UNIT – IV
Cell culture techniques

Cell culture techniques:

- In vitro cultivation and maintenance of cells outside of their natural environment
  - Design and functioning of tissue culture laboratory
  - Culture media
  - Essential components and preparation
  - Cell viability testing

Cell culture

- The term "cell culture" now refers to the culturing of cells derived from **multicellular eukaryotes**. Animal or plant cells removed from tissues will continue to grow if supplied with **a favorable artificial environment of appropriate nutrients and conditions**. When carried out in a laboratory, the process called **Cell Culture**.

- The cells may be **removed from the tissue** directly and disaggregated by **enzymatic** or mechanical means before cultivation, or they may be derived from a cell line

- It occurs ***in vitro*** (in glass) as opposed to ***in vivo*** (in life).

- The culture process allows **single cells to act as independent units**, much like a microorganism such as a bacterium or fungus. The cells are capable of dividing.

- They increase in size and, in a batch culture, can **continue to grow** until limited by some culture variable such as nutrient depletion.

- Cell culture was first successfully undertaken by **Ross Harrison in 1907** and **Roux in 1885** for the first time maintained **embryonic chick cells** in a cell culture.

Cell culture Cont…

- Cultures normally contain cells of **one type** or mixed cultures. The cells in culture may be genetically **identical (homogenous population)** or may show some genetic **variation (heterogeneous population)**.
- **A homogenous population** of cells derived from a **single parental cell** is called a **clone**. Therefore, all cells within a clonal population are genetically **identical**.
- Freshly isolated cultures from **mammalian tissues** are known as **primary cultures** until sub-cultured. At this stage, cells are usually still closely represent the **parent cell types** as well as in the **expression of tissue specific properties**.
- After several **sub-cultures** onto fresh media, the cell line will either die out or '**transform**' to become a continuous **cell line**.
- Such cell lines show many alterations from the primary cultures including change in **morphology, chromosomal variation and increase in capacity** to give rise to tumors in hosts with **weak immune systems**.
- **Animal cells** can be grown either in an unattached **suspension culture or attached to a solid surface**. Suspension cultures have been successfully developed to quite large bioreactor volumes, with successful production of viruses and **therapeutic proteins.**

Culture 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 components of culture media:

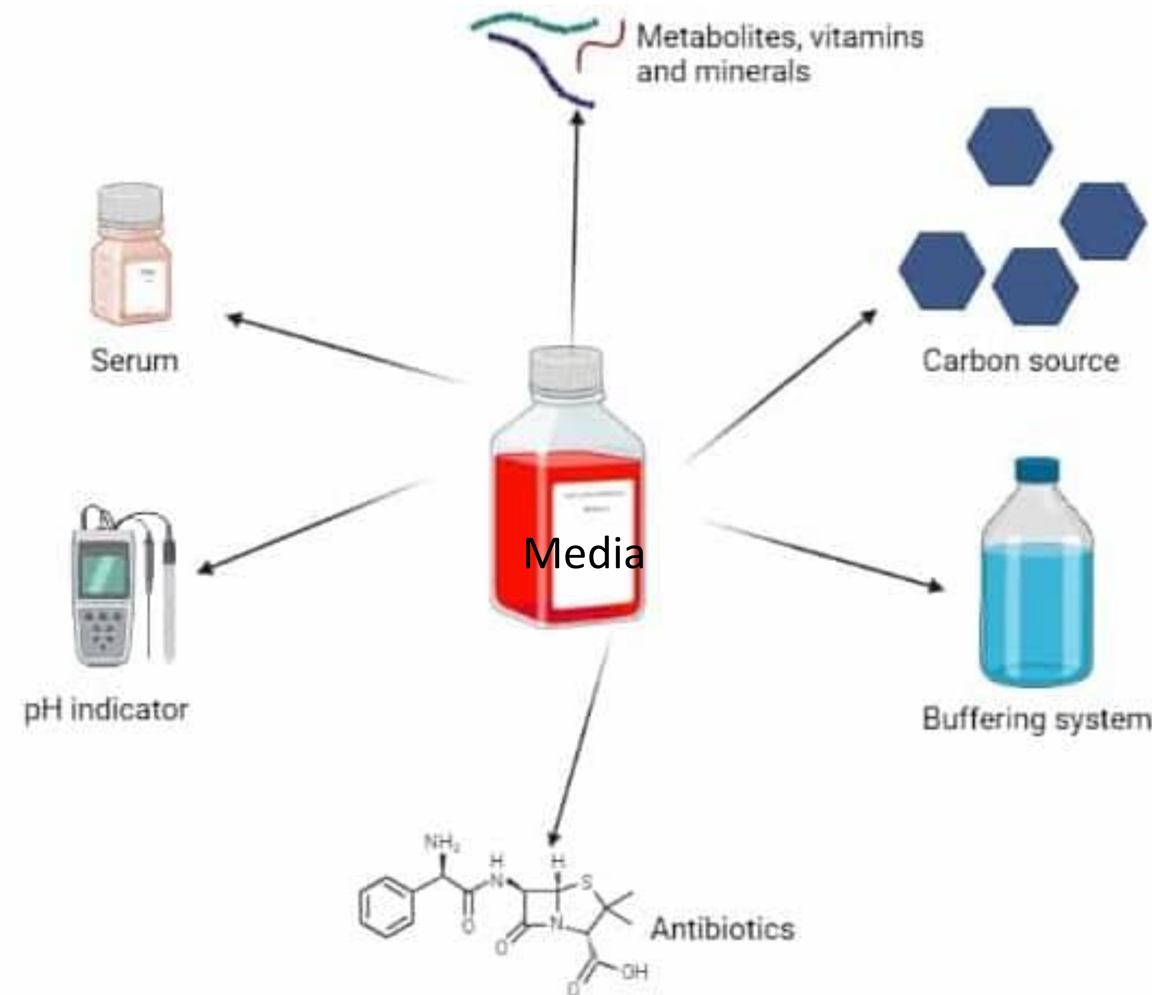
- Buffering system
- Amino acids
- Vitamins
- Carbohydrates
- Inorganic salts
- Proteins and peptides
- Fatty acids and lipids
- Trace elements
- Hormones and growth factors
- Antibiotics

Culture media

Culture Media Components

Culture Media Components	Examples
Essential ions	Na+, K+, Ca2+, Mg2+ CI, SO2, H.PO-, HCO
Trace elements:	Fe, Zn2+, Cd2+, Cu2+, Mn2+, Ni2+, Sn2+, Mo_2O_2, SeO3, SiO
Essential amino acids:	Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val
Essential fatty acids:	Linoleic acid, linolenic acid
Metabolic substrates:	D-Glucose; in some cases also D-galactose
Vitamins and cofactors:	Biotin, cobalamine, folate, nicotinamide, pantothenate, pyridoxal, thiamine, riboflavin, a-tocopherol, retinol, ascorbate, lipoic acid
Hormones:	Insulin, hydrocortisone, triiodothyronine; in some cases also progesterone, estradiol
Specific factors stimulating survival, growth, and/or differentiation:	With broad specificity, e.g., EGF, FGF, IGF, LIF, and PDGF; or with narrow specificity, e.g., interleukins, chemokines, and neurotrophins

Essential components and preparation



Culture Media Components Cont…

Antibiotics and antimycotics

- Unless good sterile conditions can be maintained (e.g., using laminar flow hoods) it is necessary to incorporate antibiotics and antimycotics into the media.
- A wide range of suitable preparations are available from relatively specific antibiotics, e.g., penicillin/streptomycin solutions, to broader spectrum antibacterial/antimycotic agents such as kanamycin or amphotericin B.

Glutamine and amino acids

- In addition to buffering the medium, there are other growth requirements including amino acids, the requirement for which may vary with cell culture type.
- Commonly the necessary amino acids include cysteine and tyrosine, but some non-essential amino acids may be needed. Glutamine is also required by most cell lines and it has been suggested that cultured cells use glutamine as an energy and carbon source in preference to glucose, although glucose is present in most defined media.

Supply and preparation of culture media

- The choice of culture media used will depend on the type of primary cell, cell line, and the incubation conditions. However, it is best to start with the medium recommended by the original supplier of the cells.

Types of media

- Although initial cell culture experiments were performed using natural media obtained from tissue extracts and body fluids, the need for standardization, media quality, and increased demand led to the development of defined media.

- The basic classes of media are basal media, reduced-serum media, and serum-free media, which differ in their requirement for supplementation with serum.

  - **Basic media**
  - **Serum**
  - **Basal Media**
  - **Reduced-Serum Media**
  - **Serum-Free Media**

Types of media Cont…

Basic media

The most basic media are balanced salt solutions (BSS),

- Eg., phosphate-buffered saline (PBS), which may be used for washing cells and for short incubations in suspension.

More complex defined media are used for growth and maintenance.

Defined media can also vary in complexity, by the addition of a number of constituents

- Eg., from Eagle's minimum essential medium (MEM) which contains essential amino acids, vitamins and salts, to McCoy's medium, which contains a larger number of different amino acids, vitamins, minerals and other extra metabolites (such as nucleosides).

Types of media Cont…

Serum

- Serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the culture of cells in basal media.
- In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients, and trace elements into the cell.
- However, using serum in media has a number of **disadvantages** including high cost, problems with standardization, specificity, variability, and unwanted effects such as stimulation or inhibition of growth and/or cellular function on certain cell cultures.

Basal Media

- The majority of cell lines grow well in basal media, which contain amino acids, vitamins, inorganic salts, and a carbon source such as glucose, but these basal media formulations must be further supplemented with serum.

Reduced-Serum Media

- Another strategy to reduce the undesired effects of serum in cell culture experiments is to use reduced-serum media.
- Reduced-serum media are basal media formulations enriched with nutrients and animal-derived factors, which reduce the amount of serum that is needed.

Serum-Free Media

- Serum-free media (SFM) circumvents issues with using animal sera by replacing the serum with appropriate nutritional and hormonal formulations.
- Serum-free media formulations exist for many primary cultures and cell lines, including recombinant protein producing lines of Chinese Hamster Ovary (CHO), various hybridoma cell lines, the insect lines Sf9 and Sf21 (Spodoptera frugiperda), and for cell lines that act as hosts for viral production (e.g., 293, VERO, MDCK, MDBK), and others.
- 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.

Advantages	Disadvantages
• Increased definition • More consistent performance • Easier purification and downstream processing • Precise evaluation of cellular functions • Increased productivity • Better control over physiological response • Enhanced detection of cellular mediators	• Requirement for cell type-specific media formulations • Need for higher degree of reagent purity • Slower growth

Types of media Cont…

Eagle's medium and derivatives thereof:

BME (Basal Medium Eagle's)

EMEM (Eagle's Minimal Essential Medium)

DMEM (Dulbecco's Modified Eagle's Medium)

GMEM (Glasgow's Modified Eagle's Medium)

Media designed for use with serum:

Liebovitz

Trowell

Williams

Media from Roswell Park Memorial Institute (RPMI):

RPMI 1630

RPMI 1640

Media designed for a specific cell line for use without serum:

CMRL 1060

Ham's F10 and F12

TC 199

IMDM (Iscove's Modified Dulbecco's Medium)

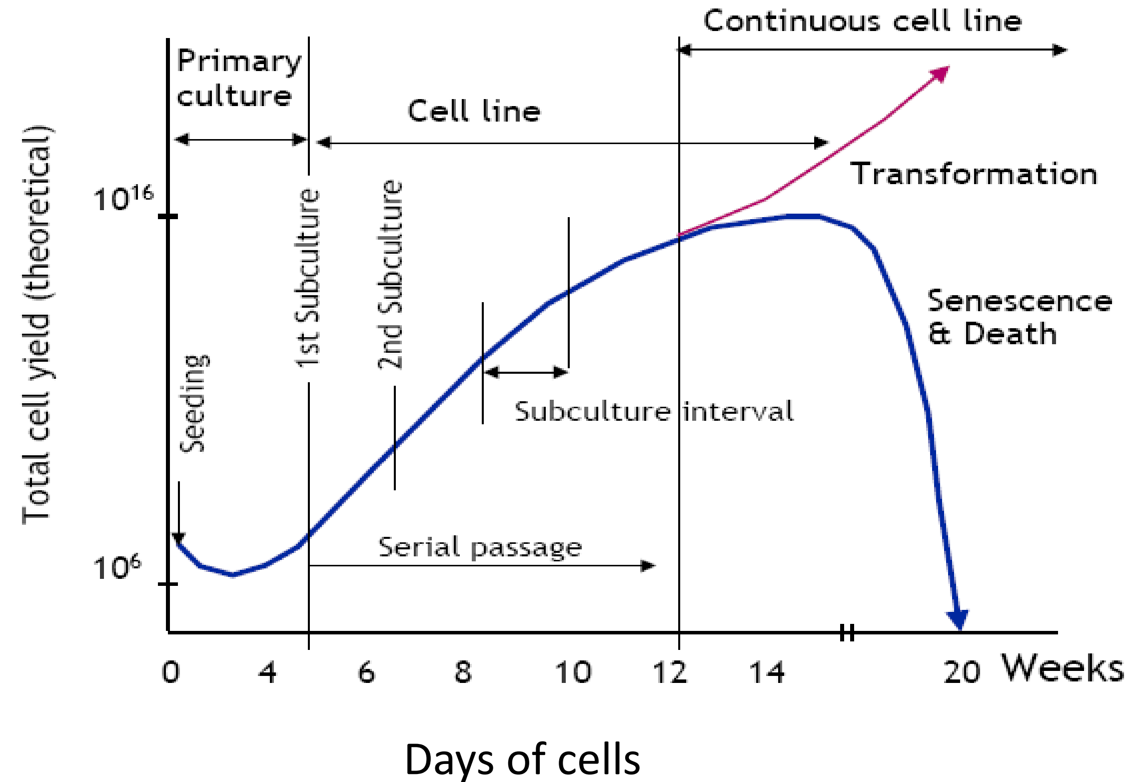
Choice of media depends on the type of cell being cultured

Types of Cell culture

1. Primary cell culture
    - Adherent cell culture
    - Suspension cell culture

2. Secondary cell culture

3. Cell line
    - finite cell line
    - continuous cell line



Types of Cell culture Cont…

Primary cell Culture

- The cells are isolated directly from the parent/animal tissue (kidney, liver, heart), Proliferated under the appropriate conditions until they occupy all of the available substrate (i.e., reach confluence), Separated by enzymatic or mechanical method

Primary cell culture is further divided into:

1. **Adherent cell**
   - Those cells which **attach to the surface** of culture flask, Form a **monolayer**, They have to be detached from the surface before they get to be subcultured, Growth limited to the surface area

2. **Suspension cell**
   - These cells do not get attached to the surface of culture flask, They are free floating, Cells in blood stream, Growth is limited to the concentration of cells

Secondary cell culture

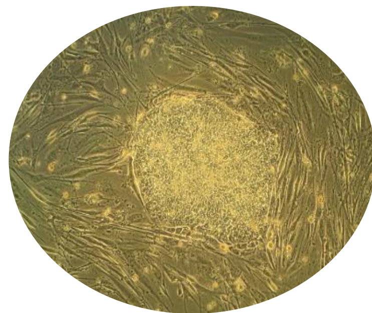
- When primary cell culture occupy all of the available substrate (i.e., reach confluence), At this stage, the cells have to be **subcultured** (i.e., passaged) by **transferring** them to a new vessel with fresh growth medium to provide more room for **continued growth then it is secondary culture**

Types of Cell culture Cont…

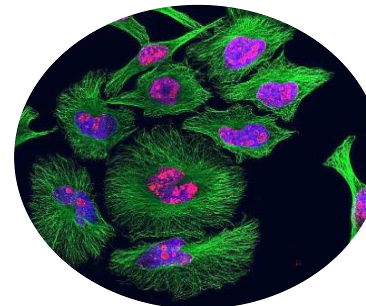
Cell Line

FINITE CELL LINE	CONTINUOUS CELL LINE
Limited life span	Grow indefinitely
Go through a limited number of generations	Grow either in monolayer or in suspension
Properties contact inhibition anchorage dependence	Properties absence contact inhibition absence anchorage dependence
Less growth rate	High growth rate
Doubling time (24 to 94 hrs)	Doubling time (12 to 24 hrs)

Eg: H9 Cell Line of Human Embryonic Stem Cells



Eg: Immortal HeLa Cells



Cell culture Maintenance

1. **pH:**
   - Most cell lines grow well at **pH 7.4**. Although the optimum pH for cell growth varies relatively little among different cell strains, some normal fibroblast lines perform best at pH 7.4 to pH 7.7, and transformed cells may do better at pH 7.0 to pH 7.4.

2. **Buffering:** Culture media must be buffered under two sets of conditions:
   - a) **Open dishes**, where the evolution of CO2 causes the **pH to rise**
   - b) **Overproduction** of CO2 and lactic acid **in transformed** cell lines at **high cell concentrations** (pH fall)

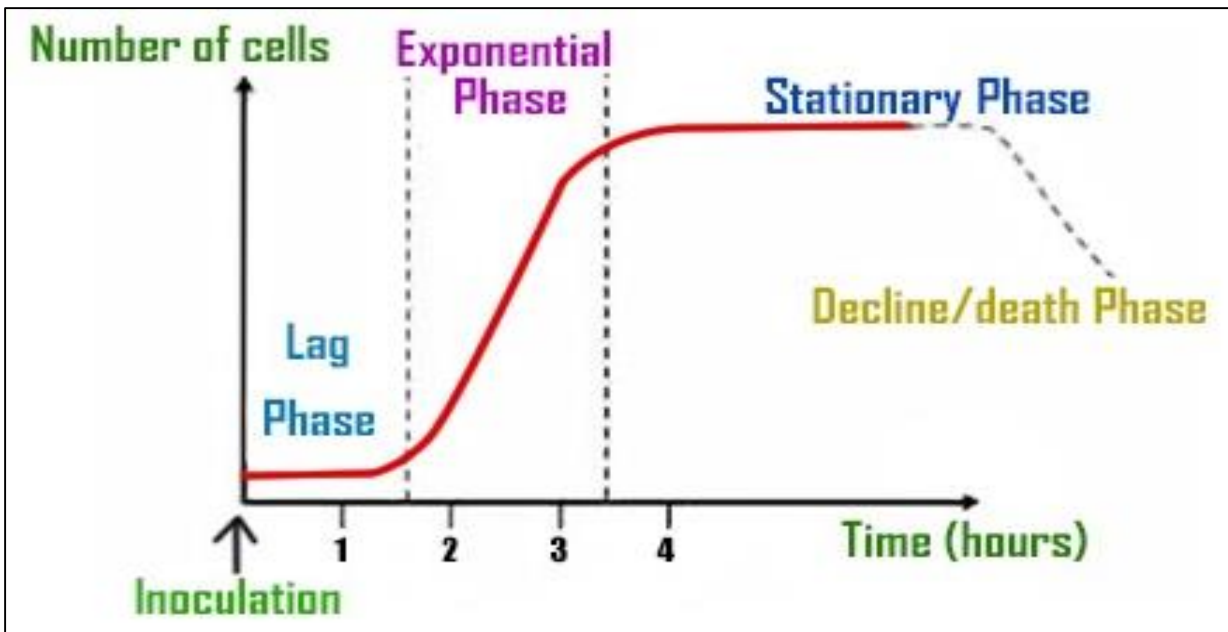
3. **Temperature:**
   - The temperature recommended for most **human and warm-blooded animal cell lines is 37°C**, closely to body heat. (Generally set a little lower for safety, because overheating may become major problem than under heating of cell lines)

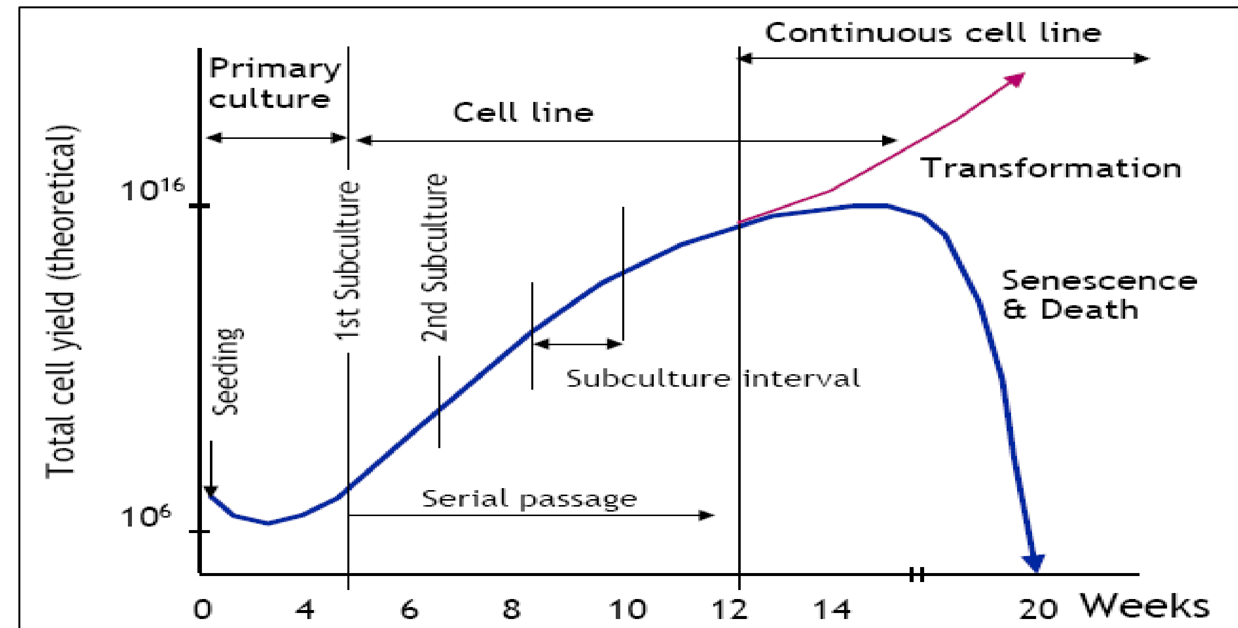
4. **Media:**
   - Although many cell lines are still propagated in medium **supplemented with serum**, in many instances cultures may now be **propagated in serum-free media**.
   - Media that have been produced commercially will have been tested for their capability of **sustaining the growth of one or more cell lines**.

5. **Growth curve:**

A growth curve gives **three parameters of measurement**

1. **Lag phase**: Cell proliferation is initiated after subculture (indicating **cells to adapt in different conditions**)
2. **Exponential (growth) phase**: Indicating the **growth promoting** capacity of the medium
3. **Maximum cell concentration** attainable indicating whether there are **limiting concentrations** of certain nutrients. In cell lines whose growth is not sensitive to density (e.g., **continuous cell lines**), the terminal cell density indicates the total yield possible and usually reflects **the total amino acid or glucose concentration**.

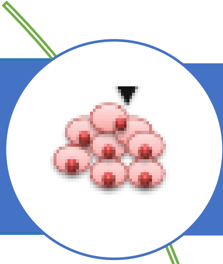




Cell culture workflow

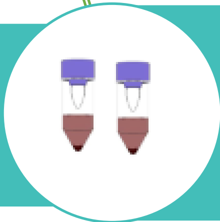
Cell culture workflow in 4 simple steps

ISOLATE



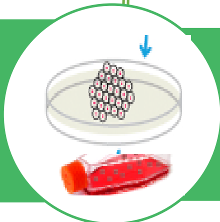
• Separate fresh tissue of interest. • Treat tissue with enzyme(s) (e.g., trypsin, collagenase, protease) and/or mechanically to isolate cells. • Wash, count, and seed cells.

VERIFY



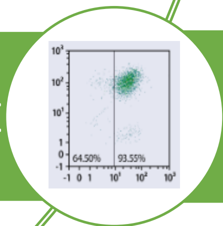
• Examine cells under a brightfield microscope to assess their growth state, attachment to culture vessels/flasks, and to check for any signs of infection. • Monitor cells for the following days until they reach confluence. • Verify isolated cell types by their morphology and expressed biomarkers.

CULTURE

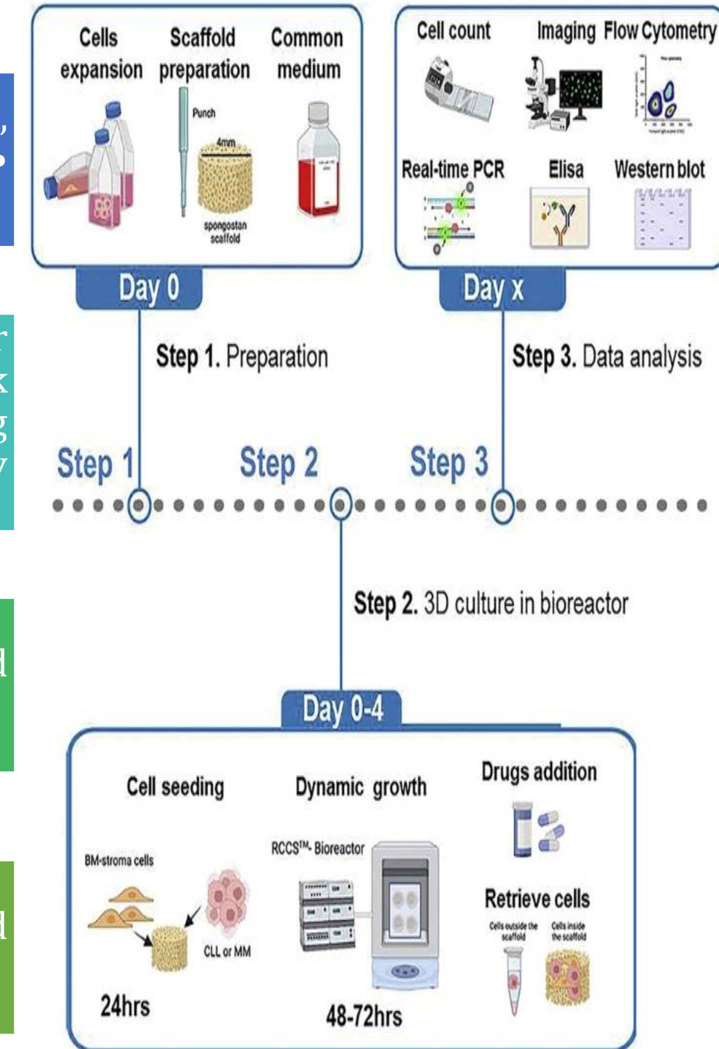


• Passage cells to propagate the cell line. • Make master and working cell banks. • Immortalize cells if necessary.

INVESTIGATE



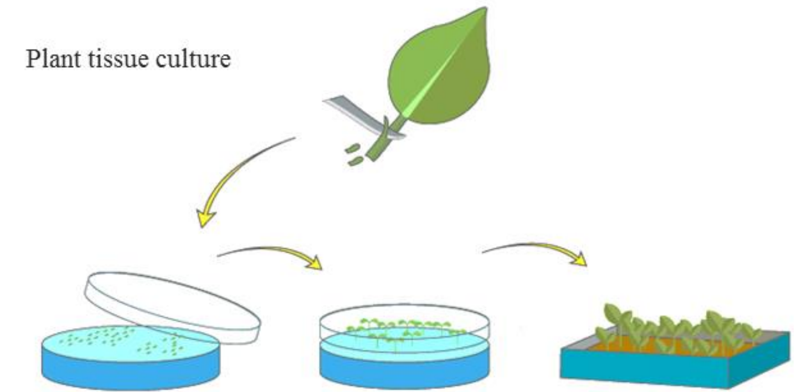
• Plan and execute experiments. • Keep monitoring cell state and possible infections using a brightfield microscope



Characteristics of tissue culture

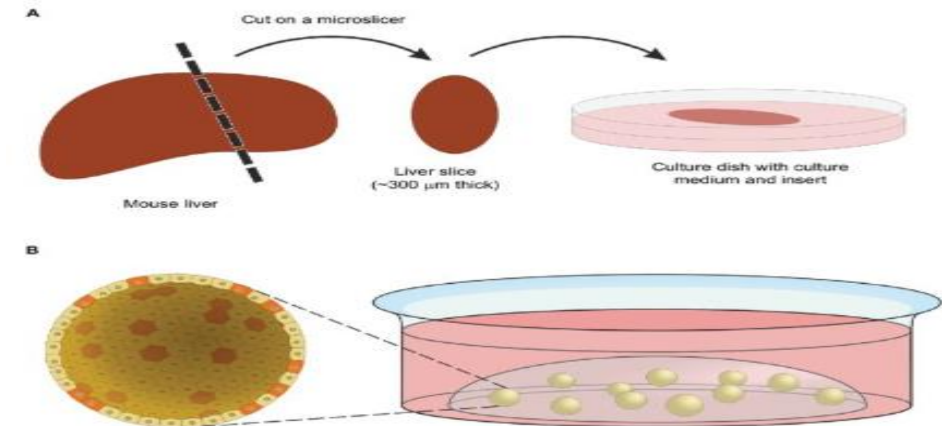
Tissue culture

- Tissue culture is used as a generic term to include the **in vitro cultivation of organs, tissues and cells**. Originally, the term is not limited to animal cells, but includes the *in vitro* cultivation of plant cells.
- Tissue culture can be subdivided into **three major** categories; organ culture, explant culture, and cell culture.



Organ culture

- Organ culture refers to a **three-dimensional culture** of tissue retaining some or all of the histological features of **the tissue in vivo**.
- The **whole organ or part of the organ is maintained** in a way that allows **differentiation and preservation of architecture**, usually by culturing the tissue at the liquid-gas interface on a grid or gel.



(A) Preparation of an organotypic tissue slice.
(B) Organoid culture, stem cells provided with stem cell, and niche factors form organoids in a three-dimensional matrix (in general hydrogel)

Characteristics of tissue culture

Tissue Culture - Concepts (continued)

➤ **Immortalized cell line:**

cell line able to **grow continuously** (i.e. over an extended time period); these arise from tumor cells or embryonic tissues. Therefore, they have specific differences in **gene expression** (e.g. telomerase) than primary cells.

Advantages:

✓ **Easier to maintain in vitro** than primary cells or cell lines;

✓Potentially lead to **highly reproducible** results;

though:

× Altered characteristics as compared to their tissue of origin or following interaction with different cell environments (need for culture condition standardization, number of passages specified, ...).

Note:

Some primary cells can be transformed into immortalized cell lines by uptake of new genetic material, which is associated with genetic instability and can lead to immortalization, aberrant growth control and malignancy.

Adult or embryonic tissue

- Cultures can be derived from adult tissue or from embryonic tissue.
- Cultures derived from embryonic tissue generally survive and grow better than those taken from adult tissue.
- Tissues from almost all parts of the embryo are easy to culture, whereas tissues from adult are often difficult or even impossible to culture.
- This presumably reflects the lower level of specialization and presence of replicating precursor or stem cells in the embryo.
- Examples of widely used embryonic cell lines are the various 3T3 lines (mouse embryo fibroblasts) and MRC-5 and other human fetal lung fibroblasts.
- Mesodermally derived cells (fibroblasts, endothelium, myoblasts) are also easier to culture than epithelium, neurons or endocrine tissue but this may reflect the extensive use of fibroblast cultures during the early years of the development of culture media together with the response of mesodermally derived cells to mitogenic factors present in serum.

Embryonic stem cells

- Embryonic stem cells (ES-cells) from the embryo during the blastocyst stage of development.
- These cells can be grown in culture for many generations and are of particular interest because they can be manipulated in culture and then re-introduced into embryos.

Normal or neoplastic tissue

- Normal tissue usually gives rise to cultures with a finite lifespan while cultures from tumors can give continuous cell lines, although there are several examples of continuous cell lines (MDCK dog kidney, 3T3 fibroblasts) which are non-tomorigenic.
- Normal cells will generally grow as an undifferentiated stem cell or precursor cell and the onset of differentiation is accompanied by a cessation in cell proliferation which may be permanent.
- Some normal cells, e.g., fibrocytes or endothelium, are able to differentiate and still dedifferentiate and resume proliferation and in turn redifferentiate, while others, e.g., squamous epithelium and many hemopoietic cells, once initiated into differentiation are incapable of resuming proliferation.

Organ culture or cell culture

- whole tissue or organ which could be maintained in vitro for only very short periods.
- Nowadays it is more usual to grow specific cell types from tissues, although there are still some situations where it is necessary to grow a whole organ (or a part of it).

Adherent or suspension culture

- Cells may grow as an adherent monolayer or in suspension.
- Adherent cells are said to be anchorage-dependent and attachment to a substratum is a prerequisite for proliferation.
- They are generally subjected to contact inhibition, which means they grow as an adherent monolayer and stop dividing when they reach such a density that they touch each other.
- Most cells, with the exception of mature hemopoietic cells and transformed cells, grow in this way.

Primary cultures or continuous cell lines

- If remove tissue from an embryo, dissociate it into a suspension of single cells, and plate them out onto a culture dish, a series of characteristic events occurs.
- Firstly, cells are in a lag phase, usually no more than 1-2 days in length, during which there is little or no increase in cell number. During this time, cells are "conditioning" the medium, undergoing internal cytoskeletal and enzyme changes and adjusting to the new medium

Morphology of Cell in Culture:

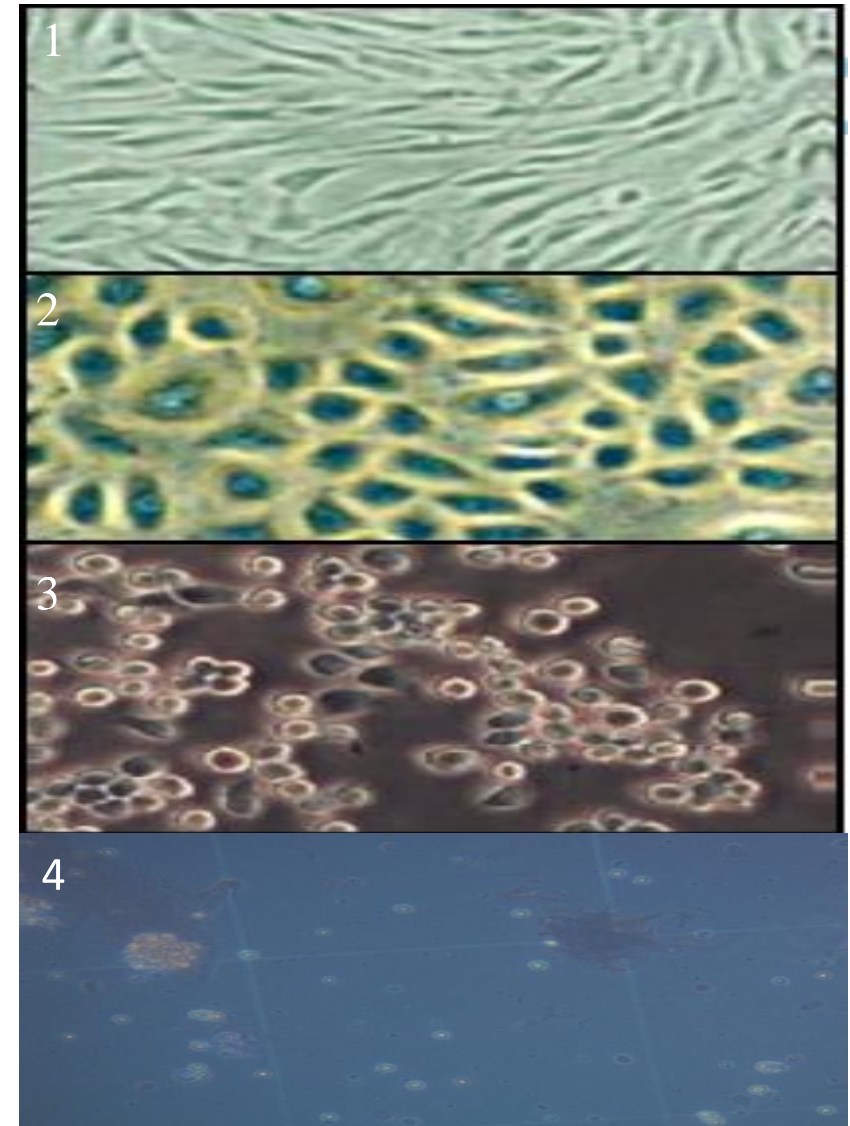
Cells in culture can be divided into three basic categories based on their shape and appearance (morphology).

1. **Fibroblastic** (or fibroblast-like) cells: they are bipolar or multipolar, have elongated shapes, and grow attached to a substrate.

2. **Epithelial-like cells:** they are polygonal in shape with more regular dimensions, and grow attached to a substrate in discrete patches.

3. **Lymphoblast-like cells**: they are spherical in shape and usually grown in suspension without attaching to a surface.

4. **Lymphocytes:** Human peripheral blood mononuclear cells (PBMCs)



Applications of Cell Culture

Model research studies:

- 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

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

Cell culture problems

As cells generally continue to divide in culture, they generally grow to fill the available area or volume.

This can generate several issues:

1. Nutrient depletion in the growth media.
2. Changes in the pH of the growth media.
3. Accumulation of apoptotic/necrotic (dead) cells.
4. Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing which known as contact inhibition.
5. Cell-to-cell contact can stimulate cellular differentiation.
6. Genetic and epigenetic alterations, with a natural selection of the altered cells potentially leading to overgrowth of abnormal, culture-adapted cells with decreased differentiation and increased proliferative capacity.
7. The choice of culture medium might affect the physiological relevance of findings from cell culture experiments due to the differences in the nutrient composition and concentrations.

Cell viability assay

- Cell-based assays are often used for screening collections of compounds to determine if the test molecules have effects on cell proliferation or show direct cytotoxic effects that eventually lead to cell death.
- For properly run experiments, it may be necessary to count the cell numbers before, after and even during the experiment.
- Day to day maintenance of cell lines also requires quantitative assessment of cell growth so that optimum cell densities for sub-culturing and storing can be determined.
- It is important to know how many viable cells are remaining at the end of the experiment.

Methods of cell viability assay

- **Tetrazolium reduction (MTT assay),**
- **Resazurin reduction (alamar blue assay and Trypan blue)**
- **Protease markers,**
- **ATP detection.**

Quantitation of cells in cell culture

- The methods available for determining cell growth into two sub-groups.

These are:

- **Direct methods:** quantitation of cells in culture Counting chambers A hemocytometer (Improved Neubaur) is the simplest and cheapest method of counting.

- **Coulter counters** Electronic particle counters consist of two electrodes separated by a small orifice. If a potential is applied to the electrodes, current will pass between them through the buffer in the orifice. The amount of current will be dependent upon the conductance (dielectric constant) of the buffer.

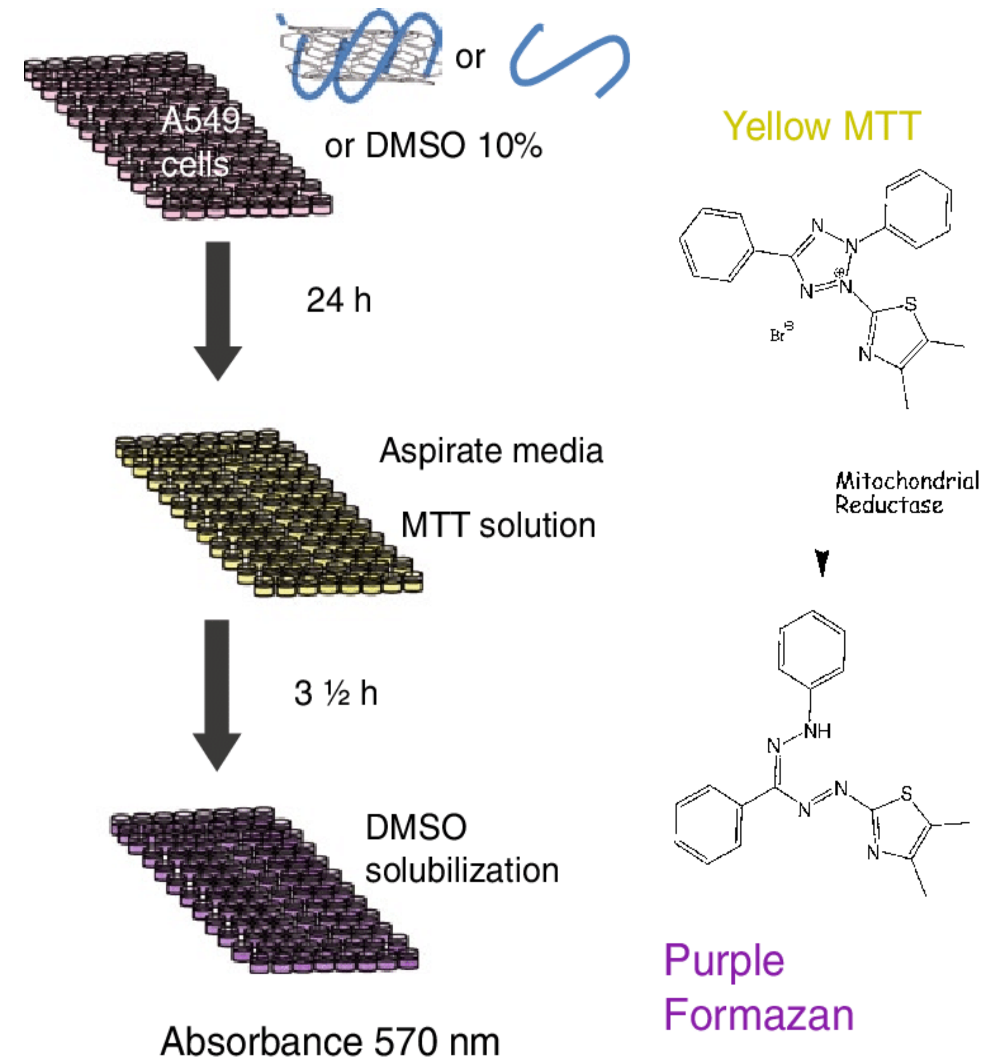
- **Indirect methods for determining cells in culture** Other methods of quantitation such as radioisotope labelling and estimation of total DNA or protein are used less frequently. They are useful when cells are grown in microwell plates or as hanging drop cultures.

In the direct method, cell numbers are determined directly either by counting using a counting chamber or by using an electronic particle counter. In the indirect methods

Cell viability testing Cont…

MTT assay

- This is a colarimetric assay that measures the reductiona* yellow 3 (4,5- dimethythiazoI2-y)-2,5-diphenyI tetrazplium bromide (MW) by mitochondrial succinate dehydrogenase.
- The MTT enters the cells and passes into the mitochondria where it is reduced to an insoluble, colDured (dark purple) farmazan product.
- The cells are then solubilised with an organic solvent (eg. isopropanal) and the released, solubtlised formazan reagent is measured spectrophatometricaily.
- Since reduction of MTT can only occur in metabolically active cells the level of activity is a measure of the viability of the cells



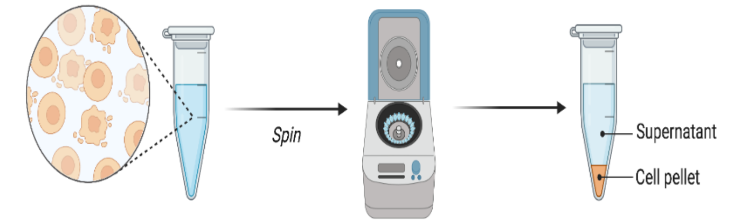
Trypan blue exclusion

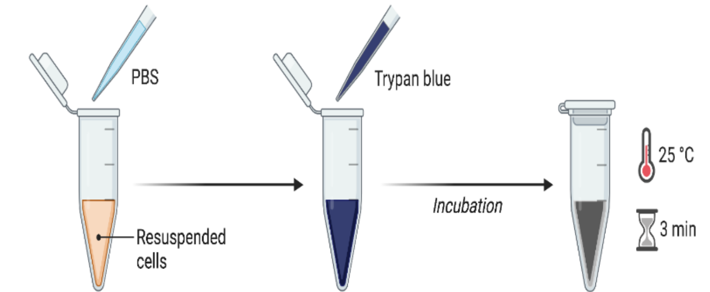
This is a rapid test for gross damage which is conveniently combined with determining cell number.

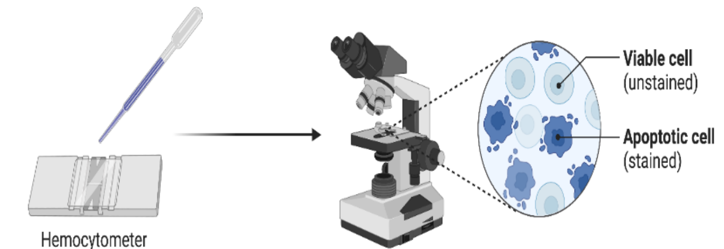
" Mix a small aliquot of cell suspension with an equal volume of 0.4% trypan blue solution. " Then, within 1-5 min, introduce the suspension into a hemocytometer chamber.

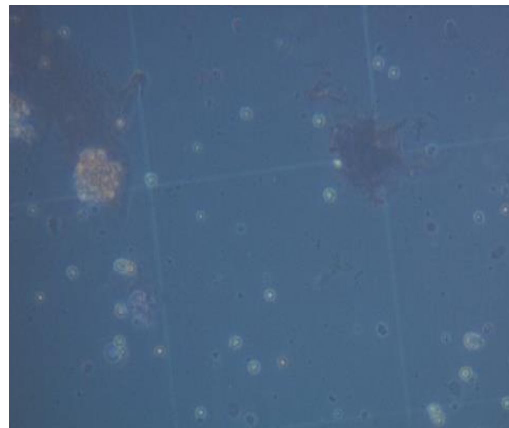
" Non-viable cells appear blue, with the nucleus staining particularly darker. " Count the viable (unstained) cells and the total number of cells.

" Express the number of viable cells as a percentage of the total. Calculate the cell number after multiplying by 2 to allow for the dilution with dye



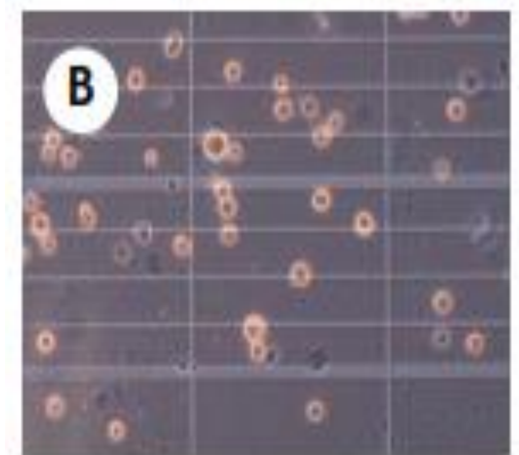






Cell viability testing Cont…

Alamar BIue



- Anther measure of cell proliferation is the metabolic activity of a population of cells.
- Tetrazolum salts or Alamar BIue are compounds that become reduced in the environment of metabolically active cells, forming a Formosan dye that subsequently changes the color of the media.
- This is caused by increased activity of the enzyme lactate dehydrogenase during proliferation
- The absorption of the media-containing dye solution can be read using a microplate reader in low- or high-throughput configurations

LDH leakage

- Leakage of cytosolic enzymes such as lactate dehydrogenase (LDH) provides a test that is similar in sensitivity to dye exclusion and can be more accurately quantitated but takes rather longer to perform.
- Centrifuge an aliquot of cell suspension at a speed suitable for sedimenting all the cells without causing cell damage (e.g., 50 x g for 2 min)
- Transfer the supernatant medium to a clean tube and keep on ice
- Add an equal volume of solubilizing agent to the cell pellet and vortex mix. Samples should be kept on ice and the assays performed on the same day.

Flow Cytometry

- Flow cytometry is the measurement of cells in a flow system, which delivers the cells singly, past a point of measurement.
- In practice the name refers to the instruments in which light is focused at the point of measurement.
- Typically light scatters at two different angles and from one to six or more fluorescence will be measured

Cell viability

- determination When cells are freshly isolated from a tissue or confluent monolayers are subcultured, the proportion of living, or viable, cells should be determined before they are used.
- This is most often determined by assessment of membrane permeability, under the assumption that a cell with a permeable membrane has suffered severe, irreversible damage.

Application Of Viability Assay

- To detect cytotoxic or growth inhibitory lymphokines
- To detect mammalian cell survival and proliferations
- To diagnose male fertility
- To screen drugs

Cell Culture Techniques:

- Key Steps: Establishing cell lines, providing suitable culture media, passaging cells, and inducing cell differentiation.
- Applications: Fundamental research, drug development, biotechnology.

Thank you

Acknowledgement:

- JSS College of Arts, Commerce and Science (Autonomous)
- Department of Zoology

Dr.Ramyashree M

Dr. Navyashree B

Dr Nijagal B.S.

Akhila M P

Mrs. Sangeetha Kademani

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