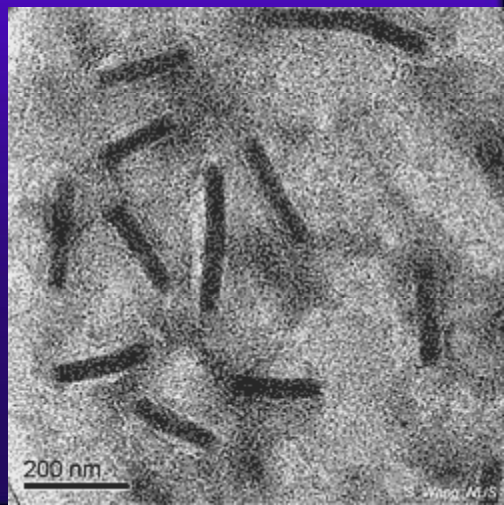
# Basics of Cell Culture



# Introduction

- Cell culture is the  process by which prokaryotic, eukaryotic or plant cells are grown under controlled conditions. But in practice it refers to the culturing of cells derived from animal cells.
- Cell culture was first successfully undertaken by Ross Harrison in 1907
- Roux in 1885 for the first time maintained embryonic chick cells in a cell culture

# Historical events in the development of cell culture

- 1878: Claude Bernard proposed that physiological systems of an organism can be maintained in a
- living system after the death of an organism.
- 1885: Roux maintained embryonic chick cells in a saline culture.
- 1897: Loeb demonstrated the survival of cells isolated from blood and connective tissue in serum
- and plasma.
- 1903: Jolly observed cell division of salamander leucocytes *in vitro*.
- 1907: Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and
- observed the growth of nerve fibers *in vitro* for several weeks. He was considered by some as
- the father of cell culture.
- 1910: Burrows succeeded in long term cultivation of chicken embryo cell in plasma clots. He made detailed observation of mitosis.

# Contd..

- 1911: Lewis and Lewis made the first liquid media consisted of sea water, serum, embryo extract, salts and peptones. They observed limited monolayer growth.
- 1913: Carrel introduced strict aseptic techniques so that cells could be cultured for long periods.
- 1916: Rous and Jones introduced proteolytic enzyme trypsin for the subculture of adherent cells.
- 1923: Carrel and Baker developed 'Carrel' or T-flask as the first specifically designed cell culture vessel. They employed microscopic evaluation of cells in culture.
- 1927: Carrel and Rivera produced the first viral vaccine - Vaccinia.
- 1933: Gey developed the roller tube technique

# Contd..

- 1940s: The use of the antibiotics penicillin and streptomycin in culture medium decreased the problem of contamination in cell culture.
- 1948: Earle isolated mouse L fibroblasts which formed clones from single cells. Fischer developed a chemically defined medium, CMRL 1066.
- 1952: Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Helen Lane) cells. Dulbecco developed plaque assay for animal viruses using confluent monolayers of cultured cells.
- 1954: Abercrombie observed contact inhibition: motility of diploid cells in monolayer culture ceases when contact is made with adjacent cells.
- 1955: Eagle studied the nutrient requirements of selected cells in culture and established the first widely used chemically defined medium.
- 1961: Hayflick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite lifespan in culture.
- 1964: Littlefield introduced the HAT medium for cell selection.
- 1965: Ham introduced the first serum-free medium which was able to support the growth of some cells.

# Contd..

- 1965: Harris and Watkins were able to fuse human and mouse cells by the use of a virus.
- 1975: Kohler and Milstein produced the first hybridoma capable of secreting a monoclonal antibody.
- 1978: Sato established the basis for the development of serum-free media from cocktails of hormones and growth factors.
- 1982: Human insulin became the first recombinant protein to be licensed as a therapeutic agent.
- 1985: Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
- 1986: Lymphoblastoid γIFN licensed.
- 1987: Tissue-type plasminogen activator (tPA) from recombinant animal cells became commercially available.
- 1989: Recombinant erythropoietin in trial.
- 1990: Recombinant products in clinical trial (HBsAG, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2).

# Major development's in cell culture technology

- First development was the use of antibiotics which inhibits the growth of contaminants.
- Second was the use of trypsin to remove adherent cells to subculture further from the culture vessel
- Third was the use of chemically defined culture medium.

# Why is cell culture used for?

Areas where cell culture technology is currently playing a major role.

- Model systems for

  Studying basic cell biology, interactions between disease causing agents and cells, effects of drugs on cells, process and triggering of aging & nutritional studies
- Toxicity testing

  Study the effects of new drugs
- Cancer research

  Study the function of various chemicals, virus & radiation to convert normal cultured cells to cancerous cells

# Contd….

- Virology

  Cultivation of virus for vaccine production, also used to study there infectious cycle.

- Genetic Engineering

  Production of commercial proteins, large scale production of viruses for use in vaccine production e.g. polio, rabies, chicken pox, hepatitis B & measles

- Gene therapy

  Cells having a functional gene can be replaced to cells which are having non-functional gene

# Equipments -Laminar-flow hood

- Most cell culture procedures are performed inside laminar-flow hoods.
- Laminarflow hoods, or biological safety cabinets, provide a clean working environment to prevent contamination of cell cultures.
- The air is filtered and cleaned of particles before blown into the cabinet.
- Additionally, the flow of air in the hood is in smooth parallel lines which creates a “curtain” to separate inside from outside.
- Some laminar hoods are equipped with a UV-germicidal lamp to sterilize the contents inside while not in use. The UV lamp must be turned off before working in the hood to prevent exposure to hazardous UV light.

# Fluorescent inverted microscopes

- Fluorescent microscopes are inverted microscopes, used to observe cells and molecules that have been labeled with fluorophores.
- **Fluorophores are molecules that can absorb energy of light at specific wavelengths and emit less energetic fluorescent light.**
- **Fluorescent microscopes are equipped with filters that will separate the absorbed light from the emitted fluorescent light.**
- **A set of filters are mounted on a block called the filter cube.**
- Fluorescent microscopes usually have several filter cubes with different sets of filters appropriate for observing fluorophores that emit light at different wavelengths.
- The filter cubes are conveniently located on a turret that can be rotated in order to place the appropriate cube in place to observe a specific fluorophore.
- Use of fluorophores and fluorescent microscopes has enabled scientists to view cellular structures and to study molecular functions and interactions.

# General rules to be followed when using a fluorescent microscope

1. For better view, turn off the lights in the room.
2. Turn on the high-intensity light source (xenon-arc or a mercury-vapor lamp) and allow about 10 minutes for warm up. **Do not turn off the lamp until you are completely done with your work. You cannot turn the light back on again until it has cooled off completely, which may take up to 1-2 hours.**
3. Block the light path to prevent your cells from overexposure to the high intensity light. Fluorophores that are exposed to continuous light will eventually lose their fluorescent properties, a phenomenon called "**photobleaching".**
4. Place your plate or flask of cells on the stage and turn on the regular microscope light.
5. Using the lower magnification objective lens, find your specimen and focus.
6. Turn to the appropriate higher magnification objective lens and adjust the focus.
7. Turn off the regular microscope light and unblock the high-intensity light path.
8. Place the appropriate filter cube in place.
9. Observe the cells and make notes.
10. Remember to provide protection against photobleaching by blocking the light path when not observing the cells.
11. Turn off the light source when your work is finished.
12. Do not put the plastic cover on the microscope until the light source has cooled down.

# Clinical Centrifuge

- Clinical centrifuges are used to concentrate the cells and to separate the cells from the media or other reagents.
- Slow-speed clinical centrifuge must be used in order to prevent damage to the cells.
- For routine spinning of the cells, speed of 80-100g (gravitational force) is sufficient. Higher speeds may damage the cells.

# Two different models of clinical centrifuges.

# General rules for using a centrifuge:

1. Transfer the liquid suspension to the appropriately sized centrifuge tubes. Not all tubes will be able to survive the forces of the centrifugation, so please use the tubes specifically manufactured for centrifuge you are using.
2. Weigh your tubes with their contents on a pan-balance to make sure that the tubes are of equal weight. Fig. -depicted two tubes that are properly balanced, i.e. weigh the same amount.
3. Sometimes you must prepare a separate balancing tube of the same size by filling it with tap water. The balancing tube must be of the same weight as the tube that needs to be centrifuged.
4. Place the two tubes that have been balanced into two opposing slots of the centrifuge.
5. Close the safety lid. 6. Set the centrifuge to the appropriate speed and time, then turn it on.
6. Stay close to the centrifuge for the first minute to make sure the centrifuge is running smoothly. If the centrifuge is not balanced properly, it will vibrate. Some centrifuges will turn off automatically if unbalanced.
7. You need to turn off the older model centrifuges manually as soon as you sense the imbalance and vibrations. Continuation of the spin while the centrifuge is imbalanced will damage the centrifuge.
8. Do not open the safety lid while the motor is running.
9. Wipe any spills that might have occurred after centrifugation.

# CO2 Incubator

- The incubators provide the appropriate environment for the cells to grow. Cell culture incubators have three main functions:

1. Constant temperature- Incubators can be set to a specific temperature appropriate for the cells. For mammalian cells the temperature is kept at 37oC, which is the optimal temperature for their growth.

# Co2 incubator conti

2. Humidity- Although cells are kept in liquid media, smaller dishes that hold less liquid require a humid environment to prevent evaporation of the media. Usually a container filled with sterile, distilled water is placed in the incubator to provide humidity. The water needs to be replaced with fresh sterile distilled water regularly to prevent growth of microorganisms and reduce the possibility of contamination.
3. CO2 gas is needed to keep the pH balanced; that is why cell culture incubators are connected to a CO2 gas tank. CO2 gas is injected inside the incubator and distributed by a fan or natural convection. CO2 levels are usually maintained at 5%. CO2 interacts with the **bicarbonate buffer in the cell culture medium.**

**This interaction stabilizes the pH at about 7.4.**

**Uncorrected changes in medium pH can damage the cells.**

**$H_2O + CO_2 \text{---.>} H_2CO_3 \leftrightarrow H^+ + HCO_3^-$**

**It is important to periodically check the tank gauges and take care that $CO_2$ levels are maintained in your incubator.**

**Keep the incubator closed at all times and avoid frequent opening of the door to prevent loss of the $CO_2$ gas.**

- **Figure 6- A cell culture incubator connected to the $CO_2$ tank. Some incubators are also able to control the amount of oxygen available to the cells.**
- **The optimal temperature and humidity provides an environment suitable for the growth of bacteria and other microorganisms. Therefore, it is necessary** to clean the incubators frequently to prevent growth and spread of contamination.

# Primary culture

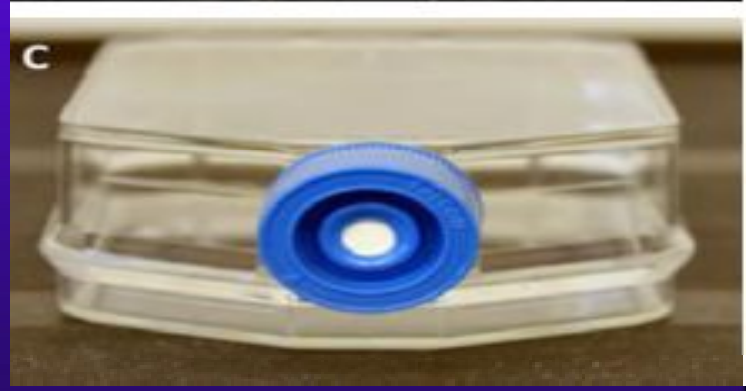
- Cells when surgically or enzymatically removed from an organism and placed in suitable culture environment will attach and grow are called as primary culture
- Primary cells have a finite life span
- Primary culture contains a very heterogeneous population of cells
- Sub culturing of primary cells leads to the generation of cell lines
- Cell lines have limited life span, they passage several times before they become senescent
- Cells such as macrophages and neurons do not divide in vitro so can be used as primary cultures
- Lineage of cells originating from the primary culture is called a cell strain

# Cell culture vessels

- Most cells in culture need to attach to a substrate in order to divide and grow.
- These cells often form a **monolayer and cover the surface available to them.**
- **Cells that require attachment for growth are said to be anchorage-dependent. Hematopoietic cells and a few other cell types can grow in liquid suspension without attachment.**
- **These cells are said to be anchorage-independent cells. The vessels used to grow anchorage-dependent and anchorage-independent cells need to be designed in a way to support the proper growth of these two cell types.**
- In most laboratories, disposable polystyrene plastic vessels are used to grow anchorage-dependent cells.
- The vessels are flat at the bottom to provide a surface for cell growth. The bottom surface of the culture vessels are coated by molecules such as poly-L-lysine, laminin, gelatin, or fibronectin.
- These mimic the natural **extracellular matrix and allow the cultured cells to attach. There are three types of commonly used culture vessels used for** anchorage-dependent cells: flasks, dishes, and multi-well plates.
- All three types can be of different sizes with different surface area. The choice of the vessel depends on the nature of the procedures and personal preference.

Cell culture dishes and multi-well plates **(A).**
**Cell culture flasks (B) with vented caps (C).**





# Pipettes

- Pipettes are used for transfer of specific volumes of liquid. Two kinds of pipettes are commonly used in laboratories:
- 1. **Serological pipettes can measure liquids between 0.1 to 50 mls. You must choose the appropriate size pipette for the volume that you are transferring. Serological pipettes are marked with calibrated lines to allow measurement of accurate volumes. The maximum volume that can be transferred and the size of the pipette's subdivisions are printed close to the top of the pipette. Serological pipettes require the use of electrical or manual pumps to draw and release liquid.**
- 2. **Micropipettes are used to transfer small volumes of liquid, between 1-1000• ls (Fig.8). You must choose the micropipette of the appropriate size to use for the volume you are transferring. The range of volumes that can be transferred is written on the micropipette. It is important to stay within the allowable range. Setting up the pipette to volumes above or below the allowable range will damage the pipette and will result in inaccurate measurements. Micropipettes fit tips that are specific for the specific volumes. The tips are kept in color-coded sterile boxes.**

When using a micropipette:

1. Choose the appropriate size micropipette.
2. Set the dial to the desired volume to be transferred.
3. Open the appropriate size tip box in a sterile environment.
4. Pick up a tip using the pipette. Do not use your fingers to fit the tip on the pipette. Remember to shut the box after the tip is removed to maintain sterility.
5. Press down gently on the plunger using your thumb to the first point where you feel resistance.
6. Place the tip inside the liquid just below the surface.
7. Gently release the plunger to draw the liquid in. If the plunger is released too rapidly the liquid will aerate into the micropipette and will increase the chances of contamination.
8. To expel the liquid, hold the tip inside the vessel, touching against the side of the vessel while holding it slightly tilted. Press down on the plunger, all the way to the bottom.
9. To dispose of the used pipette tip, hold the pipette above the disposal bucket and eject the tip into disposal by pressing the ejection button.

Three different sized micropipettes (p20, p100 and p1000) with their appropriate tips **(A). View of the micropipette tops (B).**

# Conti….

- The vessels used to grow anchorage-independent cells do not need to be treated for cell attachment.
- Sterile, stirrer bottles are normally used for agitation of the culture and to keep the cells in suspension.
- Both cell types need exchanges of gases (O2 and CO2) for growth.
- Therefore the cell culture vessels must allow gases to enter.
- Dishes and plates have loose fitting lids and the caps of the flasks must be closed loosely to allow gases to go in.
- Some flasks have vented caps with an opening that is covered by a filter to allow gases in, but prevent entrance of contamination

# Continous cell lines

- Most cell lines grow for a limited number of generations after which they ceases
- Cell lines which either occur spontaneously or induced virally or chemically transformed into Continous cell lines
- Characteristics of continous cell lines

  -smaller, more rounded, less adherent with a higher nucleus /cytoplasm ratio

  -Fast growth and have aneuploid chromosome number

  -reduced serum and anchorage dependence and grow more in suspension conditions

  -ability to grow upto higher cell density

  -different in phenotypes from donar tissue

  -stop expressing tissue specific genes

# Types of cells

On the basis of morphology (shape & appearance) or on their functional characteristics. They are divided into three.

- Epithelial like-attached to a substrate and appears flattened and polygonal in shape
- Lymphoblast like- cells do not attach remain in suspension with a spherical shape
- Fibroblast like- cells attached to an substrate appears elongated and bipolar

# Culture media

- Choice of media depends on the type of cell being cultured
- Commonly used Medium are GMEM, EMEM,DMEM etc.
- Media is supplemented with antibiotics viz. penicillin, streptomycin etc.
- Prepared media is filtered and incubated at 4 C

# 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

# Adherent cells

- Cells which are anchorage dependent
- Cells are washed with PBS (free of ca & mg ) solution.
- Add enough trypsin/EDTA to cover the monolayer
- Incubate the plate at 37 C for 1-2 mts
- Tap the vessel from the sides to dislodge the cells
- Add complete medium to dissociate and dislodge the cells
- with the help of pipette which are remained to be adherent
- Add complete medium depends on the subcu
- requirement either to 75 cm or 175 cm flask

# Suspension cells

- Easier to passage as no need to detach them
- As the suspension cells reach to confluency
- Asceptically remove 1/3$^{rd}$ of medium
- Replaced with the same amount of pre-warmed medium

# Transfection methods

- Calcium phosphate precipitation
- DEAE-dextran (dimethylaminoethyl-dextran)
- Lipid mediated lipofection
- Electroporation
- Retroviral Infection
- Microinjection

# Cell toxicity

- Cytotoxicity causes inhibition of cell growth
- Observed effect on the morphological alteration in the cell layer or cell shape
- Characteristics of abnormal morphology is the giant cells, multinucleated cells, a granular bumpy appearance, vacuoles in the cytoplasm or nucleus
- Cytotoxicity is determined by substituting materials such as medium, serum, supplements flasks etc. at atime

# 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
- 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 are established

# 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 whereas it is impermeable to this dye
- Stain the cells with trypan dye and load to haemocytometer and calculate % of viable cells

  - % of viable cells= Nu. of unstained cells x 100

    total nu. of cells

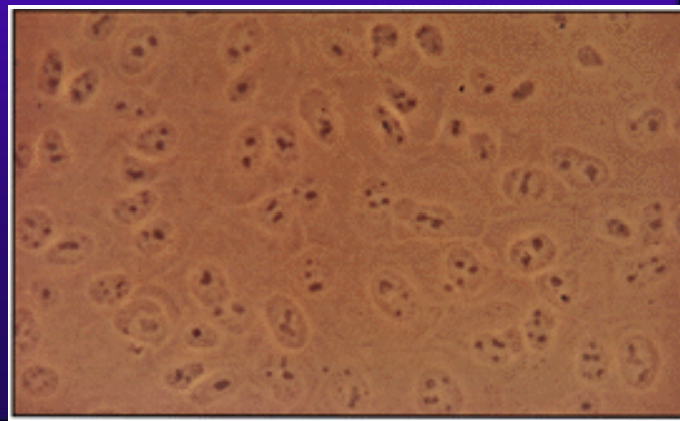
# Common cell lines

- Human cell lines
- -MCF-7 breast cancer
- HL 60 Leukemia
- HEK-293 Human embryonic kidney
- HeLa Henrietta lacks
- 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 ceses 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 and cell lysis
- 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

# Basic equipments used in cell culture

- Laminar cabinet-Vertical are preferable
- Incubation facilities- Temperature of 25-30 C for insect & 37 C for mammalian cells, co2 2-5% & 95% air at 99% relative humidity. To prevent cell death incubators set to cut out at approx. 38.5 C
- Refrigerators- Liquid media kept at 4 C, enzymes (e.g. trypsin) & media components (e.g. glutamine & serum) at -20 C
- Microscope- An inverted microscope with 10x to 100x magnification
- Tissue culture ware- Culture plastic ware treated by polystyrene

# 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)
- Continous, slow growing line
- Continous, rapidly growing lines
- Lines which may be contaminated
- Virus producing lines

# 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 104cells/ml to initiate subculture of rapidly growing cells & a higher concentration of 105cells/mlfor slowing growing cells

Thanks