated by the colour.
- Usually the culture medium should be changed/replenished if the colour turns yellow (acid) or purple (alkali).

❖ pH Indicator – Phenol Red

- Optimum cell growth approx. pH 7.4

Chemical formula	$C_{19}H_{14}O_5S$



Phenol red (pH indicator)

below pH 6.8 ⇌ *above pH 8.2*

6.8 ⇌ 8.2

Carbohydrates

- The main source of energy is derived from carbohydrates generally in the form of sugars.
- The major sugars used **are glucose and galactose, however, some media contain maltose or fructose.**
- The concentration of sugar varies from basal media containing 1g/L to 4.5g/L in some more complex media.

Amino Acids

- Amino acids are the building blocks of proteins. **‘Essential’ amino acids must be added to culture media as cells are not able to synthesize these themselves.**
- The concentration of amino acids in the culture medium will determine the maximum cell density that can be achieved - once depleted the cells will no longer be able to proliferate

L-Glutamine

- Glutamine, an essential amino acid, is particularly significant.
- In liquid media or stock solutions glutamine degrades relatively rapidly. Optimal cell performance usually requires supplementation of the media with glutamine prior to use.

- Some media formulations include L-alanyl glutamine which is a more stable form of glutamine, and do not require supplementation.
- Adding supplements of non-essential amino acids to media both **stimulates growth and prolongs the viability of the cells in culture.**

Vitamins

- Serum is an important source of vitamins in cell culture.
- However, many media are also enriched with vitamins making them consistently more suitable for a wider range of cell lines.
- Vitamins are **precursors for numerous co-factors.**

- Many vitamins, especially B group vitamins, are necessary for **cell growth and proliferation** and for some lines the presence of B12 is essential.
- Some media also have increased levels of vitamins A and E.
- The vitamins commonly used in media include **riboflavin, thiamine and biotin.**

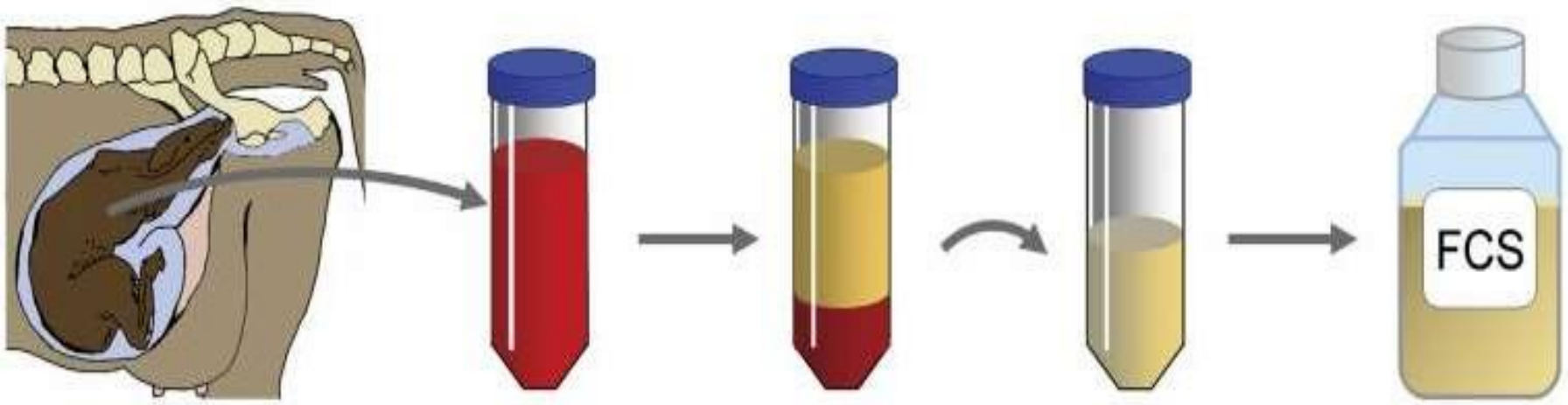
Trace Elements

- These include trace elements such as zinc, copper, selenium and tricarboxylic acid intermediates.
- **Selenium** is a detoxifier and helps remove oxygen free radicals.

Serum

- Serum is a complex mix of albumins, growth factors and growth inhibitors and is probably one of the most important components of cell culture medium.
- The most commonly used serum is **foetal bovine serum (FBS).**

A



pregnant uterus removed from slaughtered cow

blood allowed to clot in sterile container

centrifugation to remove fibrin clot and blood cells

filtration (0.1µm membrane)

- Other types of serum are available including newborn calf serum and horse serum.
- The quality, type and concentration of serum can all affect the growth of cells and it is therefore important to screen batches of serum for their ability to support the growth of cells

Serum

- Serum is also able to increase the buffering capacity of cultures that can be important for slow growing cells or where the seeding density is low (e.g. cell cloning experiments).
- It also helps to protect against mechanical damage
- In addition, serum is able to bind and neutralise toxins

Foetal Calf/Bovine Serum (FCS & FBS)

- Growth factors and hormones
- Aids cell attachment
- Binds and neutralise toxins
- Long history of use

❖ **Disadvantages**

- Infectious agents (prions)
- Variable composition
- Expensive
- Regulatory issues (to minimise risk)

❖ **Heat Inactivation (56°C for 30 mins) – why?**

- Destruction of complement and immunoglobulins
- Destruction of some viruses (also gamma irradiated serum).

Heat Inactivation

- There is also a risk of contamination associated with the use of serum.
- Heat inactivation of serum **(incubation at 56°C for 30 minutes)** can help to reduce the risk of contamination, since some viruses are inactivated by this process

Heat Inactivation

- However this process also **denatures some proteins and destroys nutrients in the serum with modern production methods for serum**
- the routine use of heat inactivated serum is not an absolute requirement for cell culture.

Origin of Serum

- Serum from a reputable supplier should have undergone various quality control tests which will be listed in the product information sheet.
- Most serum products are cell culture tested including growth promotion, cloning efficiency and plating efficiency tests.

Standard tests performed on serum

- Sterility
- Virus Contamination
- Mycoplasma Contamination
- Endotoxin
- Haemoglobin
- Total Protein
- Immunoglobulin
- Hormone Testing
- pH (at room temperature)
- Osmolality

Disadvantages of Serum

- However, using serum in media has a number of disadvantages including high cost, problems with standardization, specificity, and variability, and unwanted effects such as stimulation or inhibition of growth and/or cellular function on certain cell cultures.
- If the serum is not obtained from reputable source, contamination can also pose a serious threat to successful cell culture experiments.

Advantages of serum in media	Disadvantages of serum in media
Serum contains various growth factors and hormones which stimulates cell growth and functions.	Lack of uniformity in the composition of serum
Helps in attachment of the cells	Testing needs to be done to maintain the quality of each batch before using
Acts as a spreading factor	May contain some of the growth inhibiting factors
Acts as a buffering agent which helps in maintaining the pH of the culture media	Increase the risk of contamination
Functions as a binding protein	Presence of serum in media may interfere with the purification and isolation of cell culture products
Minimizes mechanical damages or damages caused by viscosity	

Cell Culture Media

❖ ***The three basic classes of media are:***

a. **Basal media**

b. **Reduced-serum media**

c. **Serum-free media**

Cell Culture Media

❑ Basal Media

- Contains *amino acids*, *vitamins*, *inorganic salts*, and a *carbon source* such as glucose.
- Basal media formulations **must be** further supplemented with serum

Cell Culture Media

❑ Reduced-Serum Media

- Basal media formulations enriched with nutrients and animal-derived factors with **reduced amount of serum**

Cell Culture Media

❑ Serum-Free Media

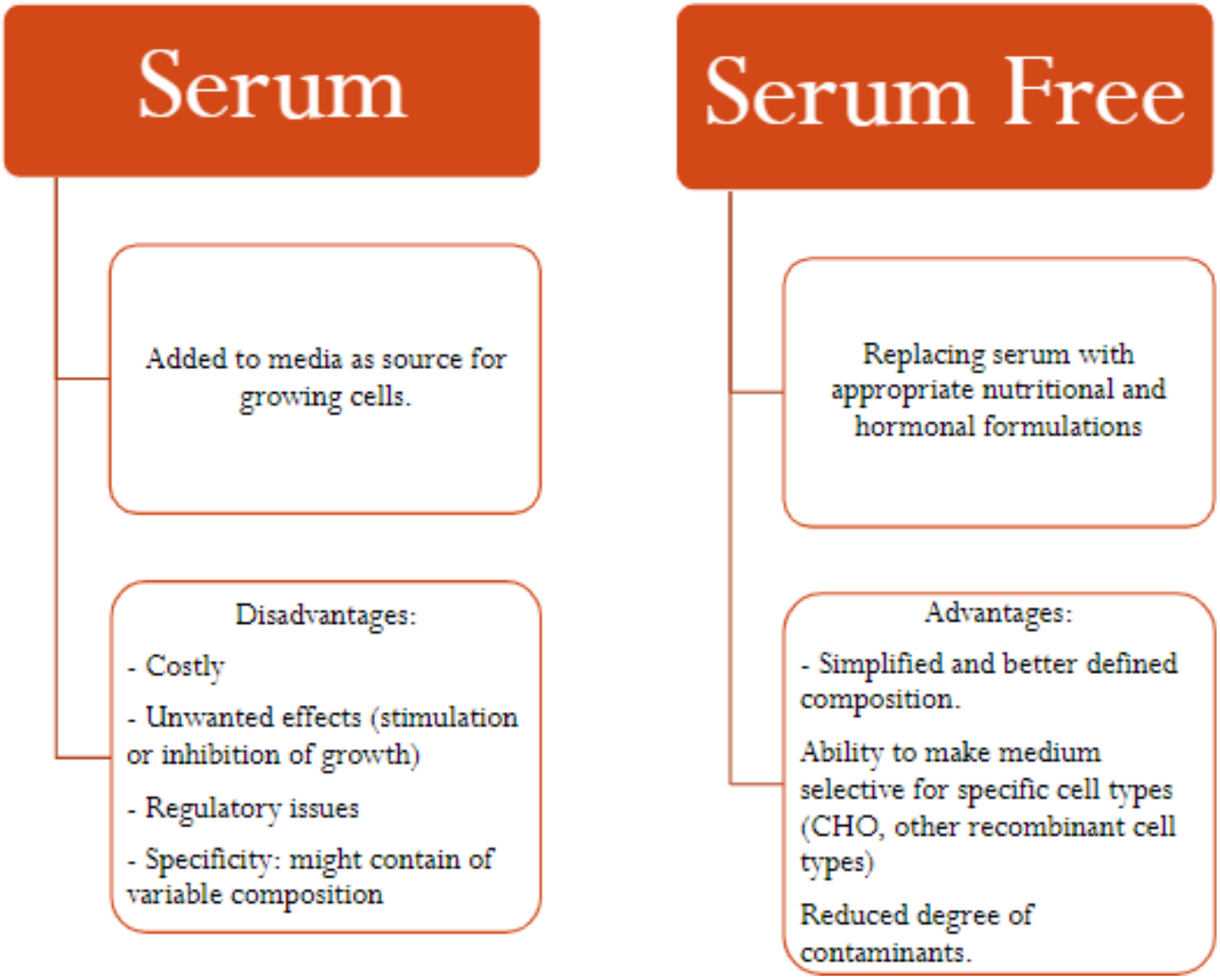
• Appropriate nutritional and hormonal formulations **replaces serum completely**

• Serum-free medium in combination with growth factors has the ability to make the **selective medium** for primary cell culture.

Serum Free Media

- One of the major advantages of using serum-free media is the ability to make the medium selective for specific cell types by choosing the appropriate combination of growth factors.

Serum VS. Serum Free Media



Cell Culture Media

- **Commonly used Medium:**
  GMEM, EMEM,DMEM, RPMI etc.

- **Media is supplemented with**
  Antibiotics (penicillin, streptomycin)
  BSA
  Na-bicarbonate
  L-glutamate
  Na-pyruvate
  HEPES
  Growth factors etc.

COMMON CELL CULTURE MEDIA

- Minimum Essential Medium (MEM)
- GMEMm (Glasgow Minimum Essential Medium)
- EMEM (Eagle's MEM)
- DMEM (Dulbecco's Modified Eagle Medium)
- Medium 199
- BME (Basal Medium Eagle)
- Ham's F-10 Medium
- Ham's F-12 Medium
- RPMI 1640 medium
- Leibovitz L-15 medium
- CMRL 1066
- Dulbecco's Modified Eagle's Medium (DMEM-001)
- MCDB 131
- McCoy's 5A

Balanced salt solution :

- Balanced salt solutions can provide an environment that maintains the structural and physiological integrity, pH and osmotic pressure of cells *in vitro*.
- Maintain osmolarity
- Regulate membrane potential (Na^+, K^+, Ca^{2+})
- Ions for cell attachment and enzyme cofactors

DPBS (Dulbecco's Phosphate-Buffered Salines)
HBSS (Hanks' Balanced Salt Solutions)
PBS (Phosphate-Buffered Salines)
EBSS (Earle's Balanced Salt Solutions)

Table 2. Different types of culture medium and their uses

Media Type	Examples	Uses
Balanced salt solutions	PBS, Hanks' BSS, Earle's salts DPBS HBSS EBSS	Form the basis of many complex media
Basal media	MEM	Primary and diploid culture
	DMEM	Modification of MEM containing increased level of amino acids and vitamins. Supports a wide range of cell types including hybridomas
	GMEM	Glasgows modified MEM was defined for BHK-21 cells
Complex media	RPMI 1640	Originally derived for human leukaemic cells. It supports a wide range of mammalian cells including hybridomas
	Iscoves DMEM	Further enriched modification of DMEM which supports high density growth
	Leibovitz L-15	Designed for CO_2 free environments
	TC 100 Graces insect medium Schneider's Insect medium	Designed for culturing insect cells
Serum free media	CHO HEK293	For use in serum free applications
	Ham F10 and derivatives Ham F12 DMEM/F12	Note: these media must be supplemented with other factors such as insulin, transferrin and epidermal growth factor. These media are usually HEPES buffered
Insect cells	Serum-Free Insect Medium 1 (Cat no. S3777)	Specifically designed for use with Sf9 insect cells

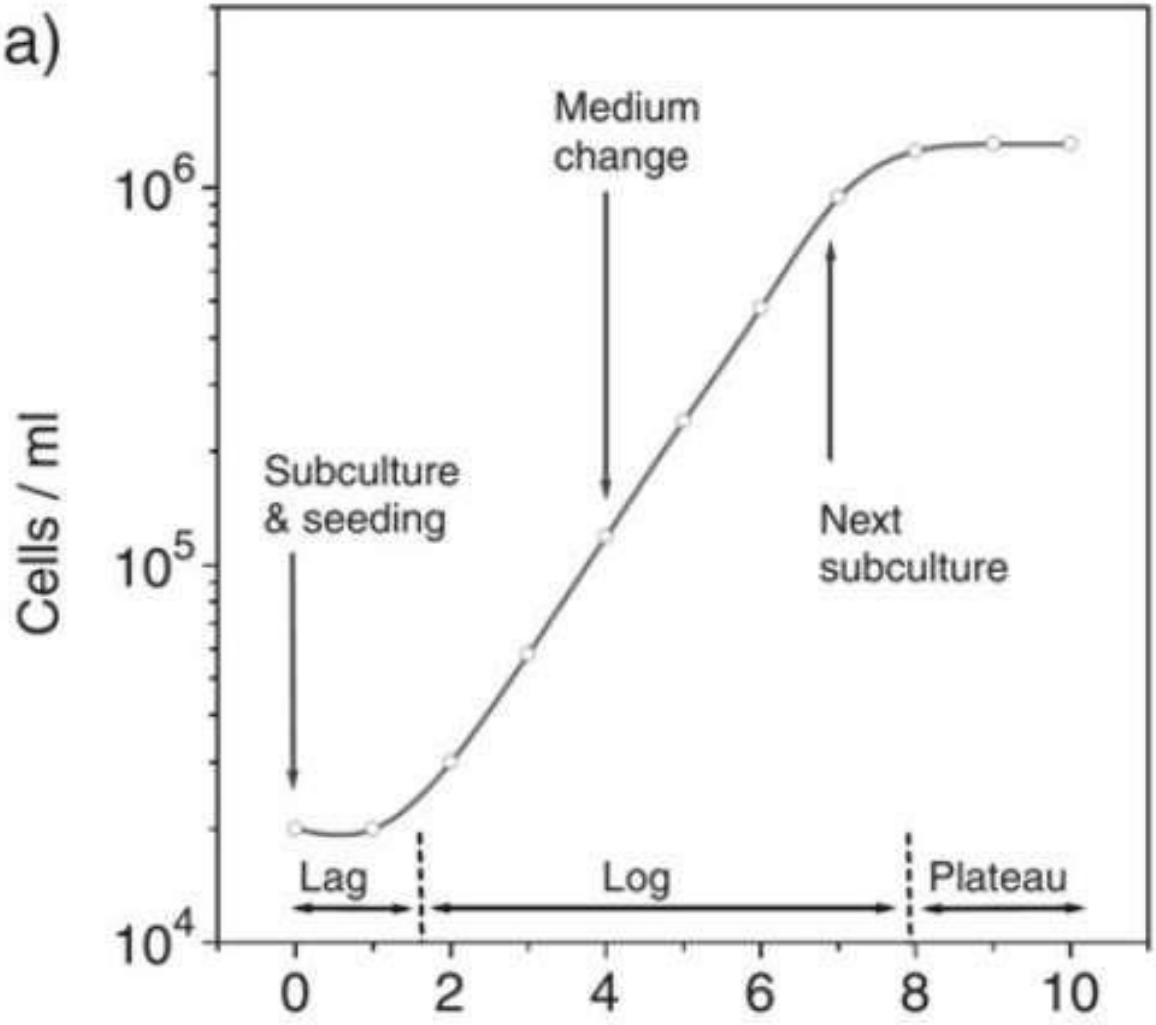
Criteria for subculture

- **Density of culture**: Normal cell should be subcultured as soon as they reach confluence.

- **Exhaustion of medium**: It indicates that the medium require replacement. Usually a drop in PH is indicate that need of subculture.

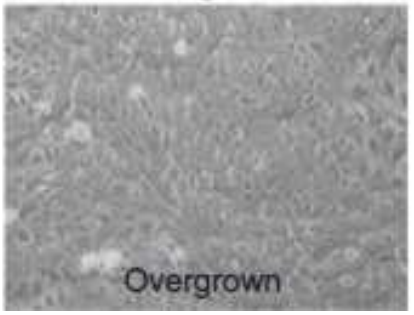
- **Time since last subculture**. Routine subculture is best performed according to a strict schedule, so that reproducible behaviour is achieved and monitored.

Subculturing Adherent Cells



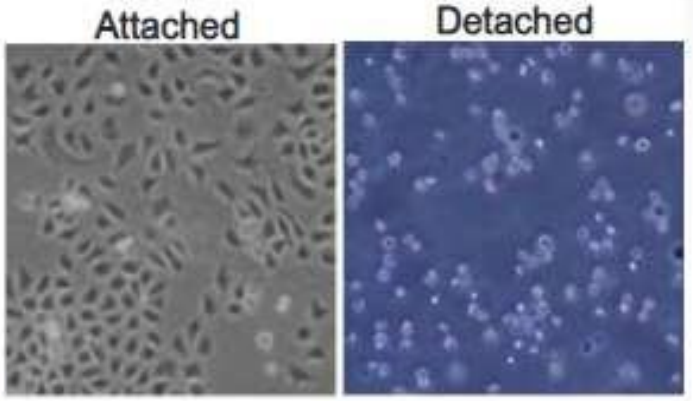
Subculturing Adherent Cells

- Examine the condition of the cell monolayer using an inverted microscope and ensure that the cells are healthy and sub-confluent
- The medium is discarded from the vessel and the cells monolayer should be washed twice with warm PBS (magnesium & calcium free)
- The cells are detached using a warm solution of Trypsin/EDTA solution and the plate is left at 37°C for ~ 5 minutes or until the cells are detached with monitoring of cells under inverted microscope
  - Cells should only be exposed to trypsin long enough to detach the cells
  - Prolonged exposure can damage surface receptors on cells



Subculturing Adherent Cells

- Gently tap the flask with the palm of the hand a couple of times to release any remaining detached cells
- A warm complete medium is added to the cells to inactivate trypsin and the cells are pipetted several times to break clumps
- Remove the cell suspension from the flask and place in to a sterile container
- Centrifuge typically at 1000 rpm for 5 min to sediment the cells



Subculturing Adherent Cells

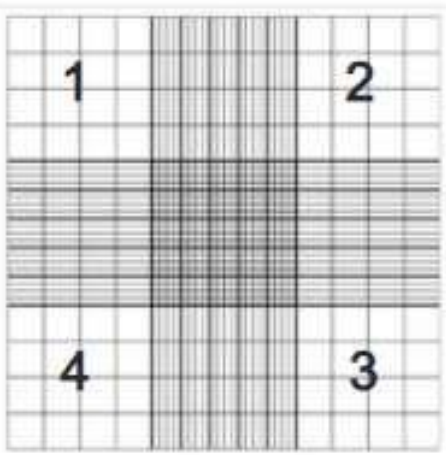
- Pour off the supernatant from the container and resuspend the pellet in complete medium
- Perform a viable cell count and reseed a flask with an aliquot of cells at the required density
- The size of culture flask used depends on the number of cells required
- An appropriate volume of complete medium is added to the flask
- A cell count may not always be necessary if the cell line has a known split ratio
- Label each flask with cell line name, passage number, and date

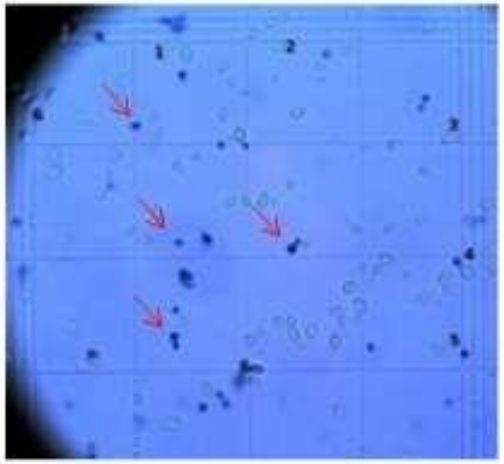
Counting of Cells

- For the majority of manipulations using cell cultures, such as cytotoxicity tests, transfections, cell fusion techniques, cryopreservation and subculture routines it is necessary to quantify the number of cells prior to use
- Trypan blue is the most commonly used vital dye in microscopy for cell counting and to measure cells viability
- The principle of trypan blue is based on the fact that the chromophore is negatively charged and does not interact with the cell unless the membrane is damaged
- Equal volumes of cell suspension and 0.4% trypan blue are mixed by pipetting and left for 5 minutes at RT
- Prepare a clean hemocytometer chamber and fill it with cell suspension

Counting of Cells

- Count the number of cells in the four large corner squares:
  - viable (seen as bright cells)
  - and non-viable cells (stained blue) Use the following formula to calculate viable number of cells

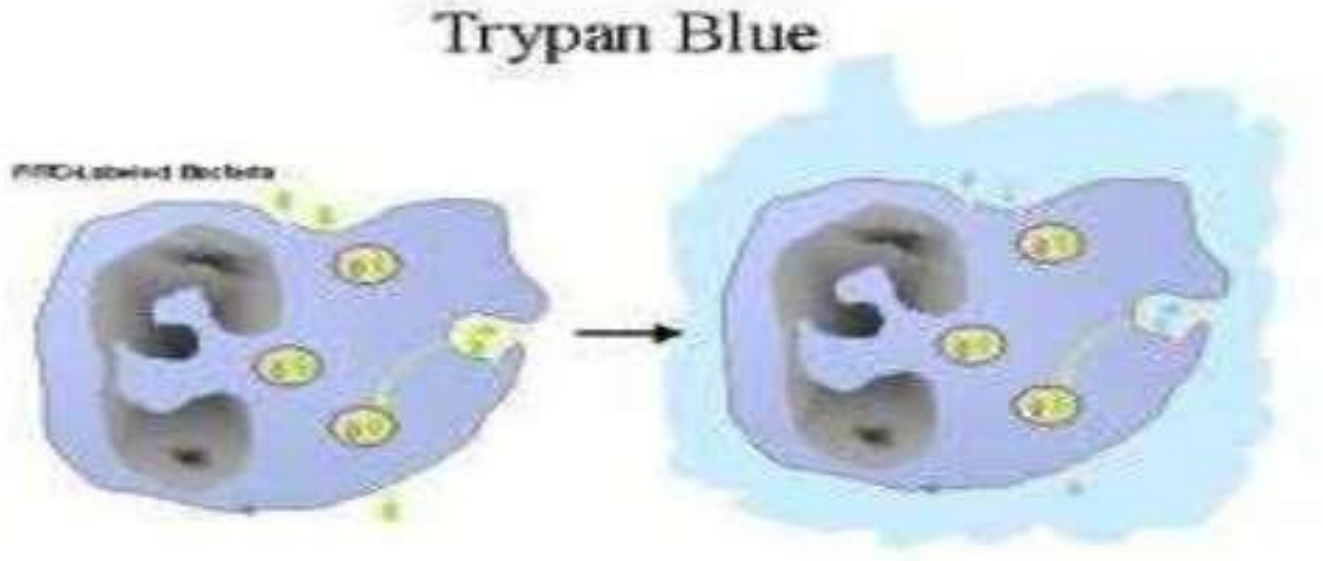




$$\text{Viable cells number/ml} = \frac{\text{Number of viable cells}}{4} \times 10^4 \times \text{dilution factor}$$

PRINCIPLE

- Dye binds to intracellular proteins of leaky cells.



procedure:

Incubation with trypan blue dye.

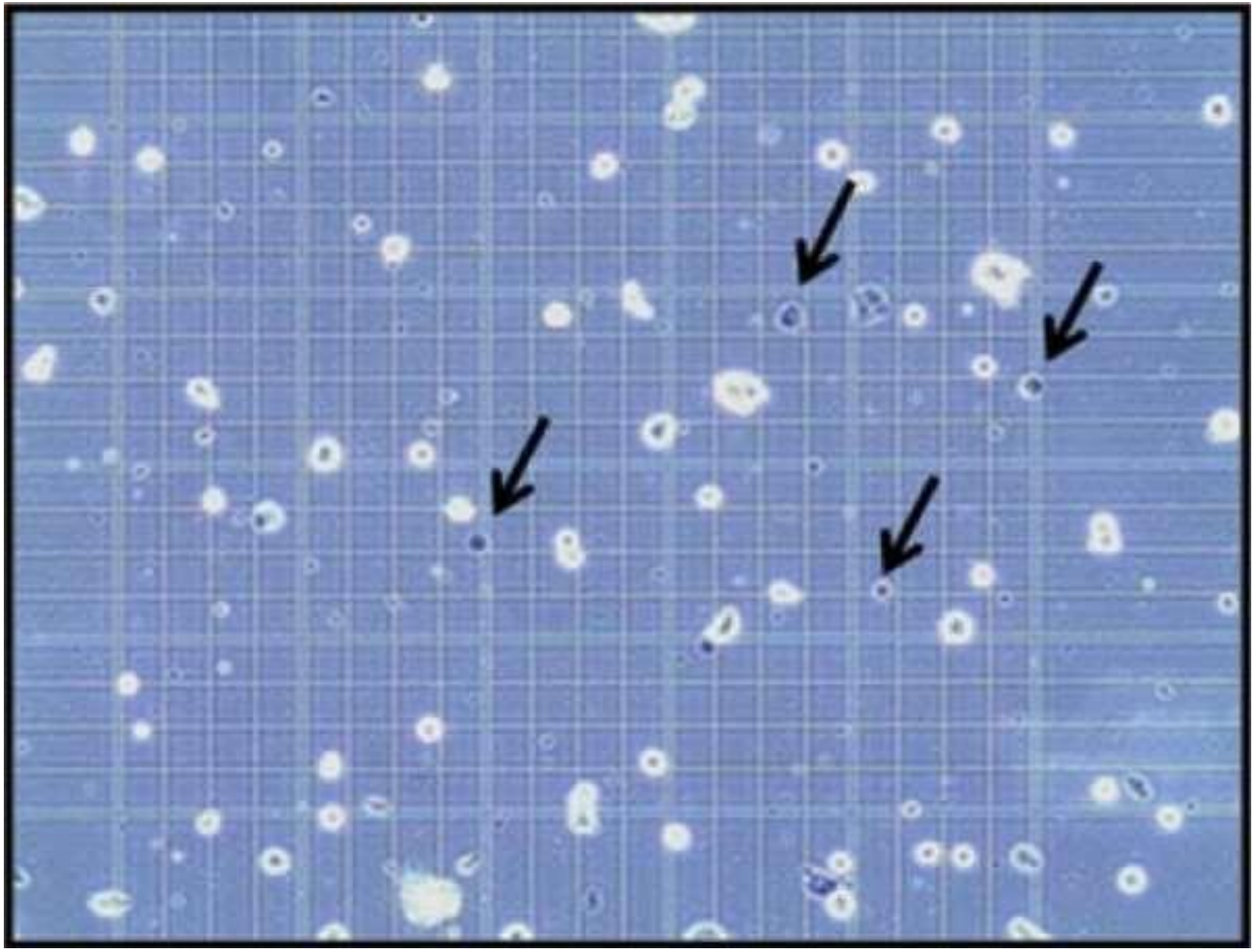
Live cells can not take up because of intact cell membranes.

Only dead cells are stained.

Visualization:

Light microscope

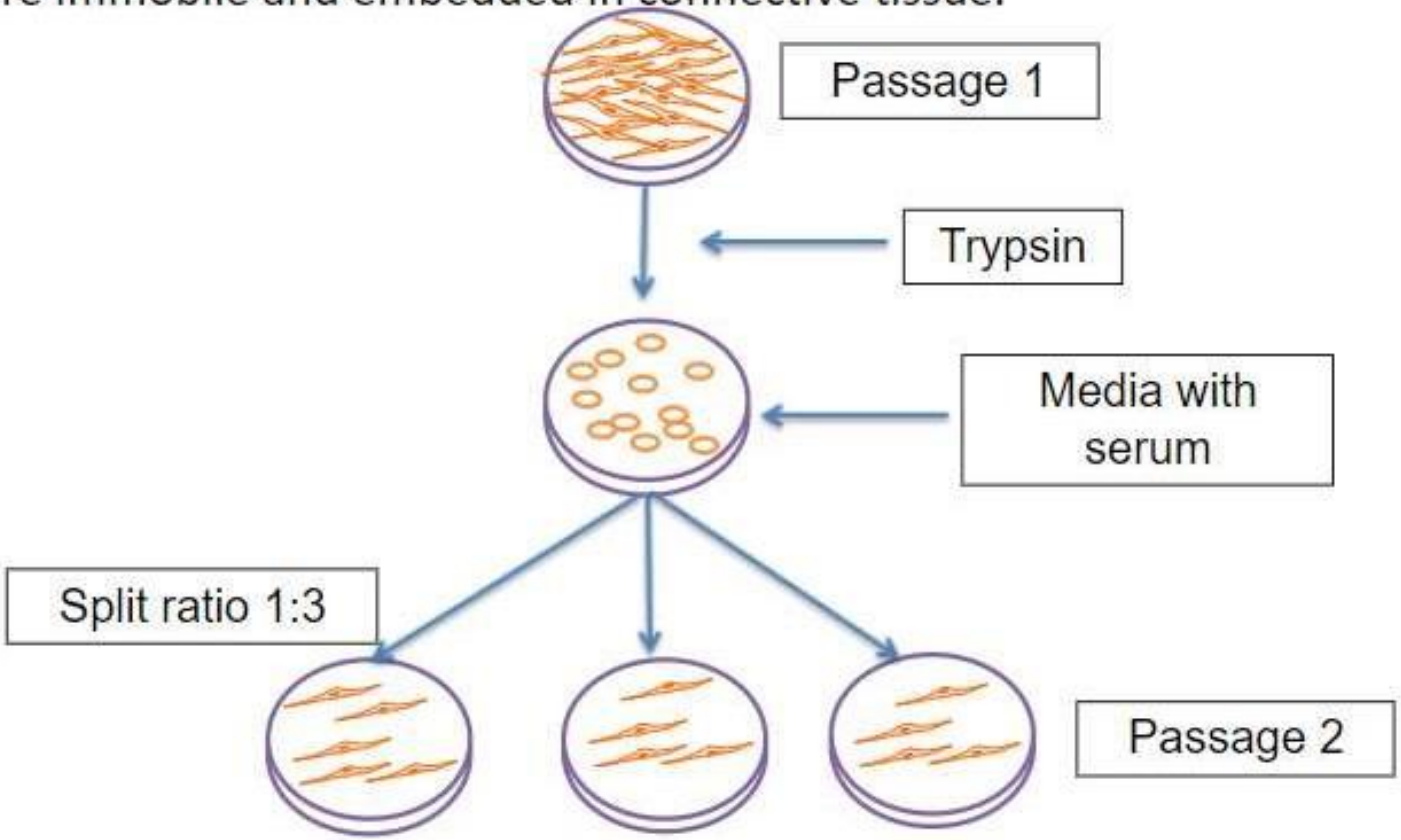
Automated cell counter and analyzer e.g; Cedex XS Analyzer or Cedex HiRes Analyzer.



Passaging

- **Adherent**

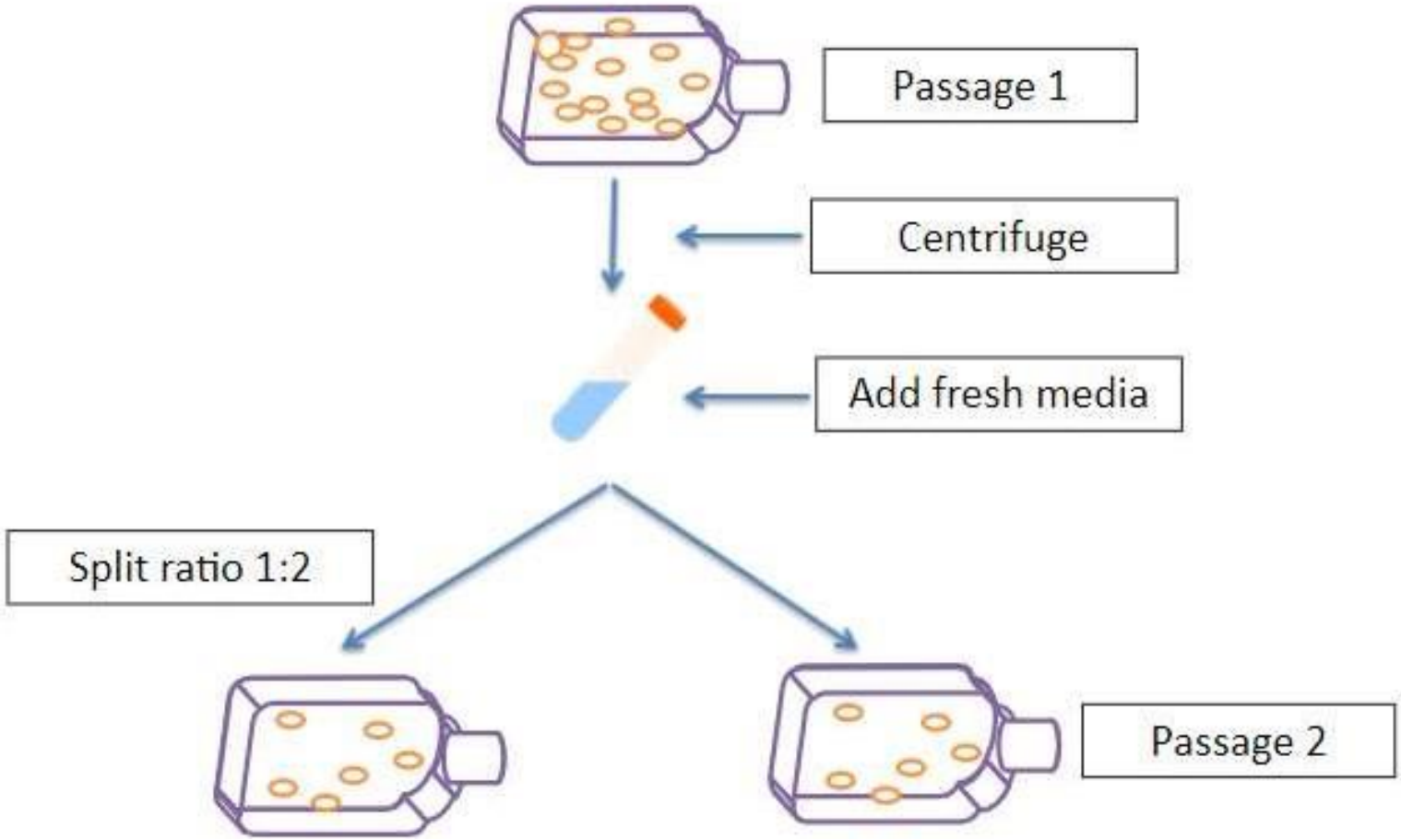
- Require a solid surface/substrate for attachment.
- Usually derived from a tissues of organs as kidneys, liver, lungs, brain etc. where they are immobile and embedded in connective tissue.



Passaging

- **Suspension**

- Grow in suspension and does not require a surface for attachment.
- Usually culture of cells from blood.



Subculturing the Cells Grown as a Monolayer

Trypsinization of a monolayer after an EDTA prewash to remove traces of medium. divalent cations. and serum.

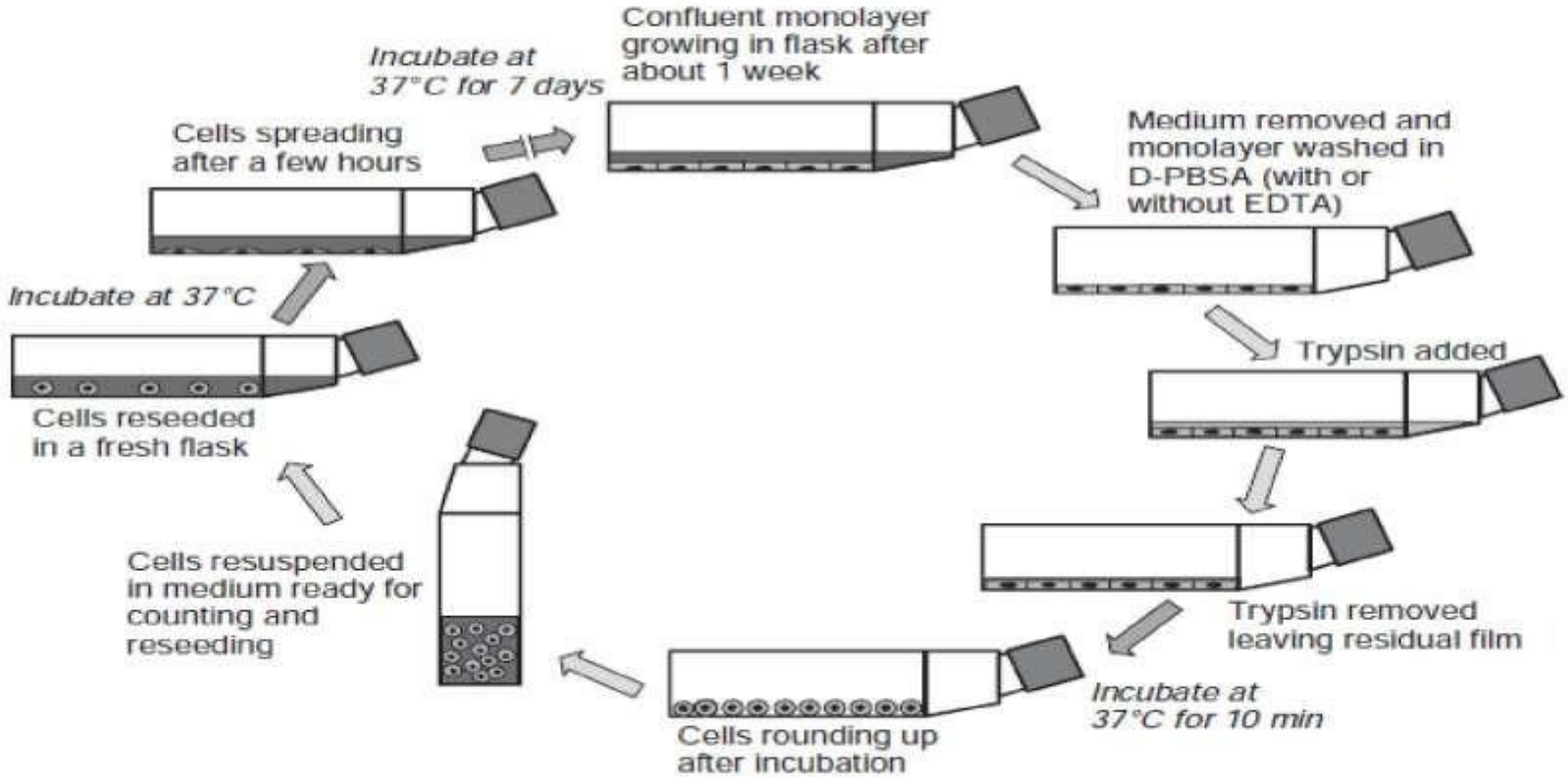


Fig. 12.3. Subculture of Monolayer. Stages in the subculture and growth cycle of monolayer cells after trypsinization (*see also* Plates 4, 5).

Ref : R. Ian Freshney (2010). 6th edition

Passaging Cells

Check confluency of cells

↓

Remove spent medium

↓

Wash with PBS

↓

Incubate with trypsin/EDTA

↓

Resuspend in serum containing media

↓

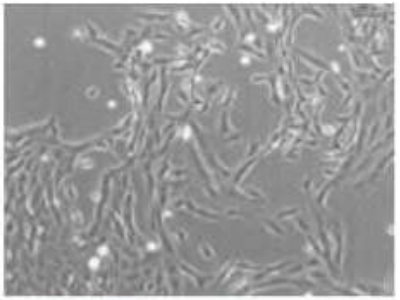
Transfer to culture flask

Why passage cells?

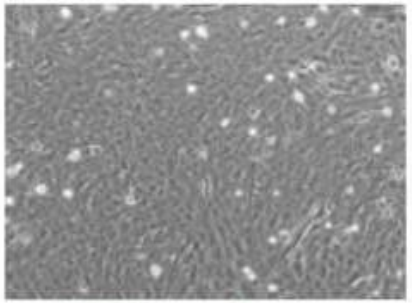
- To maintain cells in culture (i.e. don't overgrow)
- To increase cell number for experiments/storage

How?

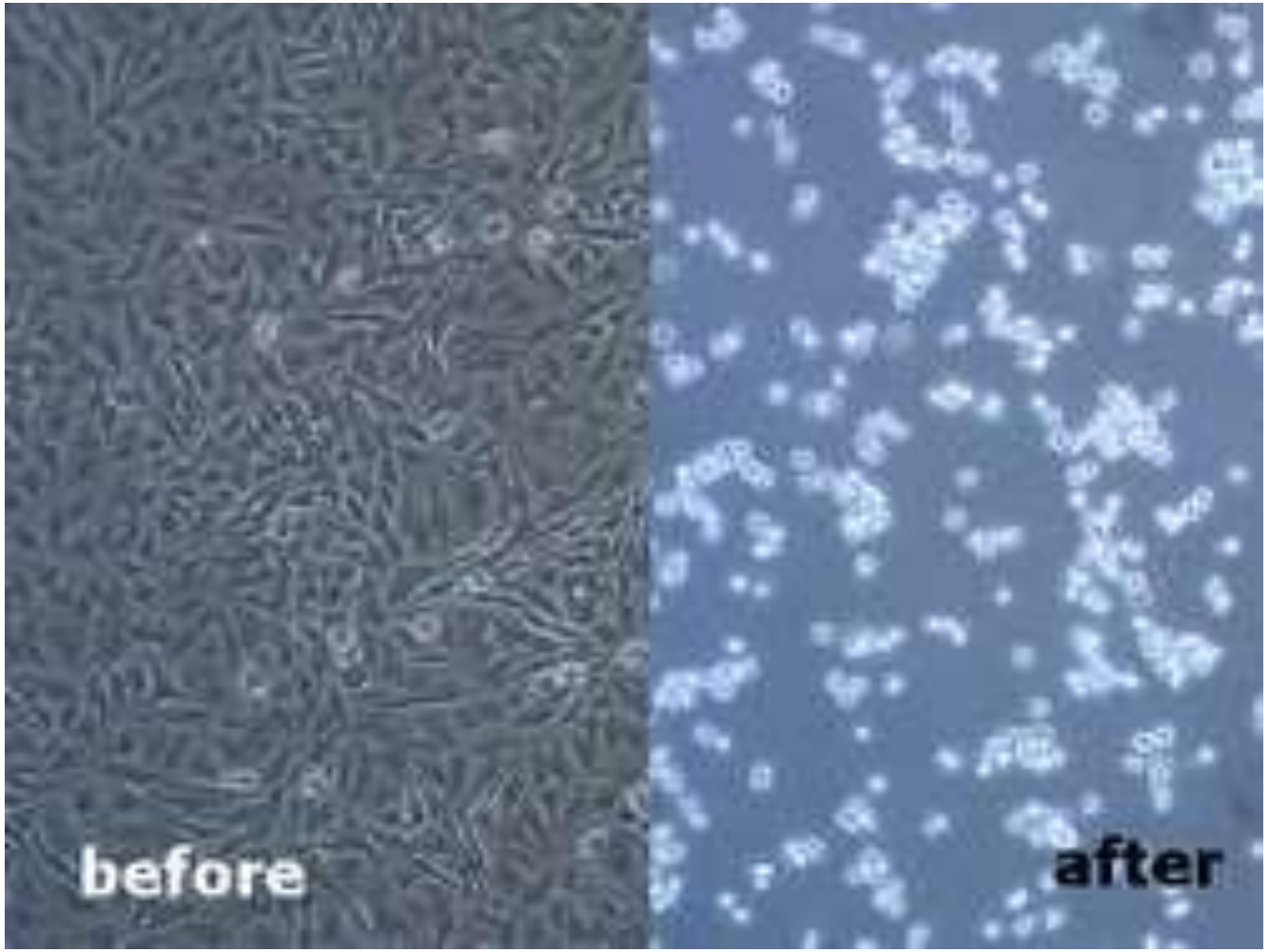
- 70-80% confluency
- Wash in PBS to remove dead cells and serum
- Trypsin digests protein-surface interaction to release cells (collagenase is also useful)
- EDTA enhances trypsin activity
- Resuspend in serum (inactivates trypsin)
- Transfer dilute cell suspension to new flask (fresh media)
- Most cell lines will adhere in approx. 3-4 hours



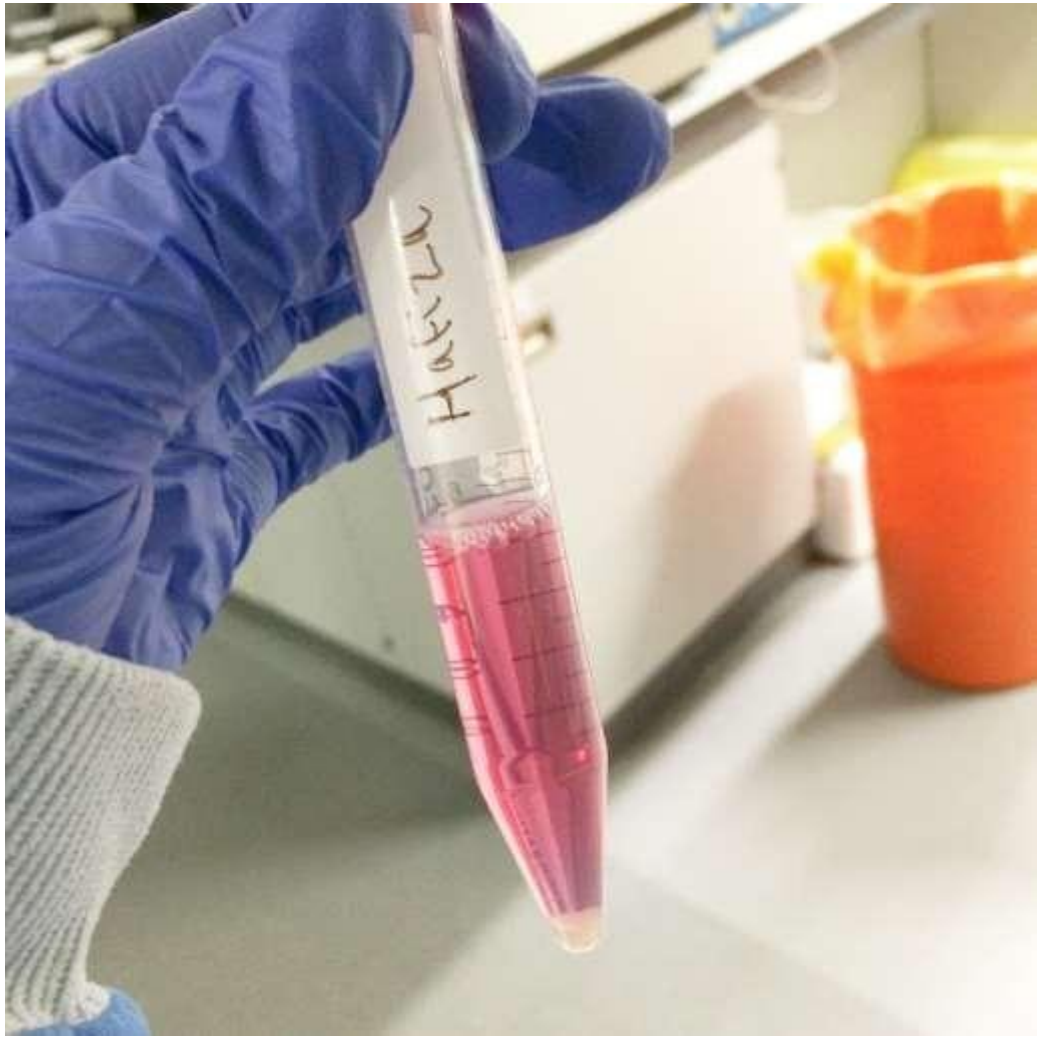
70-80% confluence

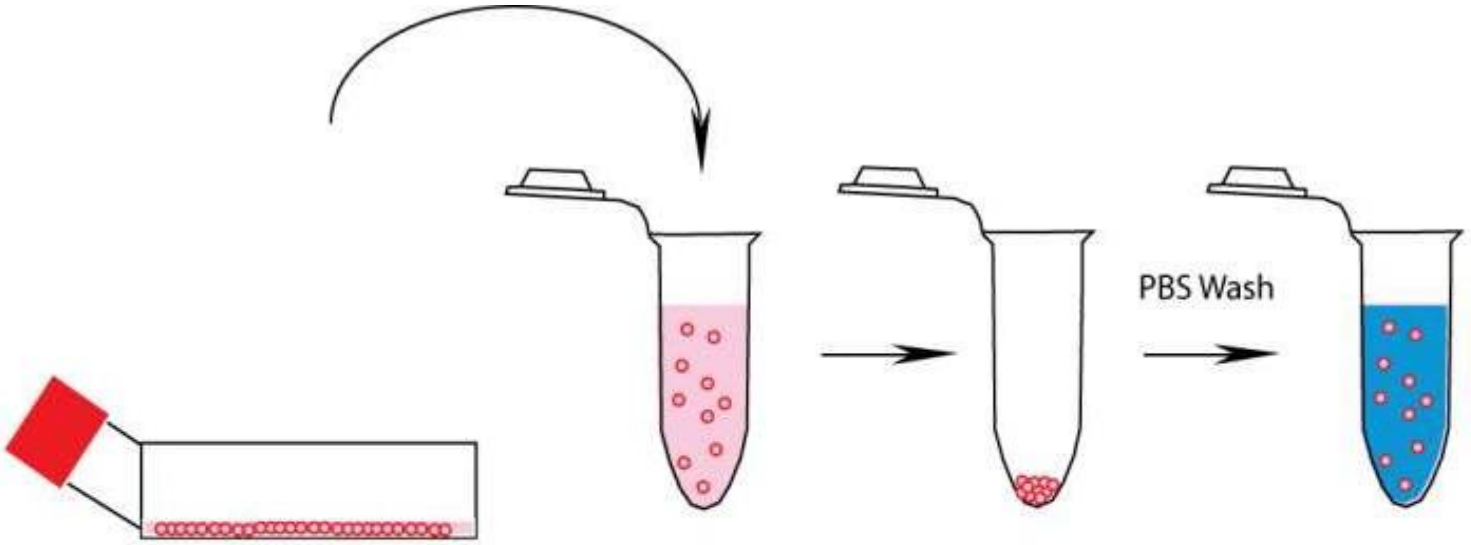


100% confluence


before
after

Cell pellets after centrifuge





Cells grown to 80% confluence

Harvest cells with EDTA, Trypsin, , or other cell dissociation solution. Minimize enzyme exposure to preserve cell surface receptors.

Centrifuge cell 250 x g to pellet, and aspirate liquid; this is important to remove trypsin and other cell dissociation factors that may affect ECM substrates or adhesion.

Resuspend cells at 1.5 x 10^5 cells/ml in warm Cell Adhesion Buffer. Buffer must be warm to prevent adverse effects to cells or plates.