Basics of Cell Culture II

Prof. Arttatrana Pal
Department of Zoology
School of Life Sciences
Mahatma Gandhi Central University, Bihar

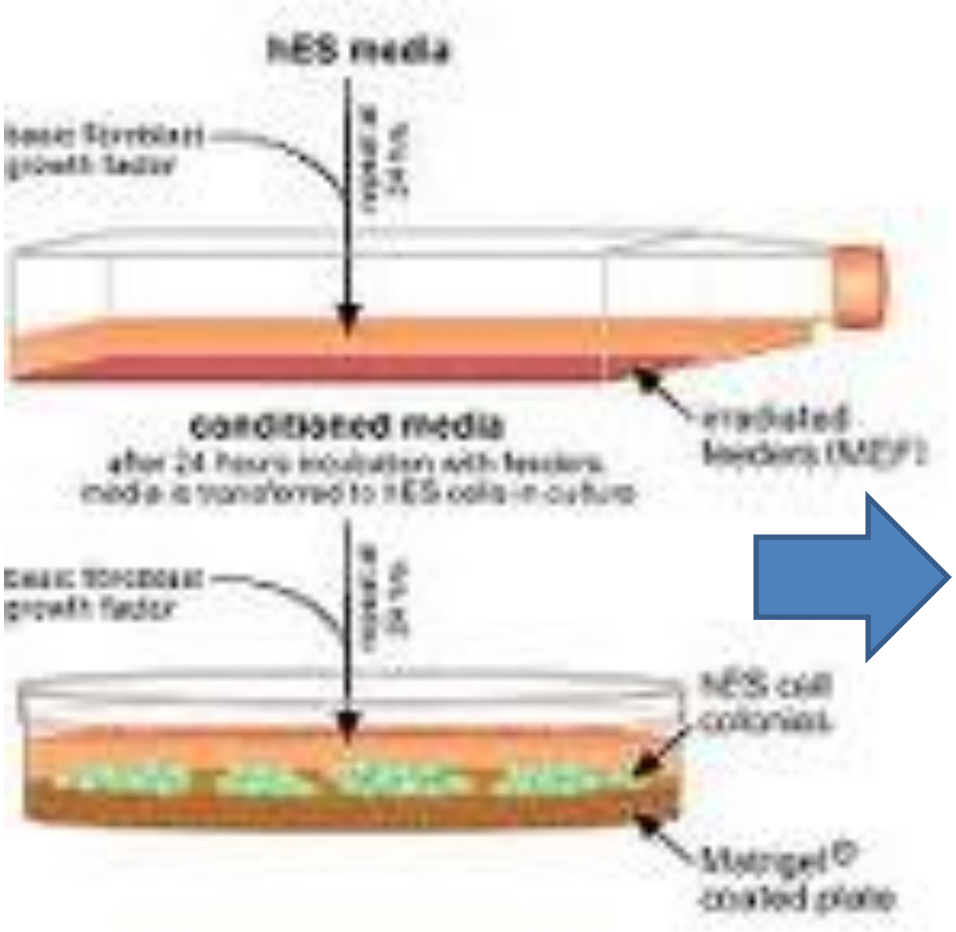
Why sub culturing?

- Once the available substrate surface is covered by cells (a confluent culture) growth slows & ceases
- Cells to be kept in healthy & in growing state have to be sub-cultured or passaged
- It's the passage of cells when they reach to 80-90% confluency in flask/dishes/plates
- Enzyme such as trypsin, dipase, collagenase in combination with EDTA breaks the cellular glue that attached the cells to the surface

Culturing of cells

- Cells are cultured as anchorage dependent or independent
- Cell lines derived from normal tissues are considered as anchorage-dependent grows only on a suitable substrate e.g. tissue cells
- Suspension cells are anchorage-independent e.g. blood cells
- Transformed cell lines either grows as monolayer or as suspension

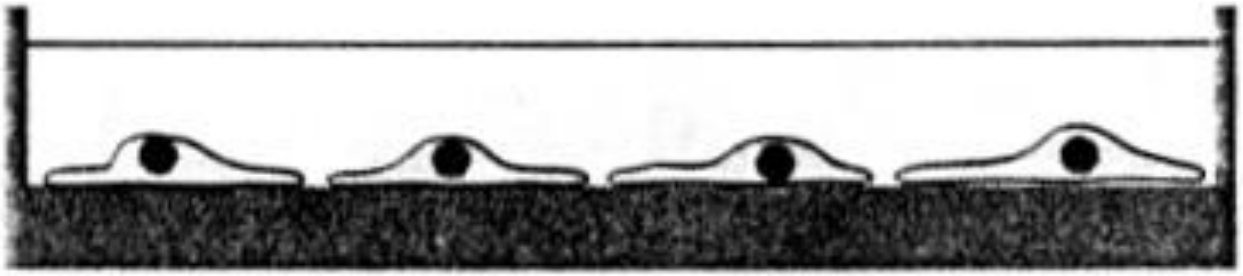
Passaging or sub-culture



Split 1 into 2 flasks

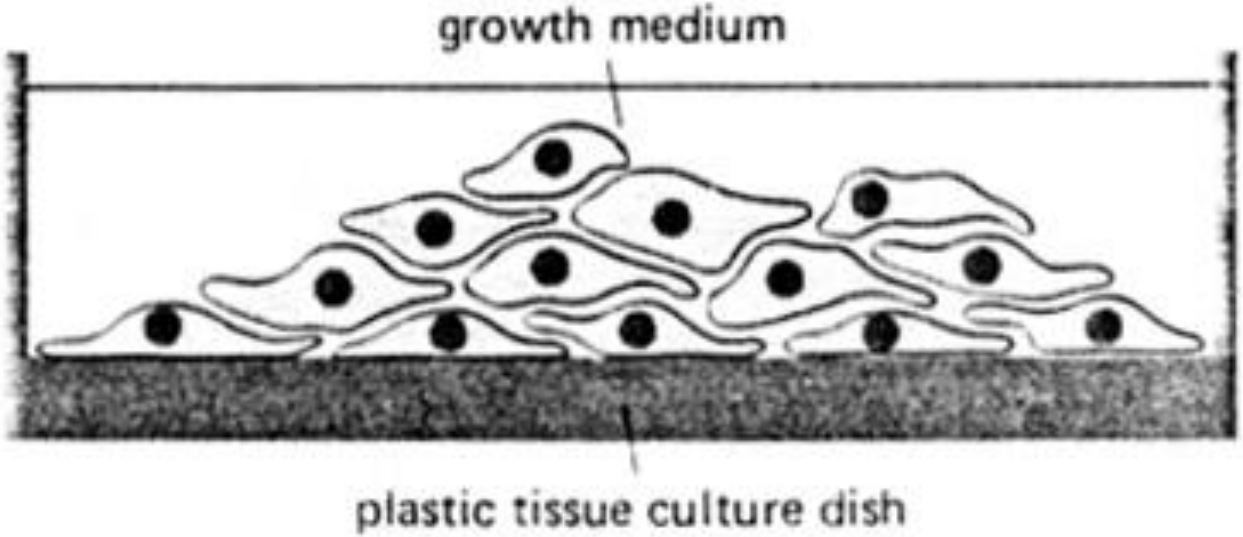
Cells dissociated from flask using enzymes

Contact inhibition



contact-inhibited monolayer of normal cells

Therefore need to split them to maintain growth



multilayer of uninhibited cancer cells

How to do passaging

Non-adherent cells

- Many cell types, in particular many microorganisms, grow in solution and not attached to a surface

- These cell types can be sub-cultured by simply taking a small volume of the parent culture and diluting it in fresh growth medium

- Cell density in these cultures is normally measured in cells per millilitre for large eukaryotic cells, or as optical density for 600 nm light for smaller cells like bacteria

- The cells will often have a preferred range of densities for optimal growth and sub-culture will normally try to keep the cells in this range

Adherent cells

- Adherent cells, for example many mammalian cell lines, grow attached to a surface such as the bottom of the culture flask

- These cell types have to be detached from the surface before they can be sub-cultured

- For adherent cells cell density is normally measured in terms of confluence

- The cells will often have a preferred range of confluences for optimal growth, for example a mammalian cell line like HeLa or Raw 264.7 generally prefer confluences over 10% but under 100%, and subculture will normally try to keep the cells in this range

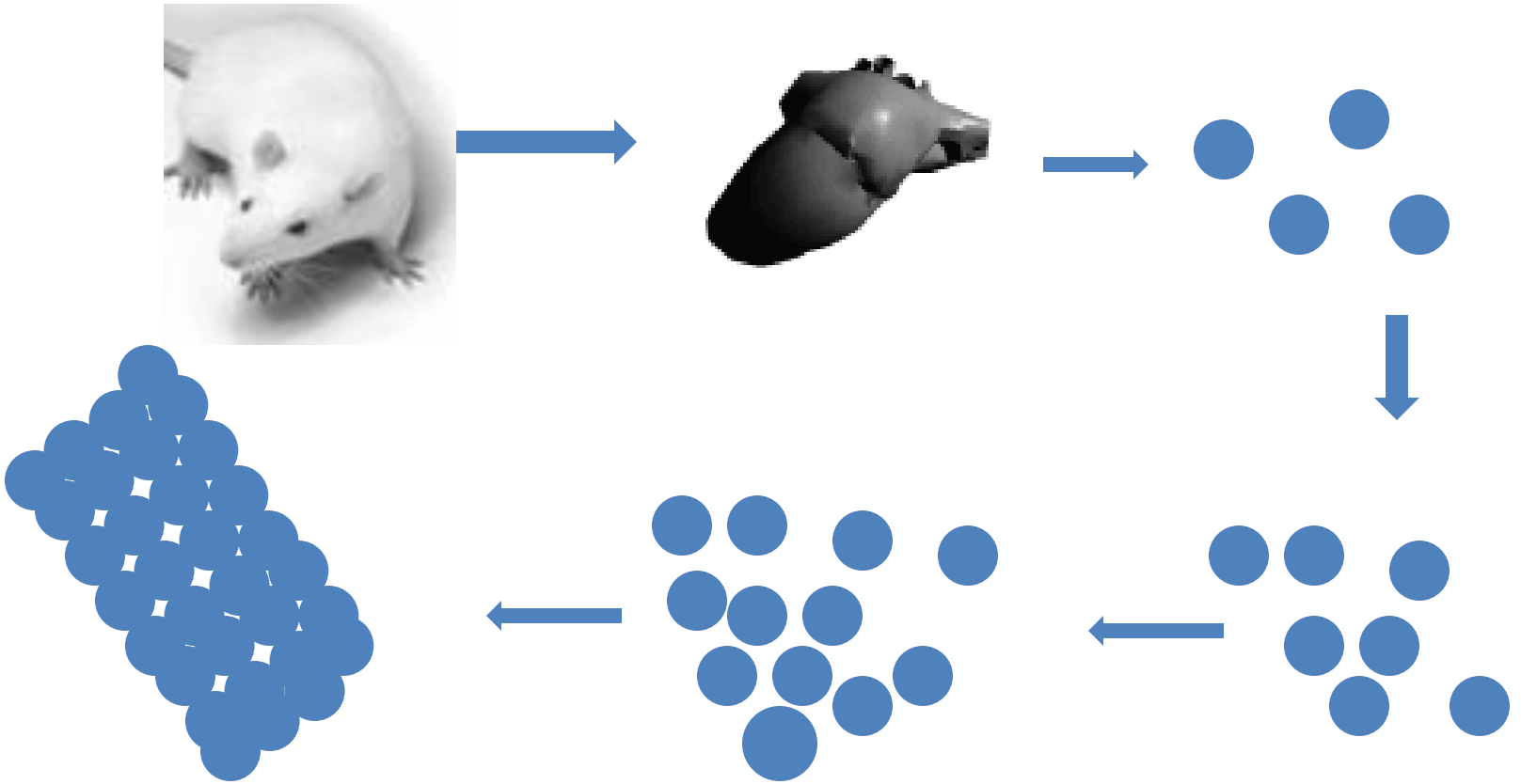
Adherent cells

- For subculture cells may be detached by one of several methods including trypsin treatment to break down the proteins responsible for surface adherence, chelating sodium ions with EDTA which disrupts some protein adherence mechanisms, or mechanical methods like repeated washing or use of a cell scraper

- The detached cells are then resuspended in fresh growth medium and allowed to settle back onto their growth surface

Steps in primary tissue culture

- Isolation of tissue
- Disaggregation of cells – initiation of culture
- Incubation and growth



Isolation of tissue

- Make sure your work is within rules
- Work safely, especially with human tissue
- If you isolate your cells far from culture place keep it on ice (4 C) for up to 72 hours

Disaggregation of cells

- Cells can be allowed to migrate out from an explant
- Mechanical dissociation (mincing)
- Enzymatic dissociation

  [Exception – hematopoietic cells do not need to be disaggregated, they already are]

Explant culture

Explant culture is a technique used for the isolation of cells from a piece or pieces of tissue. Tissue harvested in this manner is called an *explant*

Explant culture

- **Involves placing a piece of tissue into the tissue culture dish and allowing cells to migrate out from the tissue**
- **First type of cell culture developed**
- **Performed in the case of cells which are protease sensitive**
  - **– Smooth muscle cells, bone cells**
- **Or in case of small amount of tissue (such as needle biopsies)**
- **Not very effective for cells with poor adhesion (migration)**
- **Fibrinogen and thrombin used to stimulate adhesion**
- **Disadvantages – selection by speed of migration, type of attachment, localization within tissue etc**

Enzymatic disaggregation

Avoids selection of cells by migration and usually yields more representative sample

- **– But still selects by resistance to enzymatic treatment**
  - **Faster than explant**

Enzymatic disaggregation

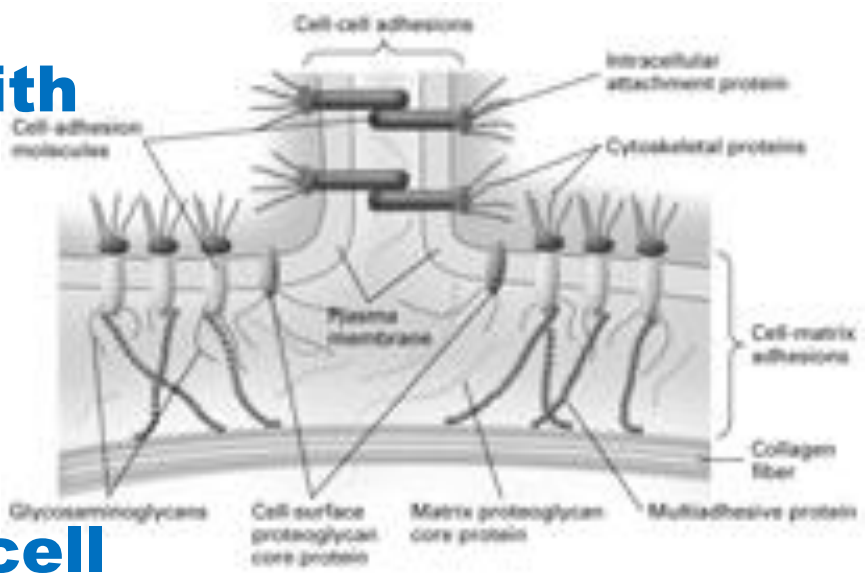
- Cell to cell adhesion is mediated by a variety of **cell adhesion molecules**
- The connections between cells and extracellular matrix have to be broken
- To break calcium dependent adhesion (cadherins and selectins) we use EDTA or EGTA (both calcium chelators)
- Extracellular matrix proteins such as fibronectin and laminin are protease sensitive
- **Proteoglycans** can be partially degraded by hyaluronidase or heparinase

Enzymes used in enzymatic disaggregation

- **Enzymes**
  - **Trypsin**
  - **Collagenase II (from *Pseudomonas perfringens*)**
  - **Elastase**
  - **Hyaluronidase**
  - **DNase**
  - **Pronase (bacterial protease)**
- **Usually a combination of enzymes**
- **Crude preparations are usually more efficient**
  - **The purer the less toxic**
  - **The cruder the more effective due to contamination with other proteases**

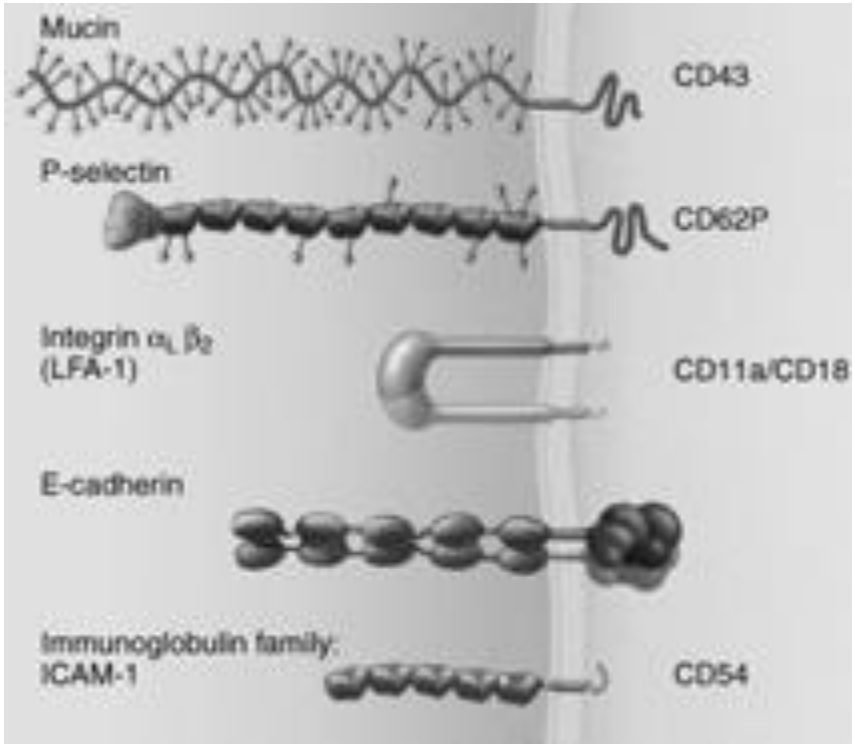
Physical connections between cells

- Cells in multicellular organisms are in contact with each other or extracellular matrix
- Cell connections involve multiple ligands and cell adhesion receptors
- The interaction between cell adhesion receptors and their ligands are relatively weak
  - A lot of weak interactions make a strong bond



Principal classes of cell-adhesion Receptors

- Cadherins
- Ig-family of cell adhesion molecules
- Integrins
- Selectins
- Others such as
  - Mucins
  - Connexins



Extracellular matrix

- Network of proteins and carbohydrates that binds cells together
  - Supports and surrounds cells
  - Regulates cells activities

- Only 5 classes of macromolecules
  - Collagens
  - Elastic fibers
  - Proteoglycans
  - Hyaluronan
  - Adhesive glycoproteins
- They can be mixed up in different proportions for different functions

How you start ???

- Start with trypsin/EDTA and than proceed to more complex enzymes
- Warm or cold trypsin
- Cold seems to give a higher yields but warm is faster (shorter exposure)
- If using warm trypsin collect cells every half hour to avoid cell death from exposure (remember to inactivate and remove trypsin before plating cells)
- Warm trypsin works better with big amounts of young tissue (mouse or chick embryos) and not too well with adult tissue (more connective tissue)

Cold trypsin

- Avoids damage by exposure to warm trypsin
- Allows for enzyme penetration with minimum of enzymatic activity
- Followed by faster 37 C digestion time
- Gives higher yield and higher survival rate
- Preserves more different cell types
- Convenient

Other enzymatic procedures

Some tissues such as fibrous connective tissue are resistant to trypsin

- **Collagenase** – particularly connective tissue and muscle
- **Hyaluronidase** – to dissolve proteoglycans
- **Pronase and dispase** – bacterial proteases
- **DNase** – to dissolve DNA aggregates from damaged cells

Mechanical disaggregation

- Produces cell suspension quicker than other methods
- But causes more mechanical damage
- Several methods
  - Mincing
  - Collecting cells when tissue is sliced
  - Pressing the tissue through a series of sieves
  - Repeated pipetting

Incubation and growth

- Appropriate medium supplemented with growth factors, cytokines and all the goodies

- Some cells require special adhesion surfaces (cover tissue culture dish with extracellular matrix proteins or synthetic attachment molecules)

- Transfer cells to final growing conditions as soon as possible

- Challenges
  - Removal of dead cells
  - Enrichment of viable cells
  - Separation of cell types

Separation of nonviable cells

- For adherent cultures first change of media

- Gradual dilution of suspension cells when proliferation starts

Separation of cell types

- Selective media
- Difference in the speed of attachment
- Use of enzymes
  - Collagenase does not easily disperse epithelial cells but works well on stroma
- Neurons need NGF while glial cells don't

Considerations

- Sensitivity to mechanical dispersal or enzymes; cell-cell contact may be required for proliferation
- Dispersed cells in culture are vulnerable
- Most primary cells require satisfactory adherence
- Some cells are not normally adherent in vivo and can be grown in liquid suspension
- In a mixed primary culture differences in growth rate may mean a loss of the cell type of interest – selection techniques
- Some cells are prone to spontaneous transformation
- Limited life span of some cultures

Factors affecting cell behaviour in the complex *in vivo* environment

The local micro-environment: metabolites, local growth factors, ECM, architecture Cell-cell interactions, Circulating proteins, cytokines, hormones

How to best mimic this in vitro?

Culture Surface

Most adherent cells require attachment to proliferate

Change charge of the surface

- **Poly-L-lysine: Coating with matrix proteins**
- **Collagen, laminin, gelatin, fibronectin**

Media formulation

- **Initial studies used body fluids**
  - **Plasma, lymph, serum, tissue extracts**

- **Early basal media**
  - **Salts, amino acids, sugars, vitamins supplemented with serum**

- **More defined media**
  - **Cell specific extremely complex PLUS SERUM**

Media formulation

- Inorganic ions
  - Osmotic balance –cell volume
- Trace Elements
  - Co-factors for biochemical pathways (Zn, Cu)
- Amino Acids
  - Protein synthesis
  - Glutamine required at high concentrations
- Vitamins
  - Metabolic co-enzymes for cell replication
- Energy sources
  - glucose

Serum provides the following [Horse serum, fetal calf serum, chick embryo extract]

Basic nutrients

- Hormones and growth factors
- Attachment and spreading factors
- Binding proteins (albumin, vitronectin, transferrin), hormones, vitamins, minerals, lipids
- Protease inhibitors
- pH buffer

N.B: A growth factor is a naturally occurring substance capable of stimulating cellular growth, proliferation and cellular differentiation. Usually it is a protein or a steroid hormone. Growth factors are important for regulating a variety of cellular processes.

For your references

Table 1. Major serum components and profile of fetal calf serum (Lindl and Bauer, ref. 12)

Component	Average concentration per litre
Na^+	137 meq
K^+	11 meq
Cl^-	103 meq
Fe^{2+}, Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, VO_3^-, $Mo_7O_{24}^{6-}$	µg to ng
SeO_3^{2+}	26 µg
Ca^{2+}	136 mg
Inorganic phosphorous	100 mg
Glucose	1250 mg
Nitrogen (urea)	160 mg
Total protein	38 g
Albumin	23 g
α-2-Macroglobulin	3 g
Fibronectin	35 mg
Uric acid	29 mg
Creatinine	31 mg
Haemoglobin	113 mg
Bilirubin (total)	4 mg
Alkaline phosphatase	255 U
Lactate dehydrogenase	860 U
Insulin	0.4 µg
TSH (thyroid stim. hormone)	1.2 µg
FSH (follicle stim. hormone)	9.5 µg
Bovine growth hormone	39 µg
Prolactin	17 µg
T_3 (triiodothyronine)	1.2 µg
Cholesterol	310 µg
Cortisone	0.5 µg
Testosterone	0.4 µg
Progesterone	80 ng
Prostaglandin E	6 µg
Prostaglandin F	12 µg
Vitamin A	90 µg
Vitamin E	1 mg
Endotoxin	0.35 µg

The gas phase

CO2 Incubator

Controlled CO2
Humidified
37oC

Oxygen

- Aerobic metabolism
- Atmospheric 20%
- Tissue levels between 1-7%

Carbon dioxide

- Buffering

pH Control

Physiological pH 7

pH can affect

- **Cell metabolism**
- **Growth rate**
- **Protein synthesis**
- **Availability of nutrients**

CO_2 acts as a buffering agent in combination with sodium bicarbonate in the media

Temperature and Humidity

Normal body temperature 37oC
Humidity must be maintained at saturating levels as evaporation can lead to changes in

- **Osmolarity**
- **Volume of media and additives**

Working with cryopreserved cells

- Vial from liquid nitrogen is placed into 37 C water bath, agitate vial continuously until medium is thawed
- Centrifuge the vial for 10 mts at 1000 rpm at RT, wipe top of vial with 70% ethanol and discard the supernatant

- Resuspend the cell pellet in 1 ml of complete medium with 20% FBS and transfer to properly labeled culture plate containing the appropriate amount of medium are established

- Check the cultures after 24 hrs to ensure that they are attached to the plate
- Change medium as the colour changes, use 20% FBS until the cells

Freezing cells for storage

- Remove the growth medium, wash the cells by PBS and remove the PBS by aspiration
- Dislodge the cells by trypsin-versene
- Dilute the cells with growth medium
- Transfer the cell suspension to a 15 ml conical tube, centrifuge at 200g for 5 mts at RT and remove the growth medium by aspiration
- Resuspend the cells in 1-2ml of freezing medium
- Transfer the cells to cryovials, incubate the cryovials at -80 C overnight
- Next day transfer the cryovials to Liquid nitrogen

Cell viability

- Cell viability is determined by staining the cells with trypan blue
- As trypan blue dye is permeable to non-viable cells or death cells
- Stain the cells with trypan dye and load to haemocytometer and calculate % of viable cells

$$\% \text{ of viable cells} = \frac{\text{No. of unstained cells} \times 100}{\text{total no. of cells}}$$

Common cell lines

Human cell lines

- MCF-7 — Breast cancer
- HL 60 — Leukemia
- HEK-293 — Human embryonic kidney
- HeLa — Henrietta lacks
- C6 — Glial cells
- HLEB3 — Human lenses epithelial cells

Primate cell lines

- Vero — African green monkey kidney epithelial cells
- Cos-7 — African green monkey kidney cells

And others such as CHO from hamster, sf9 & sf21 from insect cells

Contaminant's of cell culture

Cell culture contaminants of two types:

- **Chemical-difficult to detect caused by endotoxins, plasticizers, metal ions or traces of disinfectants that are invisible**

- **Biological-cause visible effects on the culture they are mycoplasma, yeast, bacteria or fungus or also from cross-contamination of cells from other cell lines**

Effects of Biological Contamination's

- They competes for nutrients with host cells
- Secreted acidic or alkaline by-products ceases the growth of the host cells
- Degraded arginine & purine inhibits the synthesis of histone and nucleic acid
- They also produces H_2O_2 which is directly toxic to cells

Detection of contaminants

In general indicators of contamination are

- turbid culture media
- change in growth rates
- abnormally high pH
- poor attachment
- multi-nucleated cells
- graining cellular appearance
- vacuolization
- inclusion bodies
- cell lysis

Detection of contaminants

- Yeast, bacteria & fungi usually shows visible effect on the culture (changes in medium turbidity or pH)

- Mycoplasma detected by direct DNA staining with intercalating fluorescent substances e.g. Hoechst 33258

- Mycoplasma also detected by enzyme immunoassay by specific antisera or monoclonal abs or by PCR amplification of mycoplasmal RNA

- The best and the oldest way to eliminate contamination is to discard the infected cell lines directly

Rules for working with cell culture

- Never use contaminated material within a sterile area
- Use the correct sequence when working with more than one cell lines.
  - Diploid cells (Primary cultures, lines for the production of vaccines etc.)
  - Diploid cells (Laboratory lines)
  - Continuous, slow growing line
  - Continuous, rapidly growing lines
  - Lines which may be contaminated
  - Virus producing lines

Cell Culture is a Fussy Discipline

In the tissue culture laboratory:

- **Bench tops should be kept clear and clean**
- **Wearing a long sleeve lab coat : minimises contamination from street clothing (hair, etc)**
- **Wearing gloves while doing tissue culture work: minimises contamination from skin organisms**
- **Surfaces, gloves, solutions and plasticware sprayed with 70% alcohol before placed into the biological hood**
- **Solutions, reagents and glassware used in tissue culture work should not be shared with non-tissue culture work**

Basic aseptic conditions

- If working on the bench use a **Bunsen flame** to heat the air surrounding the Bunsen
- Swab all bottle tops & necks with **70% ethanol**
- Flame all bottle necks & pipette by passing very quickly through the **hottest part of the flame**
- Avoiding placing caps & pipettes down on the bench; practice holding bottle tops with the little finger
- Work either left to right or vice versa, so that all material goes to one side, once finished
- **Clean up spills immediately** & always leave the work place neat & tidy

Safety aspect in cell culture

- Possibly keep cultures free of antibiotics in order to be able to recognize the contamination
- Never use the same media bottle for different cell lines. If caps are dropped or bottles touched unconditionally touched, replace them with new ones
- Necks of glass bottles prefer heat at least for 60 secs at a temperature of 200 C
- Switch on the laminar flow cabinet 20 mts prior to start working
- Cell cultures which are frequently used should be subcultered & stored as duplicate strains

Other key facts

- Use actively growing cells that are in their log phase of growth, which are 80-90% viable
- Keep exposure to trypsin at a minimum
- Handle the cells gently. Do not centrifuge cells at high speed or roughly re-suspend the cells
- Feeding & sub culturing the cells at more frequent intervals then used with serum containing conditions may be necessary
- A lower concentration of 10_4cells/ml to initiate subculture of rapidly growing cells & a higher concentration of 10_5cells/mlfor slowing growing cells

Thank You