Cell Culture

Cell culture is a technique by which the behavior of cells can be studied independent of whole organism. In this method cells are isolated from an animal or plant tissues and grown under controlled, favorable artificial conditions.

The process of cell culture includes

- The removal of cells from an animal or plant by enzymatic or mechanical means or may be derived from a cell line or cell strain that has already been established.
- Sub culturing or passaging (or splitting) of these cells refers so that the cells are alive and grow under cultured conditions for extended period of time.

Broadly, there are 3 types of cell culture procedures:

- **Primary cell culture:** Primary cells are extracted straight from the tissue and processed to establish them under culture conditions.
- **Secondary cell culture:** Sub-culture of primary cells results in secondary culture. Although it exhibits a few features of established cell line (below) it does not divide indefinitely.
- **Cell Line:** A cell culture developed from a single cell and having uniform genetic composition is called a cell line. On the basis of the life span, the cell lines are categorized into two types:
    - **Finite cell lines –** Finite cell lines have limited life span of about 20 – 80 population doublings. The growth is slow and the typical doubling time is 24 – 96 hours.
    - **Continuous cell lines –** Continuous cultures are comprised of a single cell type that can be serially propagated in culture for a limited number of cell divisions. To grow them indefinitely, continuous cell lines are transformed by viral oncogenes or by chemical treatments. They exhibit ploidy (aneuploidy or heteroploidy). The growth rate is rapid and the typical doubling time is 12 – 24 hours.

Primary Cell Culture

The source of primary cultures is excised animal tissue. The excised tissue is subjected to enzyme treatment and the dissociated cells are cultured under the appropriate conditions in culture medium until they reach adequate numbers. The isolated primary cells are of two types:

Adherent cells/ Anchorage Dependent cells - Cells that require attachment for growth are called anchorage dependent cells. The adherent cells are mostly derived from tissues of organs (such as kidney) where they are immobile and embedded in connective tissue.

Suspension cells/Anchorage Independent cells - Cells which do not require attachment for growth or do not attach to the surface of the culture vessels are called as anchorage independent cells/suspension cells. All suspension cultures are derived from cells of the blood system (as these cells are suspended in plasma in vitro e.g. lymphocytes).

The most popular primary cells used in research are **epithelial cells, fibroblasts, keratinocytes, melanocytes, endothelial cells, muscle cells, hematopoietic and mesenchymal stem cells**. The cultures are **initially heterogeneous** (represents a mixture of cell types present in the tissue) and can be maintained in vitro only for a limited period of time. Primary cells may be manipulated for indefinite subculture through an *in vitro* process called transformation. Transformation can occur spontaneously or can be chemically or virally induced. When a primary culture undergoes genetic transformation (provided with appropriate fresh medium and space), they divide indefinitely and become immortalized.

Culture of Primary Cells

Growth requirements

Primary cells can be grown either in **suspension** or **adherent** cultures. For the primary cells that are anchorage-dependent, adherent cells (such as solid tissues) require a surface to grow properly *in vitro*. These cells are mostly cultured in a flat un-coated plastic vessel, but sometimes a micro-carrier, which may be coated with extracellular matrix (such as collagen and laminin) components to increase adhesion properties and provide other signals needed for growth and differentiation. The cell culture media is composed of a basal medium supplemented with appropriate growth factors and cytokines. Cells are grown and maintained at an appropriate temperature and gas mixture (typically, 37 °C, 5% CO_2 for mammalian cells) in a cell incubator. The culture conditions widely vary depending up on the cell type. Growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients depending up on the cell types.

During establishment of primary cultures, it is essential to include an antibiotic like- a mixture of gentamicin, penicillin, streptomycin and amphotericin B in the growth medium to inhibit contamination introduced from the host tissue. However, long-term use of antibiotics is not recommended. Use of aseptic technique is also necessary.

Cellular confluence

Cellular confluence generally refers to the percentage of the culture vessel inhabited by attached cells. For example, 100% cellular confluence means the surface area is completely covered by cells, whereas 50% confluence means roughly half of the surface is covered. It is an important and essential parameter to track and assess in primary cell culture as various cell types require different confluence end points, at which point they need to be sub-cultured.

Maintenance and Subculture

The maintenance phase of cells begins when isolated cells are attached to the surface of the culture dish. Usually attachment takes about 24 hours after initiation of the culture. When cells reaches to a desired percent of cellular confluence and are actively proliferating, it is time to subculture. It is the best time to subculture primary cell cultures before reaching 100% confluence, since post-confluent cells may undergo differentiation and exhibit slower proliferation after passaging.

Anchorage-dependent cells grow in monolayers and need to be sub-cultured at regular intervals with appropriate culture medium to maintain exponential growth. Sub-cultivation of monolayers involves the breakage of both inter- and intracellular cell-to-surface bonds. Most adherent primary cells require the digestion of their protein attachment bonds or separation from the monolayer or relevant tissue with a low concentration of a proteolytic enzyme such as trypsin/EDTA. After the cells dissociation and dispersion into a single-cell suspension, they are counted and diluted to the appropriate concentration and transferred to fresh culture vessels (the composition of the media varies depending up on the cell types) where they will reattach and divide.

Cell counting

Hemocytometer is mostly commonly used for estimation of cell number and determination of cell viability (exclusion dye such as Trypan Blue or Erythrosin B may be used).

Automated cell counters that provide accurate and fast viable cell count are also available

NOTE- A hemocytometer is a fairly thick glass microscope slide with a rectangular indentation that creates a chamber. The chamber is engraved with a laser-etched grid of perpendicular lines and the device is

carefully crafted. The area bounded by the lines and the depth of the chamber is known. Therefore it is possible to count the number of cells in a specific volume of fluid, and thereby calculate the concentration of cells in the fluid overall.

Cryopreservation and recovery

Cryopreservation is the process to preserve structurally intact living cells using low temperatures. In case of human cells it is achieved with the use of a cryoprotectant, such as DMSO or glycerol (at correct temperature and with a controlled rate of freezing). Cryopreservation can be achieved in a mixture of 80% complete growth medium supplemented with 10% FBS and 10% DMSO for most primary cells. The freezing process is done very slowly, at a rate of -1°C per minute, to minimize the formation of ice crystals within the cells. The frozen culture needs to be stored in the vapor phase of liquid nitrogen (-196°C), or below -130°C.

Thawing cryopreserved cells is a rapid process. It is accomplished by immersing frozen cells in a 37°C water bath for about 1 to 2 minutes. Precaution should be taken as they are extremely sensitive to damage during recovery from cryopreservation. When initiating a culture of cryopreserved primary cells, it is essential to remove the spent media once the cells are attached.

Challenges faced during primary cell culture:

- Contamination: Contamination of primary tissue when carried over to culture.
- Shifts in pH: This may be caused due to incorrect salt in the culture medium, bacterial or fungal contamination, insufficient bicarbonate buffering, incorrect carbon dioxide tension etc.
- Optimum adherence: Insufficient or absence of attachment factors in the medium or contamination or overly trypsinized cells.
- Slow growth: Reasons include change in pH of the medium, depletion of essential growth-promoting components/factors, low contamination, improper storage of reagents etc.
- Cell death: Temperature fluctuation, absence of CO_2, cell damage during thawing and/or cryopreservation, increase concentration of toxic metabolite, imbalanced osmotic pressure in culture medium result in decrease in the survival of cells.
- Precipitation: (no change in pH): Precipitates in the medium without change in the pH may appear due to use of frozen medium, residual phosphate leftover while washing with detergent, which may precipitate powdered medium components.
- Cell clumping: Suspension cell may clump due to presence of calcium, magnesium ions or due to cell lysis and release of DNA (over digestion with proteolytic enzymes).
- Induced variability: Use of a variety of reagents and media induces variability in data acquired using primary cells. The handling methodology between the users may also contribute to the variability.

Relevance of primary cell culture

Primary cell culture increasingly being used as a major tool in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging, signaling studies), the effects of drugs and toxic compounds on the cells and mutagenesis and carcinogenesis. It is also used in drug screening as well as for the development of biological compounds (such as: vaccines, therapeutic proteins) on a large scale.

- **Model system:** Since primary cells are non-transformed, non-immortalized they closely simulates a living model and yield more physiologically significant results. These cells can act as a model system to study cell biology and biochemistry, to study the interaction between cell and disease causing agents (like bacteria, virus), to study the effect of drugs, to study the process of aging, to study cell signaling and metabolic regulations. In many cases the use of primary cells allows the researchers to avoid the complications (availability, cost and ethics) involved in using animal models
- **Cancer Research:** Primary cells can be exposed to radiation, chemicals and viruses to make them cancerous. Thus, the mechanism and cause of cancer and the altered signaling pathways can be

studied. It can also be used for determination of effective drugs for cancer cells. The side effects of cancer treatments (chemotherapy and irradiation) on normal cells can also be studied in this context.

- **Virology:** Detection, isolation, growth and development cycles of viruses can be studied. Primary cells are also useful to study the mode of infection.
- **Drug Screening and Toxicity Testing:** Primary cell cultures are used to study the cytotoxicity of new drug (to study the effect and safe dosage) and/or drug carriers (nanoparticles). It is useful for the synthesis of a variety of biomolecules at an industrial scale. This is particularly useful in the pharmaceutical industry. They are also used to determine the maximum permissible dosage of new drugs.
- **Vaccine Production:** Primary animal cells are used in the production of viruses and these viruses are used to produce vaccines (such as vaccines, for deadly diseases like polio, rabies, chicken pox, measles and hepatitis B are produced using animal cell culture) thus avoiding the use of animal models.
- **Genetic Engineering:** Primary (animal) cell cultures are used to produce commercially important genetically engineered proteins such as monoclonal antibodies, insulin, hormones, and much more.
- **Tissue or Organ Replacement:** Research is on-going on utilizing primary cells in the reconstruction of damaged tissue or replacement of non-functional cells or tissues. Organ culture techniques and research are being conducted on both embryonic and adult stem cell culture. These cells have the capacity to differentiate into many different types of cells and organs. By controlling the development and differentiation of these cells, we may be able to treat variety of medical conditions.
- **Prenatal diagnosis:** Amniotic fluid from pregnant women is extracted and cells are cultured for the study of chromosomes abnormalities, genes using karyotyping, and used in early detection of fetal disorders.
- **Stem Cell Therapy:** Stem cells isolated from bone marrow, blood or embryo, involve primary cell culture. Patient's own stem cells or those from a donor are grown in vitro for generating enough cells that may be used to regenerate tissue or replace functionally deficient cells. This is an area that is being explored to design therapies for genetic disorders, spinal cord injuries, degenerative diseases and cancer.

Advantages and Drawbacks of Primary Cells

Advantages	Drawbacks
Use of primary culture avoids many ethical objections raised against animal experiments Allows experiments on human tissues which otherwise could not have been done in vivo.	Primary cells takes more time to grow than other cell lines, it possess limited growth potential even under optimal growth conditions and eventually senesce and die.
The use of primary cells provides more relevant results than cell lines. Pre-screened primary cells are good models to represent the signaling in vivo very closely.	The cells taken from different donors behave differently in response to pro-inflammatory cytokines (unless they are pre-screened).2 The growth of metabolic regulatory mechanism that exist under in vivo conditions are absent in culture condition.
Primary cells are cost-effective as they help reduce the expenditure on animal models required for in vivo studies	The cost of isolation and culture is often high and prohibitive though cheaper that animal models. The tissue culture may not be always possible. The characteristics of primary cells may change with each subsequent passage if optimum culture conditions are not maintained.

Secondary culture or sub-culturing -

Sub-culturing - involves is simply taking a portion of the cells from one container and moving them to a new container with fresh media, thus providing more space and nutrients for both portions of cells to grow.

- If the cells are suspension cells, they can simply be transferred into a conical tube, spun down in a centrifuge, and then re-suspended in fresh media. This suspension can be divided into 2 or more portions and added to new containers.
- If the cells are adherent cells, they must first be detached from their container by mild treatment with Trypsin which breaks down proteins that help cells adhere to culture vessels.

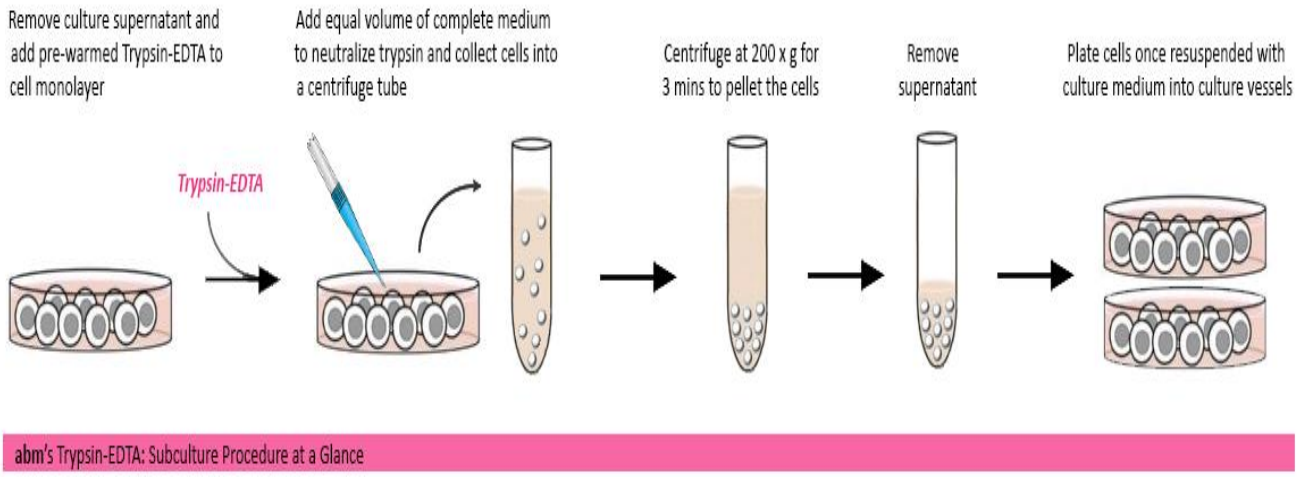


Figure 5 – Subculture procedure

Other dissociation enzymes such as dispase or collagenase (gentler than trypsin), can also be used for digestion.

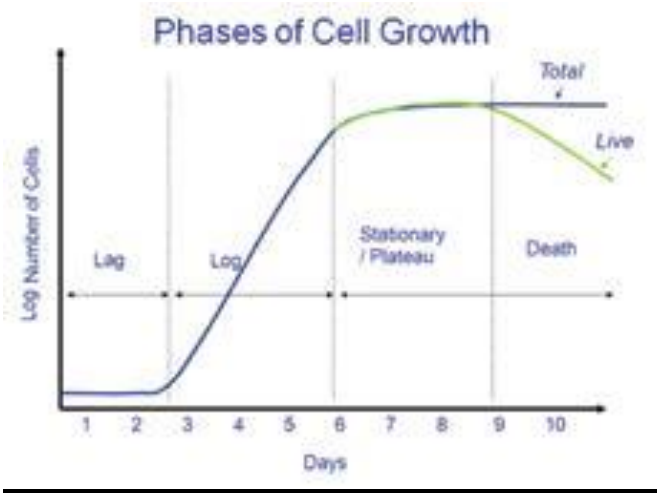
Prolonged exposure to trypsin can damage the proteins on the cell's surface and thereby affecting subsequent cell attachment and cell functioning. Therefore, trypsin must be diluted and inactivated after most cells are detached from the culture vessel. Inactivation is commonly achieved by the addition of serum and divalent cations -calcium and magnesium. In cases where serum-free conditions are used to culture cells, soybean trypsin inhibitor can be added to prevent further tryptic activity.

Before sub-culturing of cells some important aspects should be considered –

Seeding Density- it is the number of cells being added into a culture vessel. Some cell lines prefer high seeding densities while others are able to proliferate well even under low densities. Some will even change their characteristics depending on the seeding density. Eg - granulosa cells in culture will secrete estradiol at lower densities but will start secreting more progesterone as the density increases. If the cells are known to grow fast or if the cells will be cultured for a longer period of time, larger culture vessels should be used.

Propagation, Population Doubling and Passage Number

During sub-culturing of cells, at which phase of proliferation it remain is very crucial. There are four phases of cell growth and proliferation: **lag, log, stationary and decline**.



- During the lag phase, the number of cells is not increasing and the cells are simply acquiring enough resources to prepare for proliferation.
- During the log phase, the cells begin to proliferate rapidly over time.
- At the stationary phase, the rate of proliferation plateaus off and maintains a constant rate, due to the lack of nutrients and the presence of toxic metabolic waste products.
- Finally, at the decline phase cells begin to die off due to a lack of nutrients, altered pH conditions and the accumulation of toxic waste products.

So, the best option for the further seeding of cells is when they are in the lag phase so they can enter the next log phase whilst in their new media; and also they are frozen while in the log phase.

Population doubling - in cell culture is simply the time it takes for a cell population to get doubled since they were first grown in vitro. The formula used to calculate the population doubling level is - **Log^{10} (N/N_o) x 3.33**

Where N is the number of cells in the culture vessel at the end of a certain time interval, and N_o is the original number of cells plated in the vessel.

The passage number - actually describes the number of times that a culture has been sub-cultured. It reveals how old the cells may be and how suitable they would be for various assays.

Sub culturing Adherent Cells

The following protocol describes a general procedure for sub-culturing adherent mammalian cells in culture.

Materials Needed

• Culture vessels containing adherent cells
• Tissue-culture treated flasks, plates or dishes
• Complete growth medium, pre-warmed to 37°C
• Disposable, sterile 15-mL tubes
• 37°C incubator with humidified atmosphere of 5% CO2
• Balanced salt solution such as Dulbecco's Phosphate Buffered Saline (DPBS), containing no calcium, magnesium, or phenol red
• Dissociation reagent such as trypsin without phenol red
• Reagents and equipment to determine viable and total cell counts such as Automated Cell Counter, Trypan Blue and hemacytometer,

Protocol for Passaging Adherent Cells

All solutions and equipment that come in contact with the cells must be sterile and all the work should be done in a laminar flow hood.

1. Remove and discard the spent cell culture media from the culture vessel.
2. Wash cells using a balanced salt solution without calcium and magnesium (approximately 2 mL per 10 cm2 culture surface area). Gently add wash solution to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer, and rock the vessel back and forth several times.
   - ❖ *Note: The wash step removes any traces of serum, calcium, and magnesium that would inhibit the action of the dissociation reagent.*
3. Remove and discard the wash solution from the culture vessel
4. Add the pre-warmed dissociation reagent such as trypsin to the side of the flask; use enough reagent to cover the cell layer (approximately 0.5 mL per 10 cm2). Gently rock the container to get complete coverage of the cell layer.
5. Incubate the culture vessel at room temperature for approximately 2 minutes.
   - ❖ *Note that the actual incubation time varies with the cell line used.*
6. Observe the cells under the microscope for detachment. If cells are less than 90% detached, increase the incubation time a few more minutes, checking for dissociation every 30 seconds.
7. When ≥ 90% of the cells have detached, tilt the vessel for a minimal length of time to allow the cells to drain. Add the equivalent of 2 volumes (twice the volume used for the dissociation reagent) of pre-warmed complete growth medium. Disperse the medium by pipetting over the cell layer surface several times.
8. Transfer the cells to a 15-mL conical tube and centrifuge then at 200 × g for 5 to 10 minutes.
   - ❖ *Note that the centrifuge speed and time vary based on the cell type.*
9. Resuspend the cell pellet in a minimal volume of pre-warmed complete growth medium and remove a sample for counting.
10. Determine the total number of cells and percent viability using a hemacytometer, cell counter and Trypan Blue exclusion, or the Automated Cell Counter. If necessary, add growth media to the cells to achieve the desired cell concentration and recount the cells.
11. Dilute cell suspension to the seeding density recommended for the cell line, and pipet the appropriate volume into new cell culture vessels, and return the cells to the incubator.
    - ❖ *Note: If using culture flasks, loosen the caps before placing them in the incubator to allow proper gas exchange unless you are using vented flasks with gas-permeable caps.*

Cell line

Comparison between Primary Cells and Continuous cell lines

Properties	Primary cells	Continuous cell lines
Life span and cell proliferation	It is finite (i.e. limited to a less number of cell divisions)	It is infinite when handled properly (i.e. for a long period, approx. 30 cell divisions)
Consistency	Variability exists between donors and preparations	Minimal variability
Genetic Integrity	Retains in vivo tissue genetic makeup through cell doublings	Subject to genetic drift as cells divide (undefined set of mutations)
Biological relevance	More closely mimics the physiology of cells in vivo	Relevance can drift over time as cells divide (minimal biological relevance)
Ease of use (freeze-thaw & use)	Needs optimized culture conditions and careful handling	Well established conditions and robust protocols exist
Time & expense to use	Needs more time and less abundance of cells	Needs less time and more abundance of cells

Continuous Cell lines in Research

- model systems for complex biological systems for the study of analysis of the biochemistry and cell biology of mammalian cells. The property of immortality renders them suitable for reproducibility in experiments
- studies of toxicity of compounds and production of proteins in higher scale continuous cells are cost effective and easy to work with
- isolation and identification of viruses for diagnostic, research, development as well as industrial purposes
- gene expression studies as continuous cell lines are easier to clone, manipulate and maintain.

Drawbacks of Continuous Cell lines in Research

Despite the ease of handling continuous cell lines, there are distinct drawbacks that restrict the extrapolation of experimental results to in vivo conditions.

- gross mutations and chromosomal abnormalities that increase as cells divide
- poor indicators of normal cell phenotype and progression of early-stage disease
- no direct correlation of function of cell lines in relation to that of other cells, healthy or diseased
- contamination may induce problems to the existing cell line.

Characteristics of Cell lines:

Cellular features -

- Smaller,more rounded, less adherent with a higher nucleus/ctoplasm ratio.
- Fast growth.
- Grow more in suspension conditions.
- Ability to grow up to hogher cell density.
- Stop expressing tissue specific genes.

Each cell line is distinguished by characteristic features which render these cells unique and bio-medically or biotechnologically useful.

- The growth pattern and morphological appearance of the cell line should be determined and should be stable from the starting point to the end-of-production cells.
- If there are specific markers that may be useful in characterizing the cell line (such as marker chromosomes, specific surface markers), these should be characterized for stability.
- Since normal cells has limited dividing capacity, therefore after a fixed number of population doublings cell lines derived from normal tissue will die out. This is a genetically determined event which is known as senescence. If the cells have an identified finite life expectancy, the total number of population doubling levels through senescence should be determined. Some cell lines may avoid senescence and give rise to continuous cell lines which may reflects its capacity for genetic variation.
- A common feature of many human continuous cell lines is the development of a sub tetraploid chromosome number. The alteration in a culture that gives rise to a continuous cell line is commonly called in vitro transformation and may occur spontaneously or be chemically or virally induced.

Table 2: Selection in cell line Development

Stage	Factor influencing Selection	
	Primary explant	Enzymatic disaggregation
Isolation	Mechanical damage	Enzymatic damage
Primary culture	Adhesion of explant, outgrowth(migration), cell proliferation	Cell adhesion and spreading, cell proliferation
First subculture	Trypsine sensitivity, nutrient, hormone, proliferative ability	
Propagation as cell lines	Relative growth rate of different cell, selective growth rate of one lineage, nutrient, hormone and subculture limitation Effect of cell density on predominance of normal or transformed phenotypes	
Senescence, transformation	Normal cell die out, transformed cell grow	

Requirement of cell lines:

For the maintenance of Cell line some basic conditions are required. These are described as follows.

1. **pH:** Most cell lines grow well at pH 7.4. Although it may varies among different cell strains, eg.- 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, may reduce the pH.
3. **Temperature:** The temperature recommended for most human and warm-blooded animal cell lines is 37°C, although a little lower temperature is maintained for safety.
4. **Media:** Although many cell lines are propagated mostly in commercially available medium supplemented either with serum or not. Media have been tested for their capability of sustaining the growth of one or more cell lines.

Morphology of Cells in Culture

Cells in culture can be divided in to three basic categories based on their shape and appearance (i.e., morphology).

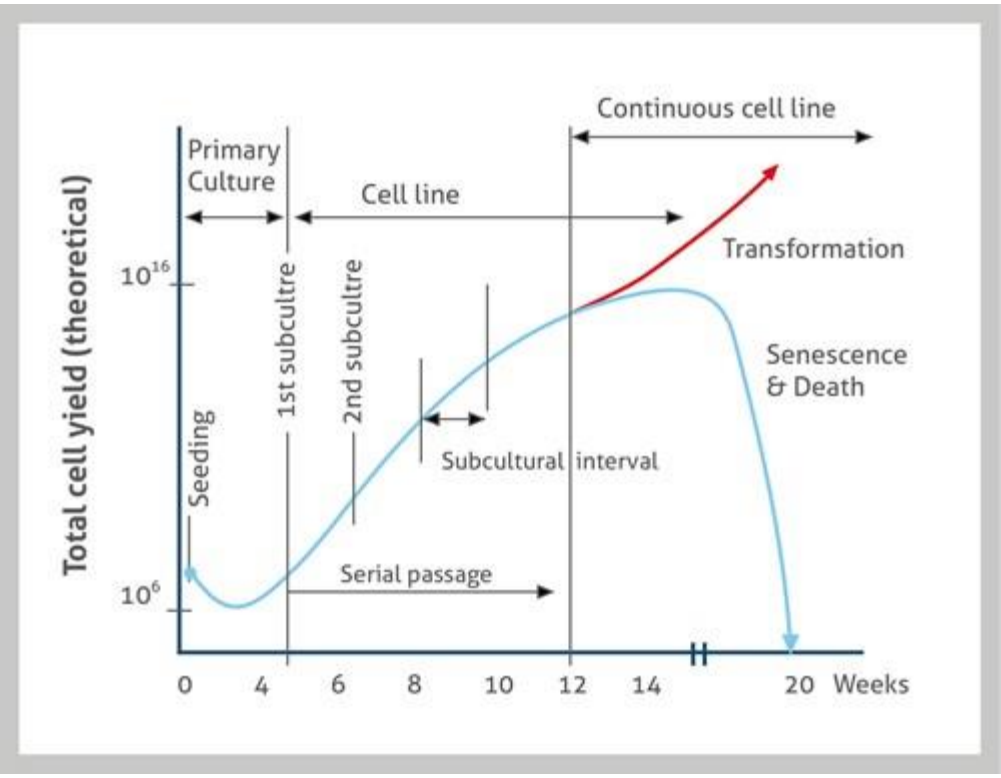
• **Fibroblastic (or fibroblast-like)** cells are bipolar or multipolar, have elongated shapes, and grow attached to a substrate.

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

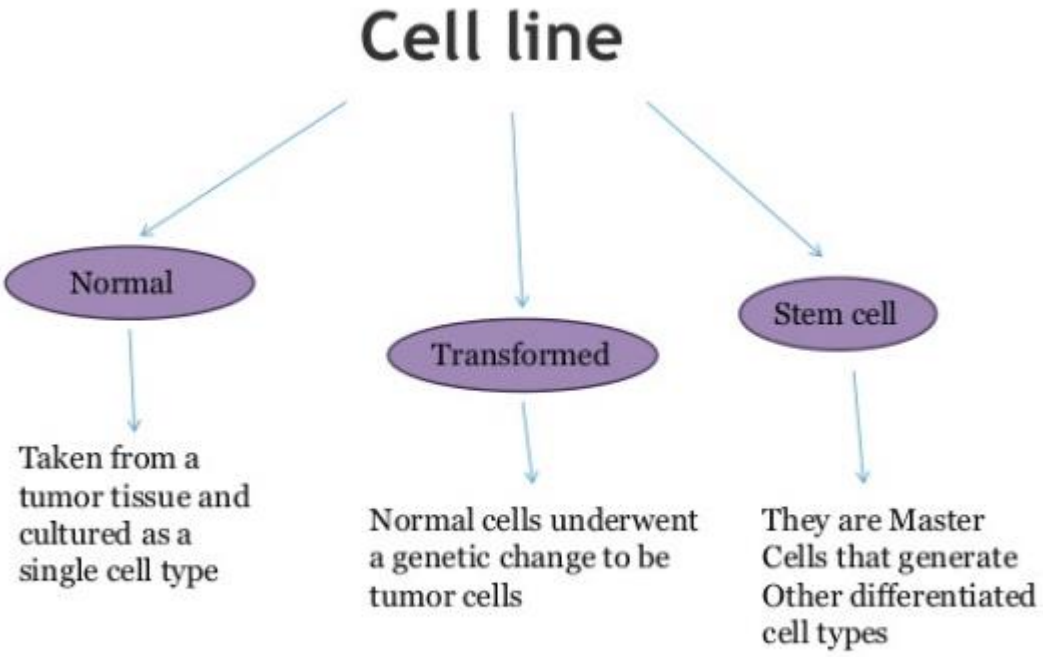
• **Lymphoblast-like** cells are spherical in shape and usually grown in suspension without attaching to a surface.

Growth curve for continuous cell line:

A growth curve gives three parameters of measurement: (1) the lag phase before cell proliferation is initiated after subculture, indicating whether the cells are having to adapt to different conditions; (2) the doubling time in the middle of the exponential growth phase, indicating the growth promoting capacity of the medium; and (3) the 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 line types- broadly the following types can be considered -



Stem cells - These cells have the unique ability to self-renew or to differentiate into various cell types in response to appropriate signals within the body. These properties provide stem cells with unique capabilities for tissue repair, replacement, and regeneration.

Cell lines derived from **primary cultures** have a limited lifespan as a consequence of being outside of their tissue niche. While cell culturing, those cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population over time.

In case of **transformed cell line** - The large T antigen from SV40 virus, or the E6 and E7 oncogenes from human papilloma virus, can turn a primary cell culture into an immortalized line as viral oncogenes essentially turn cells into a tumor and change its characteristics. Presently cell culture experts are using human telomerase reverse transcriptase (hTERT) gene instead of viral oncogenes. They behave like primary cultures but propagate like immortalized lines.

EXAMPLES OF ESTABLISHED CELL LINES

- May be derived from Normal or Tumor cells.

Cell line	Organism	Origin Tissue
HeLa	Human	Cervical cancer
293-T	Human	Kidney (embryonic)
A-549	Human	Lung carcinoma
ALC	Murine	Bone marrow
CHO	Hamster	Ovary
HB54	Hybridoma	Hybridoma
FM3	Human	Metastatic lymph node