CELL CULTURE -1

TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

Prof. Rajesh Sharma
Department of Biotechnology
Faculty of Science, V.B.S. Purvanchal University, Jaunpur
PIN-222 003, (U.P.), INDIA.
Mobile No.: +91-9415389474
E-Mail: rajeshdbtpu@gmail.com ; rajeshdbt@yahoo.co.in

VEER BAHADUR SINGH PURVANCHAL UNIVERSITY JAUNPUR-222003

E –content

Course: M.Sc.
Subject: Biochemistry; Biotechnology
Topic: Cell Culture - 1
Subtopic: **CELL CULTURE -1**
Prepared by: Prof. Rajesh Sharma
Department : Biotechnology
Faculty : Science
Email: rajeshdbtpu@gmail.com ; rajeshdbt@yahoo.co.in
Contact: +91-9415389474

Note-This E-content has been prepared as a reading material for students without any commercial interest. Original contributors are acknowledged.

ANIMAL CELL CULTURE

In vitro culture (maintain and/or proliferate) of cells, tissues or organs.

Cell culture:

Adherent monolayer on a solid substrate (various cell types) suspension in the culture medium (few cell types)

Primary explant culture:

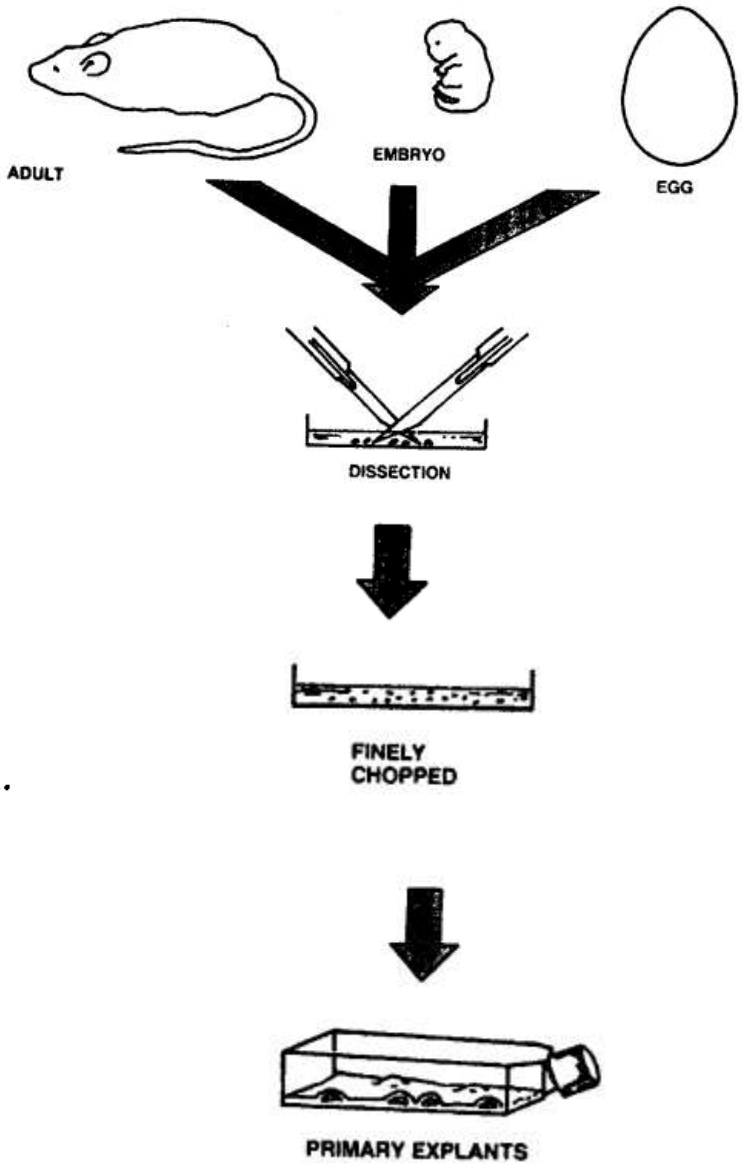
A fragment of tissue attachment and migration occurs in the plane of the solid substrate

Organ culture:

A spherical or three-dimensional shape specific histological interaction

Explant: living cells, tissues, or organs from animals or plants that transfer to a nutrient medium.

Tissue Culture



Is the growth of tissues or cells separate from the organism.

This is typically facilitated via use of a liquid, semi-solid, or solid growth medium, such as broth or agar.

Advantages

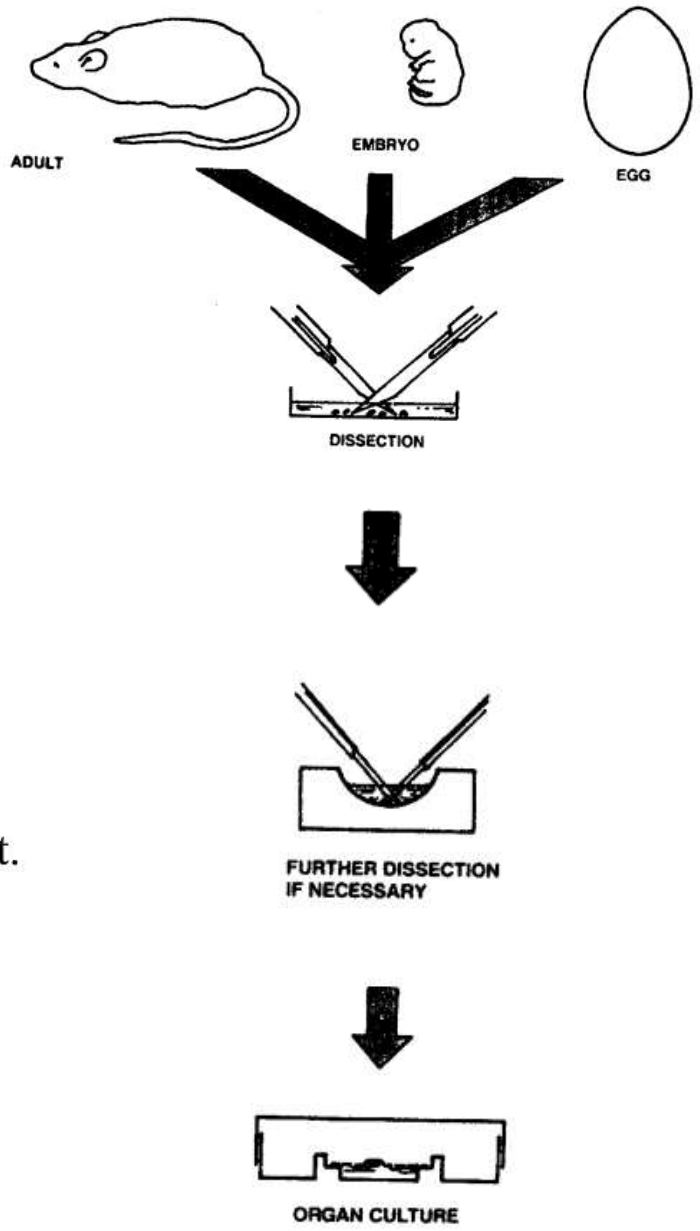
- Some normal functions may be maintained.
- Better than organ culture for scale-up but not ideal.

Disadvantages

- Original organization of tissue is lost.

Organ culture

The entire embryos or organs are excised from the body and culture



Advantages

- Normal physiological functions are maintained.
- Cells remain fully differentiated.

Disadvantages

- Scale-up is not recommended.
- Growth is slow.
- Fresh explantation is required for every experiment.

A selected list of commonly used cell lines

Cell line	*Species of origin*	*Tissue of origin*	*Morphology*	*Ploidy*	*Characteristics*
IMR-90	Human	Lung	Fibroblast	Diploid	Susceptible to human viral infections.
3T3-A31	Mouse	Connective tissue	Fibroblast	Aneuploid	Contact inhibited, readily transformed
BHK21-C13	Hamster (Syrian)	Kidney	Fibroblast	Aneuploid	Readily transformable
CHO-k1	Chinese hamster	Ovary	Fibroblast	Diploid	Simple karyotype
NRK49F	Rat	Kidney	Fibroblast	Aneuploid	Induction of suspension growth by TGF-α, β.
BRL 3A	Rat	Liver	Epithelial	Diploid	Produces IGF-2
Vero	Monkey	Kidney	Fibroblast	Aneuploid	Viral substrate and assay
HeLa-S_3	Human	Cervical carcinoma	Epithelial	Aneuploid	Rapid growth, high plating efficiency.
Sk/HEP-I	Human	Hepatoma	Endothelial	Aneuploid	Factor VIII
Caco-2	Human	Colo-rectal carcinoma	Epithelial	Aneuploid with polarised	Forms tight monolayer support.
MCF-7	Human	Breast tumor (effusion)	Epithelial	Aneuploid	Estrogen receptor positive.
Friend	Mouse	Spleen	Suspension	Aneuploid	Hemoglobin, growth hormone.

Cell Culture Media

A growth medium or culture medium is a **solid**, **liquid** or semi-**solid** designed to support the growth of microorganisms or cells, or small plants. Different types of media are used for growing different types of cells.

In the early years, the natural media obtained from various biological sources were used.

Body fluids:

Plasma, serum, lymph, amniotic fluid, ascitic and pleural fluids, aqueous humour from eyes and insect hemolymph were in common use. These fluids were tested for sterility and toxicity before their utility.

Tissue extracts:

Among the tissue extracts, chick embryo extract was the most commonly employed. The extracts of liver, spleen, bone marrow and leucocytes were also used as culture media. Some workers still prefer natural media for organ culture.

Artificial Media:

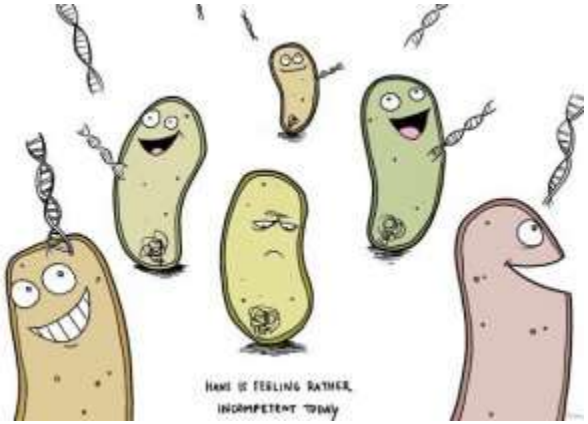
The artificial media (containing partly defined components) have been in use for cell culture since 1950.

Minimal of a medium for animal cell cultures

i. The medium should provide all the nutrients to the cells.

ii. Maintain the physiological pH around 7.0 with adequate buffering.

iii. The medium must be sterile, and isotonic to the cells.



The basis for the cell culture media was the **balanced salt solution** which was originally used to create a **physiological pH and osmolarity** required to maintain cells in vitro. For promoting growth and proliferation of cells, various constituents **(glucose, amino acids, vitamins, growth factors, antibiotics etc.**) were added, and several media developed.

BASIC COMPONENTS OF CULTURE MEDIA

Nutrient Substances	
Water	Energy Sources
Nitrogen Sources	Vitamins
Bulk Ions	Trace Elements
Lipids	Metabolites
Non Nutrient Substances	
Antibiotics	Buffers
Protective Agents	Anti Oxidants
Metabolites	High M.W Substances

PHYSICOCHEMICAL PROPERTIES OF CULTURE MEDIA

The culture media is expected to possess certain physicochemical properties (pH, O_2, CO_2, buffering, osmolarity, viscosity, temperature etc.) to support good growth and proliferation of the cultured cells.

pH:

Most of the cells can grow at a pH in the range of 7.0-7.4, although there are slight variations depending on the type of cells (i.e. cell lines).

The indicator phenol red is most commonly used for visible detection of pH of the media.

Its colouration at the different pH is shown below:

At pH 7.4 — Red

At pH 7.0 — Orange

At pH 6.5 — Yellow

At pH 7.8 — Purple

CO_2, bicarbonate and buffering:

Carbon dioxide in the medium is in a dissolved state, the concentration of which depends on the atmospheric CO_2 tension and temperature. CO_2 in the medium exists as carbonic acid (H_2CO_3), and bicarbonate (HCO^-_3) and H^+ ions as shown below.

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO^-_3$$

As is evident from the above equation, the concentrations of CO_2, HCO^-_3 and pH are interrelated. By increasing the atmospheric CO_2, the pH will be reduced making the medium acidic.

Addition of sodium bicarbonate (as a component of bicarbonate buffer) neutralizes bicarbonate ions.

$$NaHCO_3 \leftrightarrow Na^+ + HCO^-_3$$

In fact, the commercially available media contain a recommended concentration of bicarbonate, and CO_2 tension for the required pH. In recent years HEPES (hydroxyethyl piperazine 2-sulfonic acid) buffer which is more efficient than bicarbonate buffer is being used in the culture media.
However, bicarbonate buffer is preferred by most workers because of the low cost, less toxicity and nutritional benefit to the medium. This is in contrast to HEPES which is expensive, besides being toxic to the cells. The presence of pyruvate in the medium results in the increased endogenous production of CO_2 by the cells. This is advantageous since the dependence on the exogenous supply of CO_2 and HCO^-_3 will be less.

Oxygen:

A great majority of cells in vivo are dependent on the O_2 supply for aerobic respiration. This is in fact made possible by a continuous supply of O_2 to the tissues by hemoglobin. The cultured cells mostly rely on the dissolved O_2 in the medium which may be toxic at high concentration due to the generation of free radicals. Therefore, it is absolutely necessary to supply adequate quantities of O_2 so that the cellular requirements are met, avoiding toxic effects.

Some workers add free-radical scavengers (glutathione, mercaptoethanol) to nullify the toxicity. Addition of selenium to the medium is also advocated to reduce O_2 toxicity. This is because selenium is a cofactor for the synthesis of glutathione.

In general, the glycolysis occurring in cultured cells is more anaerobic when compared to in vivo cells. Since the depth of the culture medium influences the rate of O_2 diffusion, it is advisable to keep the depth of the medium in the range 2-5 mm.

Temperature:

In general, the optimal temperature for a given cell culture is dependent on the body temperature of the organism, serving as the source of the cells. Accordingly, for cells obtained from humans and warm blooded animals, the optimal temperature is 37°C.

In vitro cells cannot tolerate higher temperature and most of them **die if the temperature goes beyond 40°C**. It is therefore absolutely necessary to maintain a constant temperature (± 0.5°C) for reproducible results.

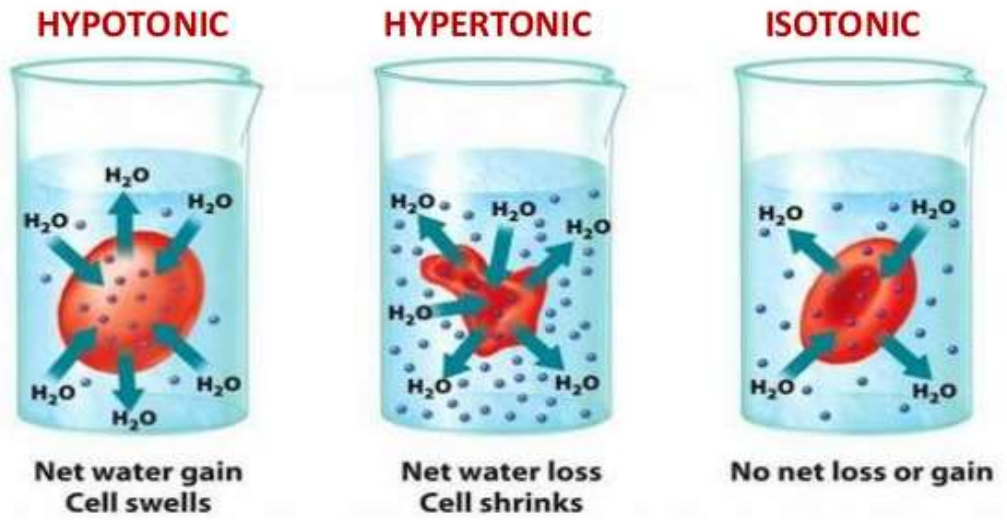
If the cells are obtained from birds, the optimal temperature **is slightly higher (38.5°C) for culturing**. For cold blooded animals (poikiltherms) that do not regulate their body heat (e.g. cold-water fish), the culture temperature may be in the range of 15-25°C. Besides directly influencing growth of cells, temperature also affects the solubility of CO_2 i.e. higher temperature enhances solubility.

Osmolality:

In general, the osmolality for most of the cultured cells (from different organisms) is in the range of 260-320 mosm/kg. This is comparable to the osmolality of human plasma (290 mosm/kg).

Osmolarity is the measure of the concentration of solute inside of a fluid or a cell

Cells can be in three types of *Osmotic Environments*:



Once an osmolality is selected for a culture medium, it should be maintained at that level (with an allowance of ± 10 mosm/kg). Whenever there is an addition of acids, bases, drugs etc. to the medium, the osmolality gets affected. The instrument osmometer is employed for measuring osmolalities in the laboratory

The terms osmolarity takes into account the total concentration of penetrating solutes *and* non-penetrating solutes, whereas tonicity takes into account the total concentration of *only* non-penetrating solutes.

BALANCED SALT SOLUTIONS:

The balanced salt solutions (BSS) are primarily composed of inorganic salts. Sometimes, sodium bicarbonate, glucose and HEPES buffer may also be added to BSS. Phenol red serves as a pH indicator.

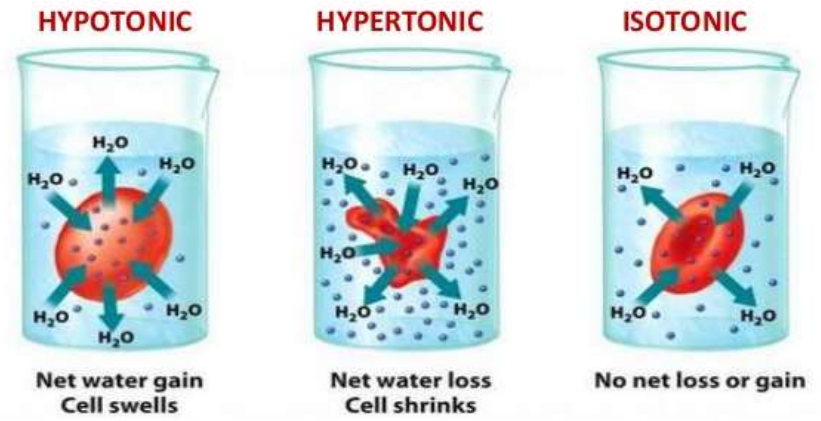
The important functions of balanced salt solutions are listed hereunder:

i. Supply essential inorganic ions.

ii. Provide the requisite pH.

iii. Maintain the desired osmolality.

iv. Supply energy from glucose.



In fact, balanced salt solutions form the basis for the preparation of complete media with the requisite additions.

Further, BSS is also useful for a short period (up to 4 hours) incubation of cells.

Composition of Balanced Salt Solutions (BSS)

Ingradient	*Earle's BSS*	*Hank's BSS*
NaCl	6.68	8.0
KCl	0.4	0.4
$CaCl_2$ (anhydrous)	0.02	0.14
$MgSO_4.7H_2O$	0.2	0.1
$NaHCO_3$	2.2	0.35
$NaH_3PO_4.H_2O$	0.14	—
$Na_2HPO_4.7H_2O$	—	0.09
KH_2PO_4	—	0.06
D-Glucose	1.0	1.0
Phenol red	0.01	0.01
HEPES, Na salt (buffer)	13.02	2.08

The composition of two most widely used BSS namely Earle's BSS and Hank's BSS

COMPLETE CULTURE MEDIA

The complete media, in general, contains a large number of components amino acids, vitamins, salts, glucose, other organic supplements, growth factors and hormones, and antibiotics, besides serum. Depending on the medium, the quality and quantity of the ingredients vary.

In the early years, balanced salt solutions were supplemented with various nutrients (amino acids, vitamins, serum etc.) to promote proliferation of cells in culture. Eagle was a pioneer in media formulation. He determined (during 1950-60) the nutrient requirements for mammalian cell cultures. Many developments in media preparation have occurred since then. There are more than a dozen media now available for different types of cultures.

Some of them are stated below:

EMEM—Eagle's minimal essential medium

DMEM—Dulbecco's modification of Eagle's medium

CMEM—Glasgow's modification of Eagle's medium

RPMI 1630 and RPMI 1640—Media from Rosewell Park Memorial Institute.

COMPOSITION OF THREE COMMONLY USED CULTURE MEDIA

Component	Eagle's MEM	RPMI 1640	Ham's F 12
Amino acids			
L-Alanine			8.91
L-Arginine HCl	105	200	211
L-Asparagine H_2O		50	15.0
L-Aspartic acid		20	13.3
L-Cystine	24	50	24.0
L-Glutamic acid		20	14.7
L-Glutamine	292	300	146.2
Glycine		10	7.51
L-Histidine HCl H_2O	31	15	21.0
L-Isoleucine	52	50	3.94
L-Leucine	52	50	13.12
L-Lysine	58	40	36.54
L-Methionine	15	15	4.48
L-Phenylalanine	32	15	4.96
L-Proline		20	34.5
L-Serine		30	10.51
L-Threonine	48	20	11.91
L-Tryptophan	10	5	2.042
L-Tyrosine	36	20	5.43
L-Valine	46	20	11.7
Glutathione (red)		1	
L-Hydroxyproline		20	

Component	Eagle's MEM	RPMI 1640	Ham's F 12
Inorganic salts			
$CaCl_2.2H_2O$	200		44.1
$CaNO_3.4H_2O$		100	
$CuSO_4.5H_2O$			0.0025
$FeSO_4.7H_2O$			0.83
KCl	400	400	223
$MgSO_4.7H_2O$	220	100	133
NaCl	6800	6000	7599
$NaHCO_3$	2000	2000	1176
$Na_2HPO_4.7H_2O$		1512	268
$NaH_2PO_4.2H_2O$	150		
Other components			
D-Glucose	1000	2000	1801
Phenol red		5.0	1.2
Sodium pyruvate			110
Lipoic acid			0.21
Linoleic acid			0.084
Hypoxanthine			4.08
Putrescine 2HCl			0.16

Component	Eagle's MEM	RPMI 1640	Ham's F 12
Vitamins			
D-Biotin		0.2	0.007
Ca D-pantothenate	1	0.25	0.26
Choline chloride	1	3.0	13.96
Folic acid	1	1.0	1.32
i-Inositol	2		18.02
Nicotinamide	1	35	0.037
p-Aminobenzoic acid		1.0	
Pyridoxine HCl		1	0.062
Pyridoxal HCl	1		
Riboflavin	0.1	0.2	0.038
Thiamine HCl	1	1.0	0.34
Vitamin B_{12}		0.005	1.36

Amino acids:

All the essential amino acids (which cannot be synthesized by the cells) have to be added to the medium. In addition, even the non-essential amino acids (that can be synthesized by the cells) are also usually added to avoid any limitation of their cellular synthesis. Among the non-essential amino acids, glutamine and/or glutamate are frequently added in good quantities to the media since these amino acids serve as good sources of energy and carbon.

Vitamins:

The quality and quantity of vitamins depends on the medium. For instance, Eagle's MEM contains only water soluble vitamins (e.g. B-complex, choline, inositol). The other vitamins are obtained from the serum added. The medium M 199 contains all the fat soluble vitamins (A, D, E and K) also. In general, for the media without serum, more vitamins in higher concentrations are required.

Hormones and growth factors:

For the media with serum, addition of hormones and growth factors is usually not required. They are frequently added to serum-free media.

Other organic supplements:

Several additional organic compounds are usually added to the media to support cultures. These include certain proteins, peptides, lipids, nucleosides and citric acid cycle intermediates. For serum-free media, supplementation with these compounds is very useful.

Antibiotics:

In the early years, culture media invariably contained antibiotics. The most commonly used antibiotics were ampicillin, penicillin, gentamycin, erythromycin, kanamycin, neomycin and tetracycline. Antibiotics were added to reduce contamination. However, with improved aseptic conditions in the present day tissue culture laboratories, the addition of antibiotics is not required. In fact, the use of antibiotics is associated with several disadvantages.

i. Possibility of developing antibiotic-resistant cells in culture.

ii. May cause anti-metabolic effects and hamper proliferation.

iii. Possibility of hiding several infections temporarily.

iv. May encourage poor aseptic conditions.

The present recommendation is that for the routine culture of cells, antibiotics should not be added. However, they may be used for the development of primary cultures.

SERUM

Serum is a natural biological fluid, and is rich in various components to support cell proliferation. The major constituents found in different types of sera are listed in next slide. The most commonly used sera are calf serum (CS), fetal bovine serum (FBS), horse serum and human serum. While using human serum, it must be screened for viral diseases (hepatitis B, HIV).

Approximately 5-20% (v/v) of serum is mostly used for supplementing several media. Some of the important features of the serum constituents are briefly described.

Proteins:
The in vitro functions of serum protein are not very clear. Some of them are involved in promoting cell attachment and growth e.g. fetuin, fibronectin. Proteins increase the viscosity of the culture medium, besides contributing to buffering action.

Nutrients and metabolites:
Serum contains several amino acids, glucose, phospholipids, fatty acids, nucleosides and metabolic intermediates (pyruvic acid, lactic acid etc.). These constituents do contribute to some extent for the nutritional requirements of cells. This may however, be insignificant in complex media with well supplemented nutrients.

MAJOR CONSTITUENT OF SERUM

Proteins
- Albumin
- Globulins
- Fetuin
- Fibronectin
- Transferrin
- Protease inhibitors (α_1-antitrypsin)

Amino acids
- Almost all the 20

Lipids
- Cholesterol
- Phospholipids
- Fatty acids

Carbohydrates
- Glucose
- Hexosamine

Other organic compounds
- Lactic acid
- Pyruvic acid
- Polyamines
- Urea

Vitamins
- Vitamin A
- Folic acid

Growth factors
- Epidermal growth factor
- Platelet-derived growth factor
- Fibroblast growth factor

Hormones
- Hydrocortisone
- Thyroxine
- Triiodothyronine
- Insulin

Inorganics
- Calcium
- Sodium
- Potassium
- Chlorides
- Iron
- Phosphates
- Zinc
- Selenium

Growth factors:

There are certain growth factors in the serum that stimulate the proliferation of cells in the culture:

i. Platelet-derived growth factor (PDGF).

ii. Fibroblast growth factor (FGF).

iii. Epidermal growth factor (EGF).

iv. Vascular endothelial growth factor (VEGF).

v. Insulin-like growth factors (IGF-1, IGF-2).

In fact, almost all these growth factors are commercially available for use in tissue culture.

Hormones:

Hydrocortisone promotes cell attachment, while insulin facilitates glucose uptake by cells. Growth hormone, in association with somatomedins (IGFs), promotes cell proliferation.

Inhibitors:

Serum may also contain cellular growth inhibiting factors. Majority of them are artefacts e.g. bacterial toxins, antibodies. The natural serum also contains a physiological growth inhibitor namely transforming growth factor β (TGF-β). Most of these growth inhibitory factors may be removed by heat inactivation (at 56°C for 30 minutes).

Selection of Medium and Serum:

As already stated, there are around a dozen media for the cell cultures. The selection of a particular medium is based on the cell line and the purpose of culturing. For instance, for *chick embryo fibroblasts and HeLa cells, EMEM is used*.

The medium DMEM can be used for the cultivation of neurons. A selected list of cells and cell lines along with the media and sera used is given in Table 34.4. In fact, information on the selection of appropriate medium for a particular cell line is available from literature.

A Selected List of the Cells/Cell Lines Along with the Media and Serum Used for them

Cells or cell line	*Medium*	*Serum*
Chick embryo fibroblasts	EMEM	CS
Chinese hamster ovary (CHO)	EMEM, Ham's F12	CS
HeLa cells	EMEM	CS
Human leukemia	RPMI 1640	FB
Mouse leukemia	Fischer's medium, RPMI 1640	FB, HoS
Neurons	DMEM	FB
Mammary epithelium	RPMI 1640, DMEM	FB
Hematopoietic cells	RPMI 1640, Fischer's medium	FB
Skeletal muscle	DMEM, F 12	FB, HoS
Glial cells	MEM, F 12, DMEM	FB
3T3 cells	MEM, DMEM	CS

The selection of serum is also based on the type of cells being cultured.

The following criteria are taken into consideration while choosing serum:

i. Batch to batch variations.

ii. Quality control.

iii. Efficiency to promote growth and preservation of cells.

iv. Sterility.

v. Heat inactivation.

In recent years, there is a tendency to discontinue the use of serum, and switch over to more clearly defined media.

Supplementation of the Medium with Tissue Extracts:

Besides serum, the culture media can also be supplemented with certain tissue extracts and microbial culture extracts. The examples are—chick embryo extract, proteolytic digests of beef heart, bactopeptone, lactalbumin hydrolysate, tryptose. The chick embryo extract was found to contain both high molecular weight and low molecular weight compounds that support growth and proliferation of cells.

Advantages of Serum:

- Provides various components
- Modulates physiological properties of medium
- Protease inhibitors
- Provides nutrients not present in basal medium
- Carrier proteins for low molecular weight substances (e.g. transferrin)
- Help in solubilization of poorly dissolved substances (e.g. apolipoprotein)
- Cell substrate attachment (fibronectin, vironectin)
- Various enzymes
- Proteins which prevent non specific adsorption (e.g. albumin)
- Neutralization of detergents
- Prevents essential nutrients e.g. fatty acids

Disadvantages of Serum in culture medium:

iv. Potential introduction of animal viruses

vi. Antibodies against viruses, to which host cell is exposed.

viii. Availability of high quality

x. Undesirable contaminants

xii. High running costs & capital requirements

xiv. Shelf Life & Storage – always purchased in bulk

xvi. Physiological variability & consistency

xviii. Downstream Processing

xx. Characterization of final product laborious.

HEPES Buffer

4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

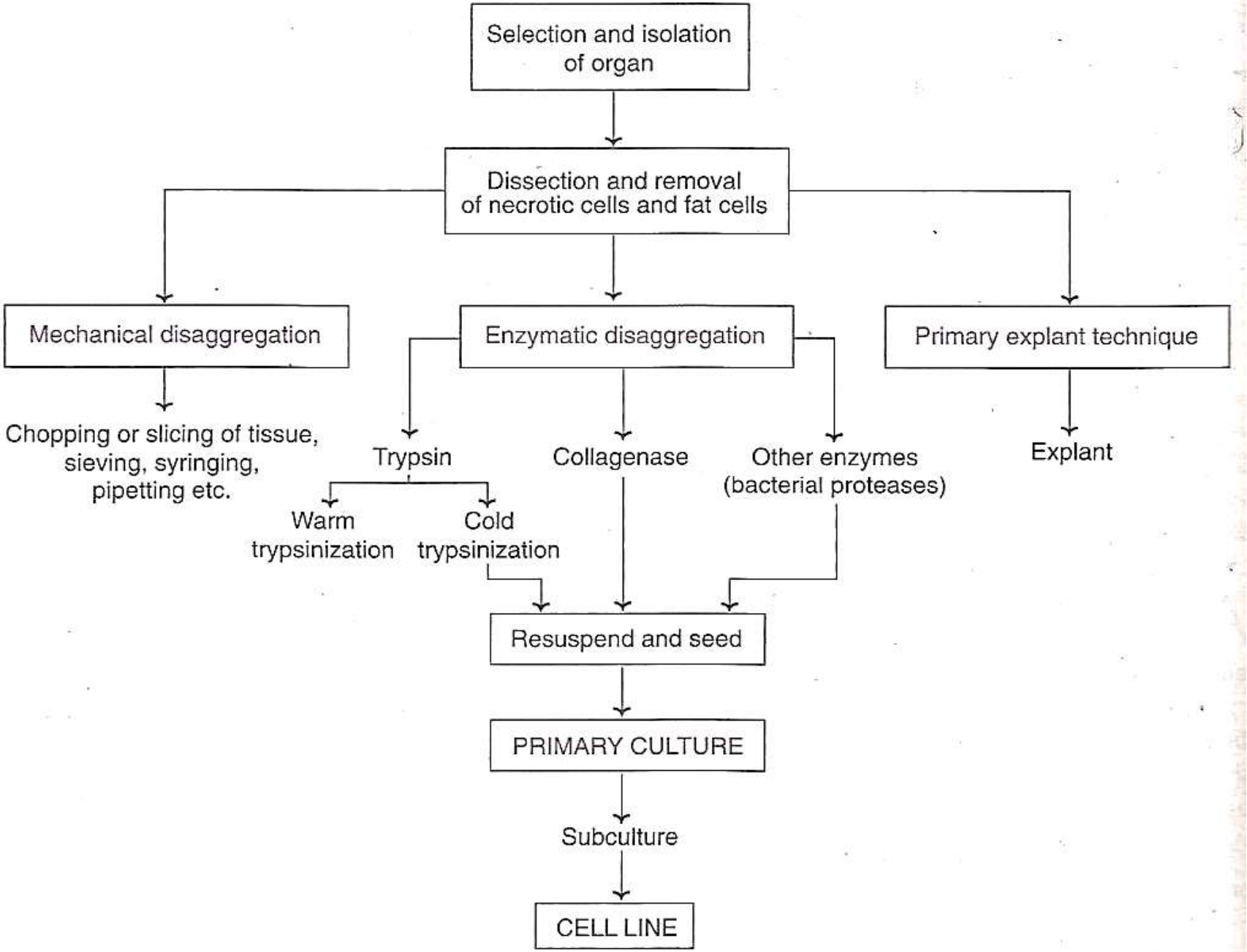
- Chemical buffering using a zwitterion, HEPES, has a superior buffering capacity in the pH range 7.2-7.4 and does not require a controlled gaseous atmosphere
- HEPES is relatively expensive and toxic at a higher concentration for some cell types.
- HEPES has also been shown to greatly increase the sensitivity of media to phototoxic effects induced by exposure to fluorescent light

TECHNIQUE USED FOR PRIMARY CELL CULTURE

The three types of technique are:

(1) Mechanical Disaggregation
(2) Enzymatic Disaggregation
(3) Primary Explant Technique.

Primary culture broadly involves the culturing techniques carried following the isolation of the cells, but before the first subculture. Primary cultures are usually prepared from large tissue masses. Thus, these cultures may contain a variety of differentiated cells e.g. fibroblasts, lymphocytes, macrophages, epithelial cells.


Selection and isolation of organ
Dissection and removal of necrotic cells and fat cells
Mechanical disaggregation
Enzymatic disaggregation
Primary explant technique
Chopping or slicing of tissue, sieving, syringing, pipetting etc.
Trypsin
Collagenase
Other enzymes (bacterial proteases)
Explant
Warm trypsinization
Cold trypsinization
Resuspend and seed
PRIMARY CULTURE
Subculture
CELL LINE

TECHNIQUE # 1. MECHANICAL DISAGGREGATION:

For the disaggregation of soft tissues (e.g. spleen, brain, embryonic liver, soft tumors), mechanical technique is usually employed. This technique basically involves careful chopping or slicing of tissue into pieces and collection of spill out cells.

The cells can be collected by two ways:

i. Pressing the tissue pieces through a series of sieves with a gradual reduction in the mesh size.

ii. Forcing the tissue fragments through a syringe and needle.

Although mechanical disaggregation involves the risk of cell damage, the procedure is **less expensive**, **quick and simple**. This technique is particularly useful when the **availability of the tissue is in plenty**, and the **efficiency of the yield is not very crucial**. It must however, be noted that the viability of cells obtained from mechanical techniques is much lower than the enzymatic technique.

TECHNIQUE # 2. ENZYMATIC DISAGGREGATION

Enzymatic disaggregation is mostly used when high recovery of cells is required from a tissue. Disaggregation of embryonic tissues is more efficient with higher yield of cells by use of enzymes. This is due to the presence of less fibrous connective tissue and extracellular matrix. Enzymatic disaggregation can be carried out by using trypsin, collagenase or some other enzymes.

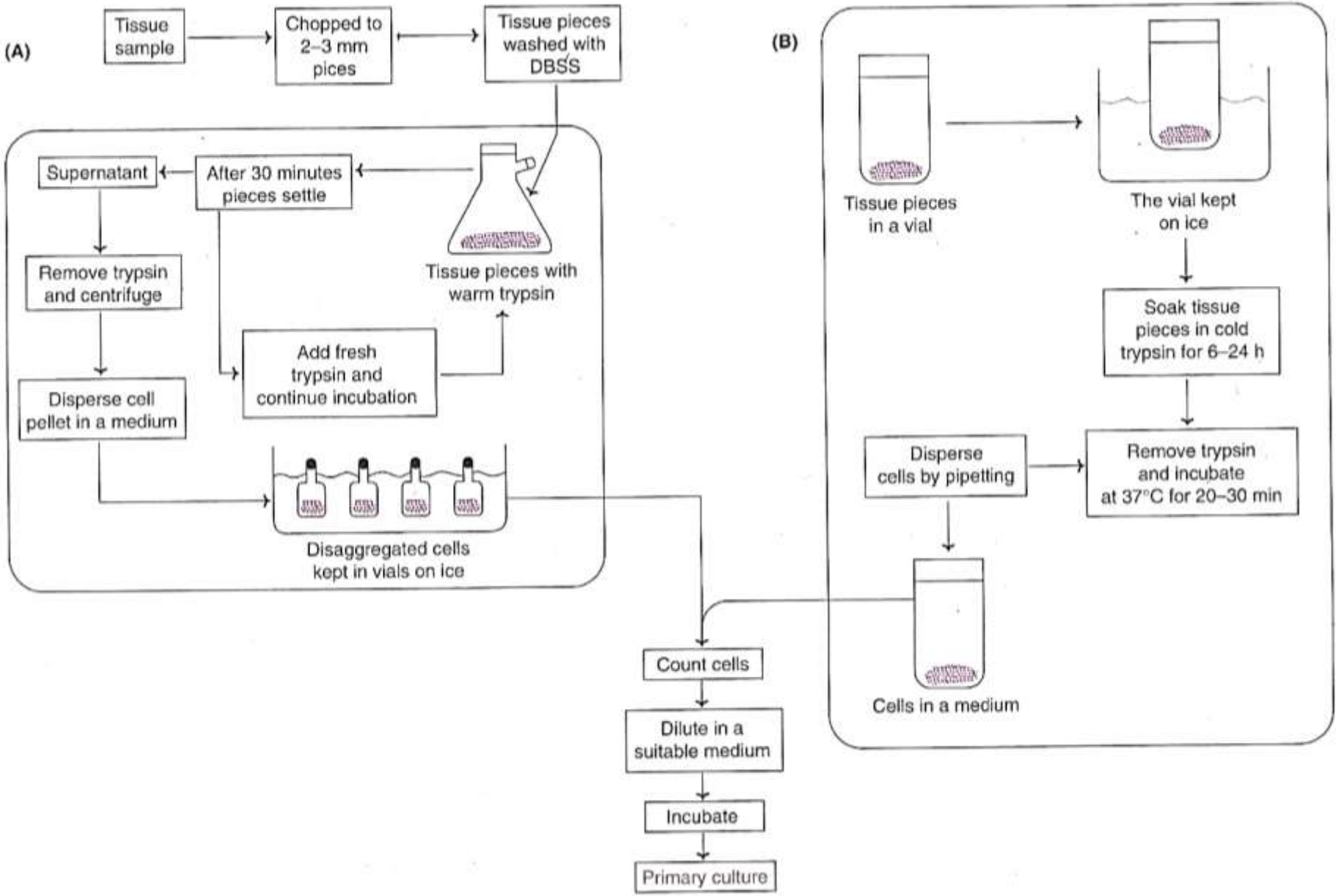
Disaggregation by trypsin:

The term trypsinization is commonly used for disaggregation of tissues by the enzyme, trypsin.

Many workers prefer to use crude trypsin rather than pure trypsin for the following reasons:

i. The crude trypsin is more effective due to the presence of other proteases
ii. Cells can tolerate crude trypsin better.
iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization



*Preparation of primary culture by trypsin disaggregation (**A**) Warm trypsinization (**B**) Cold trypsinization (DBSS–Dissection basal salt solution).*

WARM TRYPSINIZATION

This method is widely used for disaggregation of cells. The chopped tissue is washed with dissection basal salt solution (DBSS), and then transferred to a flask containing warm trypsin (37° C). The contents are stirred, and at an interval of every thirty minutes, the supernatant containing the dissociated cells can be collected. After removal of trypsin, the cells are dispersed in a suitable medium and preserved (by keeping the vial on ice).

The process of addition of fresh trypsin (to the tissue pieces), incubation and collection of dissociated cells (at 30 minutes intervals) is carried out for about 4 hours. The disaggregated cells are pooled, counted, appropriately diluted and then incubated.

COLD TRYPSINIZATION

This technique is more appropriately referred to as trypsinization with cold pre-exposure. The risk of damage to the cells by prolonged exposure to trypsin at 37°C (in warm trypsinization) can be minimized in this technique.

After chopping and washing, the tissue pieces are kept in a vial (on ice) and soaked with cold trypsin for about 6-24 hours. The trypsin is removed and discarded. However, the tissue pieces contain residual trypsin. These tissue pieces in a medium are incubated at 37°C for 20-30 minutes. The cells get dispersed by repeated pi-pettings. The dissociated cells can be counted, appropriately diluted and then used.

The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory.

The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

LIMITATIONS OF TRYPSIN DISAGGREGATION

Disaggregation by trypsin may damage some cells (e.g. epithelial cells) or it may be almost ineffective for certain tissues (e.g. fibrous connective tissue). Hence other enzymes are also in use for dissociation of cells.

DISAGGREGATION BY COLLAGENASE

Collagen is the most abundant structural protein in higher animals. It is mainly **present in the extracellular matrix of connective tissue** and **muscle**. The enzyme collagenase (usually a crude one contaminated with non-specific proteases) can be effectively used for the **disaggregation of several tissues (normal or malignant)** that may be sensitive to trypsin.

Highly **purified grades of collagenase have been tried, but they are less effective when compared to crude collagenase.** The important stages in collagenase disaggregation, depicted in Fig. 36.3, are briefly described hereunder.

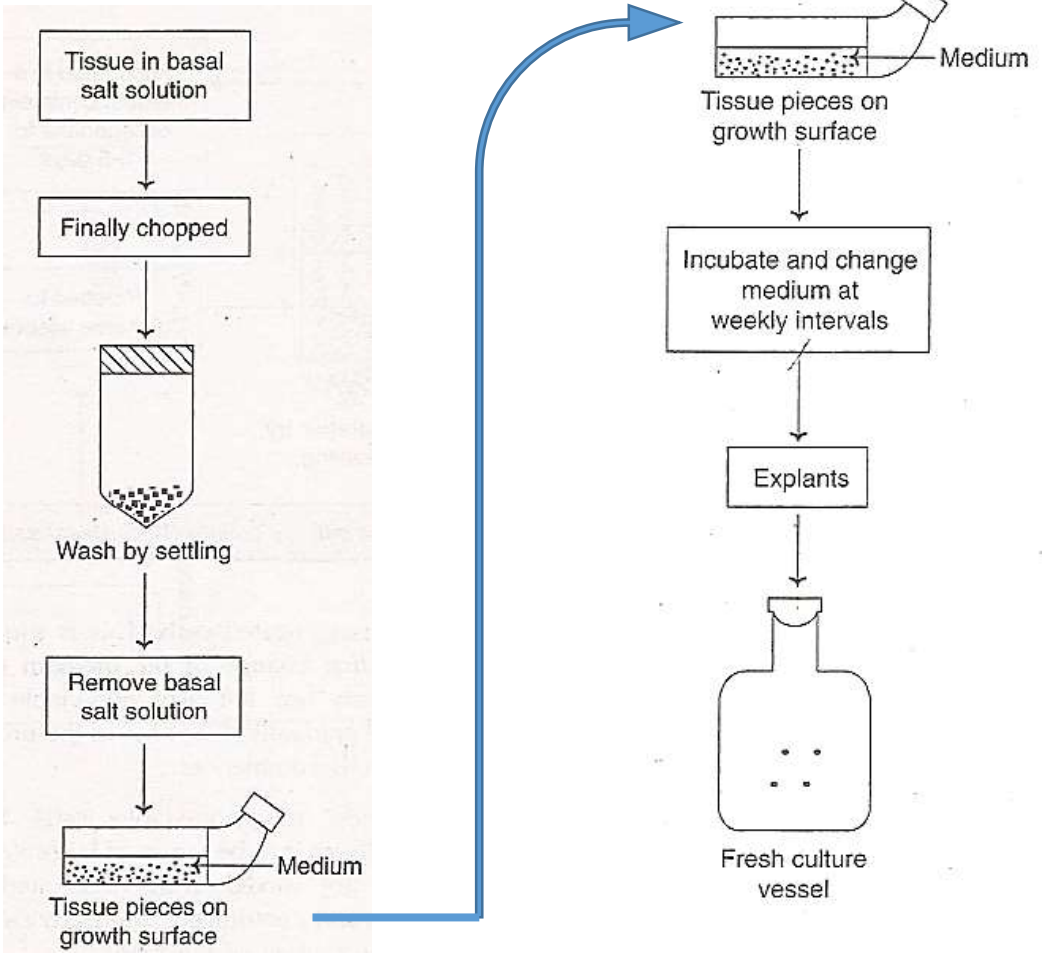
The desired tissue suspended in basal salt solution, containing antibiotics is chopped into pieces. These pieces are washed by settling, and then suspended in a complete medium containing collagenase. After incubating for 1-5 days, the tissue pieces are dispersed by pipetting. The clusters of cells are separated by settling. The epithelial cells and fibroblastic cells can be separated.

Collagenase disaggregation has been successfully used for **human brain**, **lung and several other epithelial tissues**, besides various human tumors, and other animal tissues. Addition of another enzyme **hyaluronidase (acts on carbohydrate residues on cell surfaces) promotes disaggregation**.

Collagenase in combination with hyaluronidase is found to be very effective for dissociating rat or rabbit liver. This can be done by per-fusing the whole organ in situ. Some workers use collagenase in conjunction with trypsin, a formulation developed in chick serum, for disaggregation of certain tissues.

Technique # 3. PRIMARY EXPLANT TECHNIQUE

The primary explant technique was, in fact the original method, developed by **Harrison in 1907**. This technique has undergone several modifications, and is still in use. The simplified procedure adopted for primary explant culture is depicted in Fig. 36.4, and briefly described below.



Primary explant technique for primary culture.

The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is changed at weekly intervals until a substantial outgrowth of cells is observed.

Now, the explants are removed and transferred to a fresh culture vessel.
The primary explant technique is particularly useful for **disaggregation of small quantities of tissues** (e.g. **skin biopsies**). The other two techniques mechanical or enzymatic disaggregation however, are not suitable for **small amounts of tissues, as there is a risk of losing the cells**.

The limitation of explant technique is the **poor adhesiveness of certain tissues to the growth surface**, and the selection of cells in the outgrowth. It is however, observed that the primary explant technique can be used for a majority of embryonic cells e.g. fibroblasts, myoblasts, epithelial cells, glial cells.

SEPARATION OF VIABLE AND NON-VIABLE CELLS:

It is a common practice to **remove the nonviable cells while the primary culture is prepared from the disaggregated cells**. This is usually done when the **first change of the medium is carried out**. The very few left over **non-viable cells get diluted and gradually disappear as the proliferation of viable cells commences.**

Sometimes, the non-viable cells from the primary cultures may be removed by centrifugation. The cells are mixed with ficoll and sodium metrizoate, and centrifuged. The dead cells form a pellet at the bottom of the tube.

References

1. Satyanarayana, U. “Biochemistry” 5th Edition, Elsevier India 2017, ISBN: 9788131249406
2. Voet D, Voet J. .G “Biochemistry”, 4th Edition, John Wiley & Sons, Inc.,2010. ISBN: 978-0-470-57095-1
3. David L. Nelson; Michael M. Cox “Lehninger Principles of Biochemistry” Seventh Edition, Macmillan 2017, ISBN:9781464187957
4. Wilson, K., Walker, J. (eds.); Cambridge University Press, Cambridge, 2000, 784 pp., ISBN 0-521-65873-X (paperback)

All the original contributors of the concept and findings published are gratefully acknowledged while preparing the e-content for the students of Biotechnology and allied sciences

CELL CULTURE -2

TEJASVI NAVADHITAMASTU

"Let our (the teacher and the taught) learning be radiant"

Let our efforts at learning be luminous and filled with joy, and endowed with the force of purpose

Prof. Rajesh Sharma
Department of Biotechnology
Faculty of Science, V.B.S. Purvanchal University, Jaunpur
PIN-222 003, (U.P.), INDIA.
Mobile No.: +91-9415389474
E-Mail: rajeshdbtpu@gmail.com ; rajeshdbt@yahoo.co.in

VEER BAHADUR SINGH PURVANCHAL UNIVERSITY JAUNPUR-222003

E –content

Course: M.Sc.
Subject: Biochemistry; Biotechnology
Topic: Cell Culture- 2
Subtopic: **CELL CULTURE -2**
Prepared by: Prof. Rajesh Sharma
Department : Biotechnology
Faculty : Science
Email: rajeshdbtpu@gmail.com ; rajeshdbt@yahoo.co.in
Contact: +91-9415389474

Note-This E-content has been prepared as a reading material for students without any commercial interest. Original contributors are acknowledged.

PRIMARY CELL CULTURE

Derived directly from excised tissue and cultured either as:

Outgrowth of excised tissue in culture

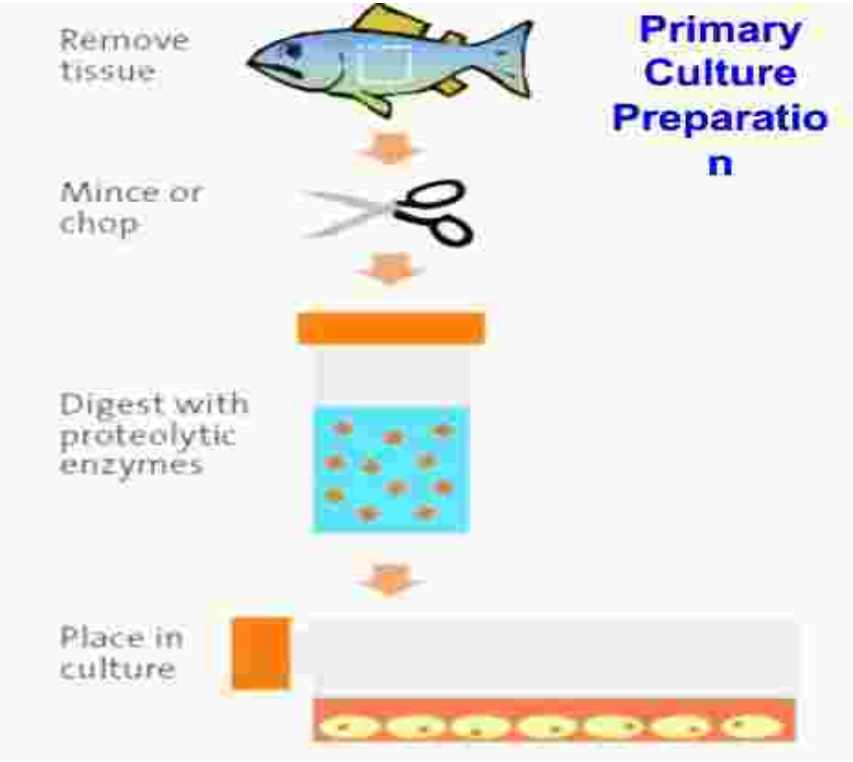
Dissociation into single cells (by enzymatic digestion or mechanical dispersion).

Characteristics:

Morphologically similar to the parent tissue

Limited number of cell divisions

Best experimental models for *in vivo* situations



Advantages:

- usually retain many of the *differentiated characteristics* of the cell *in viv*

Disadvantages:

- initially *heterogeneous* but later become dominated by *fibroblasts*.
- the preparation of primary cultures is labor intensive
- can be maintained in vitro only for a limited period of time.
- Difficult to obtain
- Relatively short life span in culture
- Very susceptible to contamination
- May not fully act like tissue due to complexity of media

CRITERIA/ CHARACTERISTICS FOR EFFICIENT DEVELOPMENT OF PRIMARY CULTURES:

1. **Embryonic tissues rather than adult tissues** are preferred for primary cultures. This is due to the fact that the embryonic cells can be disaggregated easily and yield more viable cells, besides rapidly proliferating in vitro.

2. The **quantity of cells used in the primary culture should be higher** since their survival rate is substantially lower (when compared to subcultures).

3. The tissues should be processed with **minimum damage to cells for use in primary culture**. Further, the dead cells should be removed.

4. Selection of an **appropriate medium** (preferably a nutrient rich one) is advisable. For the addition of serum, fetal bovine source is preferred rather than calf or horse serum.

5. It is **necessary to remove the enzymes used for disaggregation** of cells by centrifugation.

CELL SEPARATION

They are broadly categorized into two groups:
1. Physical fractionation for cell separation.
2. Chemical blockade for cell separation.

Cell Separation by Physical Means:
Physical fractionation or cell separation techniques, based on the following characteristics are in use:
a. Cell density.
b. Cell size.
c. Affinity of antibodies on cell surface epitopes.
d. Light scatter or fluorescent emission by labeled cells.

The two commonly used techniques namely centrifugal elutriation and fluorescence-activated cell separation are briefly described hereunder.

Cell Differentiation:
The various cell culture conditions favour maximum cell proliferation and propagation of cell lines.

Among the factors that promote cell proliferation, the following are important:
i. Low cell density
ii. Low Ca^{2+} concentration
iii. Presence of growth factors
For the process of cell differentiation to occur, the proliferation of cells has to be severely limited or completely abolished.

Cell differentiation can be promoted (or induced) by the following factors:
i. High cell density.
ii. High Ca^{2+} concentration.
iii. Presence of differentiation inducers (e.g. hydrocortisone, nerve growth factor).

As is evident from the above, different and almost opposing conditions are required for cell proliferation, and for cell differentiation. Therefore if cell differentiation is required two distinct sets of conditions are necessary.
1. To optimize cell proliferation.
2. To optimize cell differentiation.

METHODS OF CELL TRANSFORMATION AND CHARACTERISTICS OF TRANSFORMED CELLS

Cell transformation due to changes in the genetic material, and cell cloning involving the production of a population single cell are described here.

The four aspects of Cell Transformation are:
(1) Genetic Instability
(2) Immortalization
(3) Aberrant Growth Control and
(4) Tumorigenicity.

Transformation of Cells:

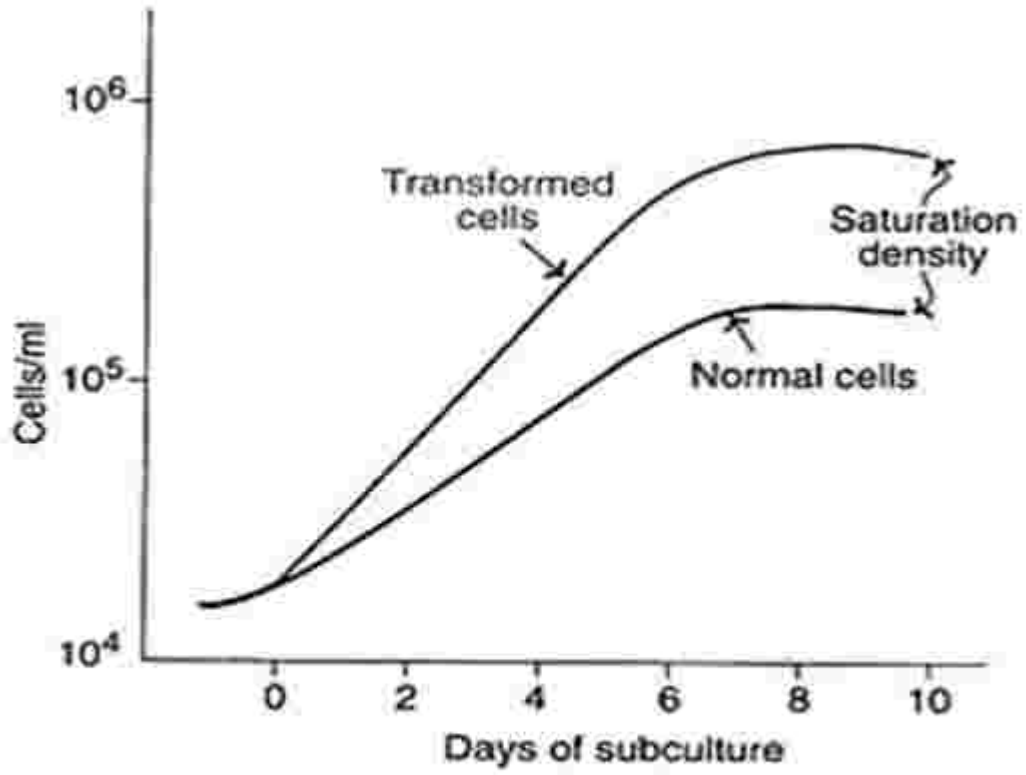
Transformation broadly refers to the change in phenotype of a cell due to a new genetic material. As regards the cultured cells, transformation involves spontaneous or induced permanent phenotypic alterations as a result of heritable changes in DNA, and consequently gene expression.

Transformed Cells:
Transformation is the phenomenon of the change in phenotype due to the acquirement of new genetic material. Transformation is associated with promotion of genetic instability.

The transformed and cultured cells exhibit alterations in many characters with reference to

1. Growth rate
2. Mode of growth
3. Longevity
4. Tumorigenicity
5. Specialized product formation.

While characterizing the cell lines, it is necessary to consider the above characters to determine whether the cell line has originated from tumor cells or has undergone transformation in culture



*Growth curves of transformed and normal cells (**Note** : The cell concentration is expressed in semilog plot).*

Transformation of cells may occur due to any one of the following causes that ultimately result in a changed genetic material:

i. Spontaneous.
ii. Infection with transforming virus.
iii. From gene transfection.
iv. Exposure to chemical carcinogens.
v. Exposure to ionizing radiations.

The normally occurring genetic variations in the cultured cells are due to the following causes:

1. High rate of spontaneous mutations in the in vitro conditions, possibly due to high rate of cell proliferation.

2. The continued presence of mutant cells in the culture, as they are not normally eliminated.

CHARACTERISTICS OF TRANSFORMED CELLS

The general characters of transformed cells are given in Table.They are grouped as genetic, structural, growth and neoplastic, and listed.

Transformation is associated with genetic instability, immortalization, aberrant growth control and malignancy. These aspects are briefly described.

Genetic Instability:

In general, the cell lines in culture are prone to genetic instability. A majority of normal finite cell lines are usually genetically stable while cell lines from other species (e.g. mouse) are genetically unstable, and can get easily transformed. The continuous cell lines derived from tumors of all species are unstable.

Genetic characters
Aneuploid
Heteroploid
High spontaneous mutation rate
Overexpressed oncogenes
Mutated or deleted suppressor genes
Structural characters
Altered cytoskeleton
Changed extracellular matrix
Modified expression of cell adhesion molecules
Disrupted cell polarity
Growth characters
Immortalized cells
Loss of contact inhibition
Anchorage independent
Density limitation of growth reduced
Growth factor independent
Low serum requirement
Shorter population doubling time
Neoplastic characters
Tumorigenic
Invasive
Increased protease secretion

Immortalization

The acquisition of an infinite life span by a cell is referred to as immortalization. Most of the normal cells (from different species) have a finite life span of 20-100 generations. But some cells from mouse, most of the tumor cells have infinite life span, as they go on producing continuous cell lines.

Control of finite life span of cells

The finite life span of cultured cells is regulated by about 10 senescence genes. These dominantly acting genes synthesize products which inhibit the cell cycle progression. It is strongly believed that immortalization occurs due to inactivation of some of the cell cycle regulatory genes e.g. Rb, p^{53} genes.

Immortalization of cells by viral genes:

Several viral genes can be used to immortalize cells. Some of these genes are listed below.

1. SV40LT
2. HPV16E6/E7
3. hTRT
4. Ad5E1a
5. EBV.

Among the above viral genes, SV40LT is most commonly used to induce immortalization. The product of this gene (T antigen) binds to senescence genes such as Rb and p^{53}. This binding restricts surveillance activity of senescence genes. The result is an increased genomic instability and activity, leading to further mutations favouring immortalization.

For the process of immortalization, the cells are infected with retroviruses containing immortalizing gene before they enter senescence. By this way, the life span of the cells can be extended by 20-30 population doublings. Thereafter, the cells cease to proliferate, and enter a crisis phase that may last for several months. At the end of the crisis phase, a small portion of cells can grow, and eventually become immortalized.

Immortalization of human fibroblasts:

The human fibroblasts are most successfully immortalized by the viral gene namely SV40LT. The process of fibroblast immortalization is complex and indirect with a very low probability i.e. about 1 in 10^7cells.

Immortalization of cells by telomerase-induction:

The most important cause of finite life span of cells (i.e. senescence) is due to telomeric shortening, followed by cell death (apoptosis). If the cells are transfected with telomerase gene htrt, the life span of the cells can be extended. And a small proportion of these cells become immortal.

CELL CULTURE

The cell culture can be initiated by the cells derived from a tissue through enzymatic or mechanical treatments. Primary culture is a selective process that finally results in a relatively uniform cell line. The selection occurs by virtue of the capacity of the cells to survive as monolayer cultures (by adhering to substrates) or as suspension cultures.

Among the cultured cells, some cells can grow and proliferate while some are unable to survive under the culture environment. The cells continue to grow in monolayer cultures, till the availability of the substrate is occupied.

The term confluence is used when the cultured cells make close contact with one another by fully utilizing the available growth area. For certain cells, which are sensitive to growth limitation due to density, the cells stop growing once confluence is reached. However, the transformed cells are insensitive to confluence and continue to overgrow.

When the culture becomes confluent, the cells possess the following characters:

1. The closest morphological resemblance to the tissue of origin (i.e. parent tissue).
2. The expression of specialized functions of the cells comparable to that of the native cells.

BIOLOGY OF CULTURED CELLS.

Development of continuous cell lines:

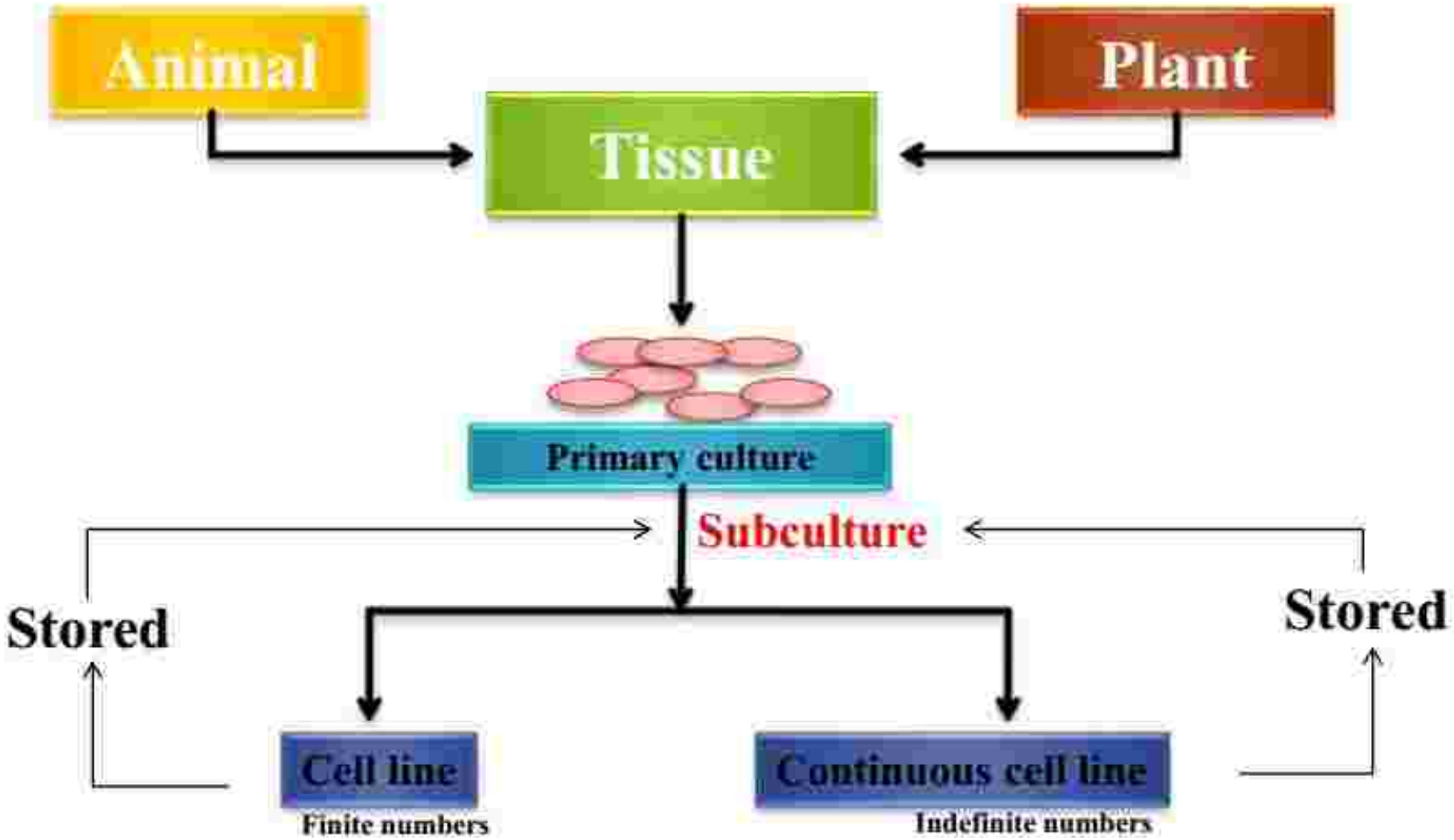
Certain alterations in the culture collectively referred to as transformation, can give rise to continuous cell lines. Transformation may be spontaneously occurring, chemically or virally- induced. Transformation basically involves an alteration in growth characteristics such as loss of contact inhibition, density limitation of growth and anchorage independence. The term immortalization is frequently used for the acquisition of infinite life span to cultured cells.

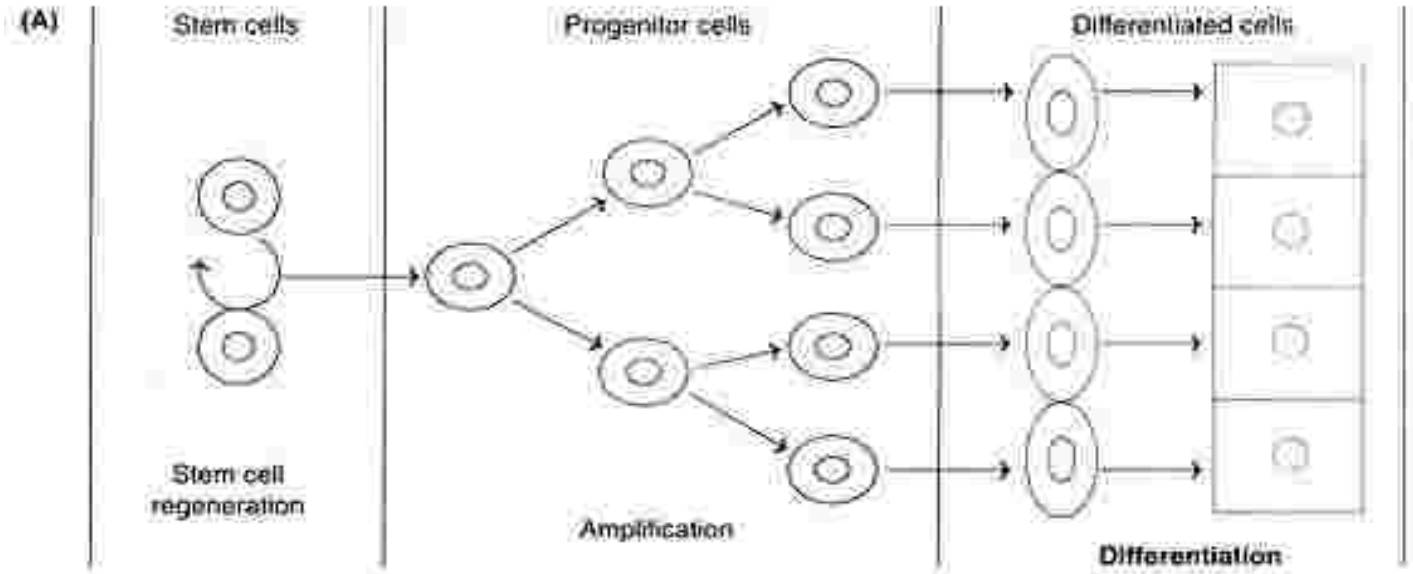
Genetic variations:

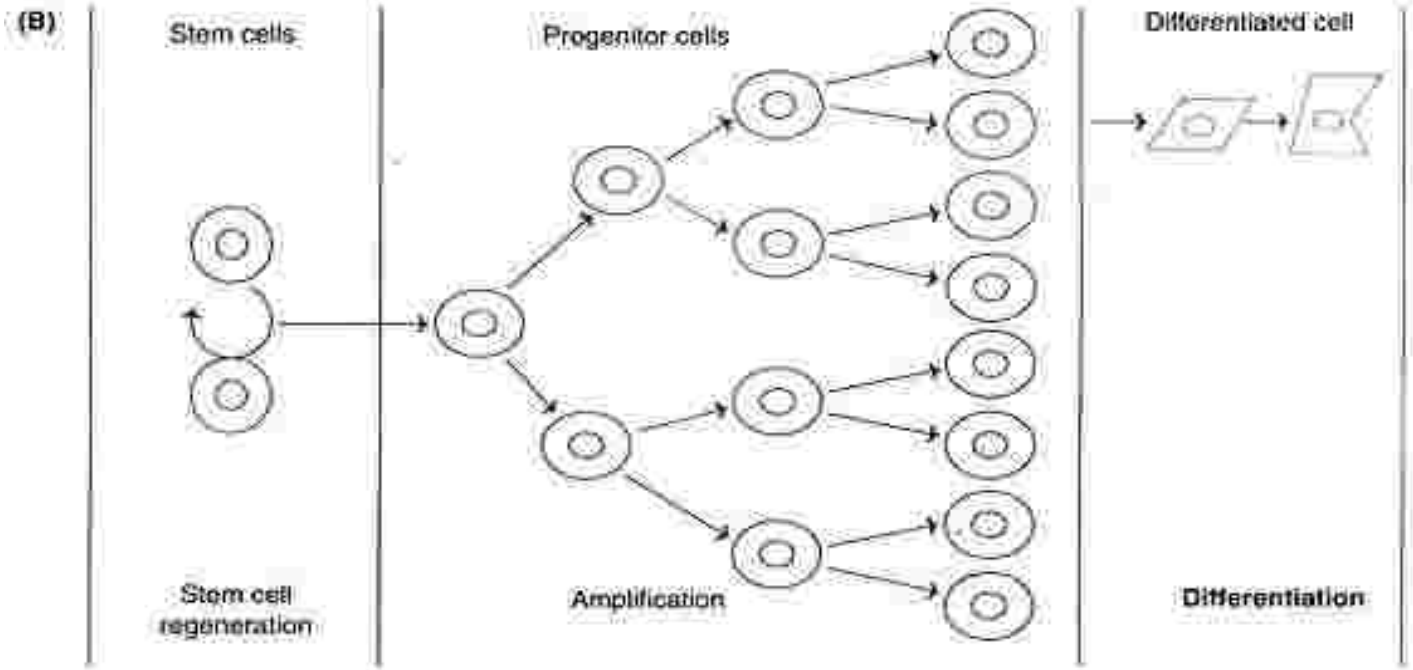
The ability of the cells to grow continuously in cell lines represents genetic variation in the cells. Most often, the deletion or mutation of the p^{53} gene is responsible for continuous proliferation of cells. In the normal cells, the normal p^{53} gene is responsible for the arrest of cell cycle. Most of the continuous cell lines are aneuploid, possessing chromosome number between diploid and tetraploid value.

Normal cells and continuous cell lines:

A great majority of normal cells are not capable of giving rise to continuous cell lines. For instance, normal human fibroblasts go on proliferating for about 50 generations, and then stop dividing. However, they remain viable for about 18 months. And throughout their life span, fibroblasts remain euploid. Chick fibroblasts also behave in a similar fashion. Epidermal cells and lymphoblastic cells are capable of forming continuous cell lines.


Animal
Plant
Tissue
Primary culture
Subculture
Stored
Stored
Cell line
Finite numbers
Continuous cell line
Indefinite numbers

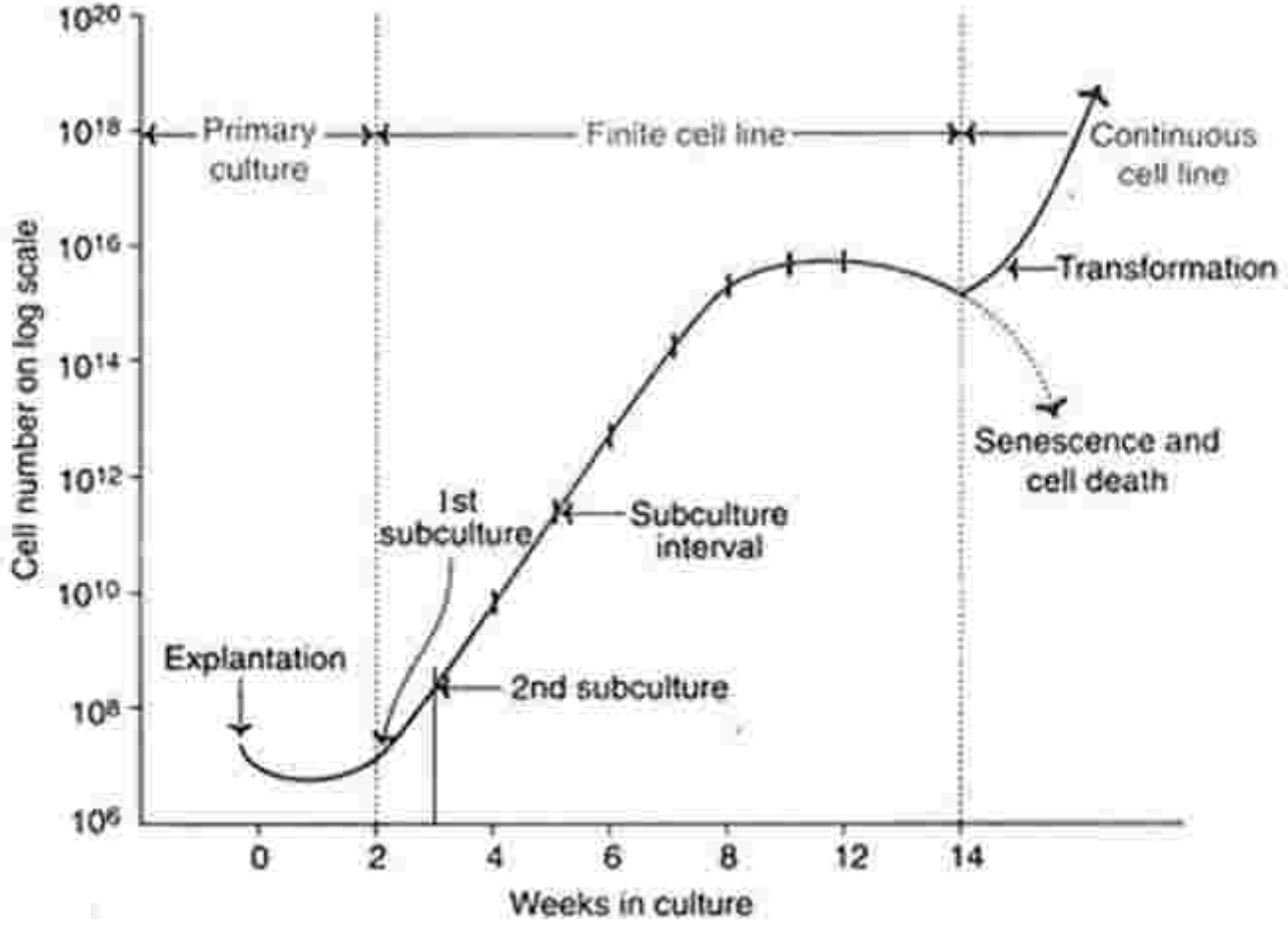

(A)
Stem cells
Progenitor cells
Differentiated cells
Stem cell regeneration
Amplification
Differentiation


(B)
Stem cells
Progenitor cells
Differentiated cell
Stem cell regeneration
Amplification
Differentiation

Evolution and Development of Cell Lines: The primary culture grown after the first subculture is referred to as cell line. A given cell line may be propagated by further sub culturing. As the subcultures are repeated, the most rapidly proliferating cells dominate while the non- proliferating or slowly proliferating cells will get diluted, and consequently disappear.

Senescence: The genetically determined event of cell divisions for a limited number of times (i.e. population doublings), followed by their death in a normal tissue is referred to as senescence. However, germ cells and transformed cells are capable of continuously proliferating. In the in vitro culture, transformed cells can give rise to continuous cell lines. The evolution of a continuous cell line is depicted in Fig. 35.4. The cumulative cell number in a culture is represented on Y-axis on a log scale, while the X-axis represents the time in weeks. The time for development of a continuous cell line is variable. For instance, for human diploid fibroblasts, the continuous cell line arises at about 14 weeks while the senescence may occur between 10 to 20 weeks; usually after 30 and 60 cell doublings.

Dedifferentiation: Dedifferentiation refers to the irreversible loss of specialized properties of cells when they are cultured in vitro. This happens when the differentiated in vitro cells lose their properties In the in vivo situation, a small group of stem cells give rise to progenitor cells that are capable of producing differentiated cell pool. On the other hand, in the in vitro culture system, progenitor cells are predominantly produced which go on proliferating. Very few of the newly formed cells can form differentiated cells. The net result is a blocked differentiation. Dedifferentiation implies an irreversible loss of specialized properties of the cells. On the other hand, de-adaptation refers to the re-induction of specialized properties of the cells by creating appropriate conditions.



Diagrammatic representation of evolution of a cell line.

GROWTH CYCLE OF CULTURED CELLS: The growth cycle of cultured cells is represented by three phases — the lag phase, the log (exponential) phase and the plateau phase

The lag phase: The lag phase represents a period of adaptation during which the cell forms the cell surface and extracellular matrix (lost during trypsinization), attaches to the substrate and spreads out. There is an increased synthesis of certain enzymes (e.g. DNA polymerase) and structural proteins, preparing the cells for proliferation. The production of specialized products disappears which may not reappear until the cell proliferation ceases. The lag phase represents preparative stage of the cells for proliferation following subculture and reseeding.

The log phase: The log phase is characterized by an exponential growth of cells, following the lag phase. During the log phase, the cultured cells are in the most uniform and reproducible state with high viability. This is an ideal time for sampling. The log phase terminates after confluence is reached with an addition of one or two population doublings.

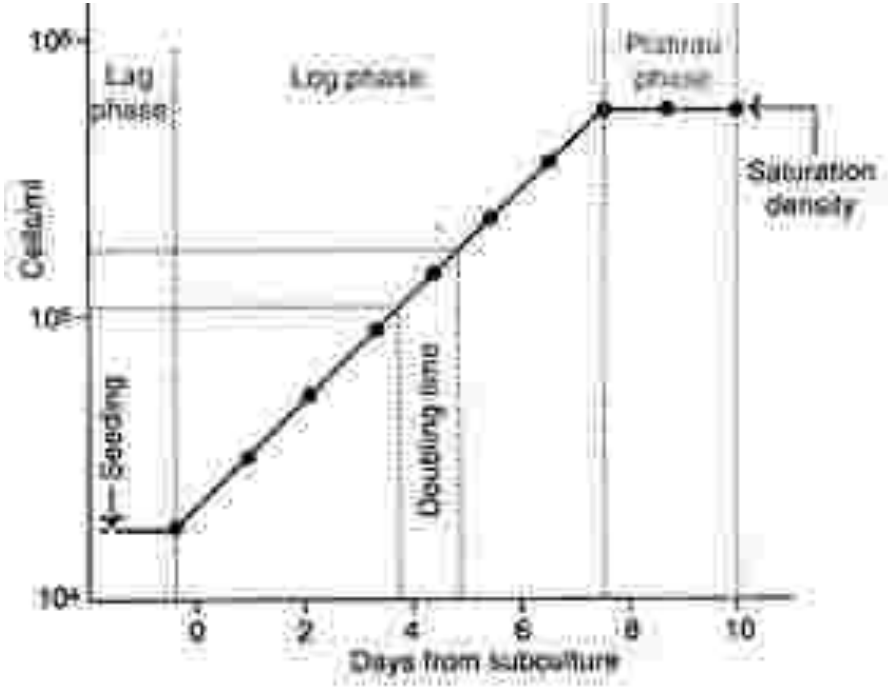
The duration of log phase depends on the cells with reference to:

a. Seeding density.
b. Growth rate.
c. Density after proliferation.

The plateau phase: As the cells reach confluence, the growth rate is much reduced, and the proliferation of cultured cells almost stops.

This stage represents plateau or stationary phase, and is characterized by:

a. Low motility of cells.
b. Reduced ruffling of plasma membrane.
c. Cells occupying minimum surface area.
d. Contact inhibition.
e. Saturation density.
f. Depletion of nutrients and growth factors.
g. Reduced synthesis of structural proteins.
h. Increased formation of specialized products.



PLATING EFFICIENCY OF CULTURED CELLS

1. Plating efficiency, representing colony formation at low cell density, is a measure used for analyzing cell proliferation and survival.
2. When the cells, at low densities, are cultured in the form of single cell suspensions, they grow as discrete colonies. Plating efficiency is calculated as follows.
3. Plating efficiency = No. of colonies formed/No. of cells seeded × 100
4. The term cloning efficiency is used (instead of plating efficiency) when each colony grows from a single cell.
5. Seeding efficiency representing the survival of cells at higher densities is calculated as follows.

Seeding efficiency = No. of cells recovered/No. of cells seeded × 100

Cell Synchronization: Synchronization literally means to make two or more things happen exactly simultaneously. For instance, two or more watches can be synchronized to show exactly the same time. The cells at different stages of the cell cycle in a culture can be synchronized so that the cells will be at the same phase. Cell synchrony is required to study the progression of cells through cell cycle. Several laboratory techniques have been developed to achieve cell synchronization.

Some Highlights of Cell Synchronization:

1. Cell separation by physical methods is more effective than chemical procedures.
2. Chemical blockade is often toxic to the cells.
3. Transformed cells cannot be synchronized by nutritional deprivation.
4. A high degree of cell synchrony (>80%) can be obtained in the first cycle, and in the second cycle it would be <60%. The cell distribution may occur randomly in the third cycle.

Cellular Senescence: The growth of the cells is usually measured as population doublings (PDs). The PDs refer to the number of times the cell population doubles in number during the period of culture and is calculated by the following formula.

$$\log_{10}(\text{No. of cells harvested}) - \log_{10}(\text{No. of cells seeded}) / \log 10^2$$

The phenomenon of senescence has been mostly studied with human fibroblast cultures. After 30-60 populations doublings, the culture is mainly composed of senescent fibroblasts. These senescent fibroblast are unable to divide in response to mitotic stimuli. It must be noted that the cells do not appear suddenly, but they gradually accumulate and increase in number during the life span of the culture.

CHARACTERIZATION OF CULTURED CELLS

Characterization of cultured cells or cell lines is important for dissemination of cell lines through cell banks, and to establish contacts between research laboratories and commercial companies.

Characterization of cell lines with special reference to the following aspects is generally done:

1. Morphology of cells, 2. Species of origin, 3. Tissue of origin.
4. Whether cell line is transformed or not, 5. Identification of specific cell lines.

Morphology of Cells: A simple and direct identification of the cultured cells can be done by observing their morphological characteristics. However, the morphology has to be viewed with caution since it is largely dependent on the culture environment. For instance, the epithelial cells growing at the center (of the culture) are regular polygonal with clearly defined edges, while those growing at the periphery are irregular and distended (swollen).
The composition of the culture medium and the alterations in the substrate also influence the cellular morphology. In a tissue culture laboratory, the terms fibroblastic and epithelial are commonly used to describe the appearance of the cells rather than their origin.

Fibroblastic cells: For these cells, the length is usually more than twice of their width. Fibroblastic cells are bipolar or multipolar in nature.

Epithelial cells: These cells are polygonal in nature with regular dimensions and usually grow in monolayers. The terms fibroblastoid (fibroblast-like) and epitheloid (epithelial-like) are in use for the cells that do not possess specific characters to identify as fibroblastic or epithelial cells.

Measurement of Growth Parameters of Cultured Cells:

Information on the growth state of a given culture is required to:

a. Design culture experiments.
b. Routine maintenance of culture.
c. Measurement of cell proliferation.
d. Know the time for subculture.
e. Determine the culture response to a particular stimulus or toxin.

Some of the commonly used terms in relation to the measurement of growth of cultured cells are explained.

Population doubling time (PDT):

The time interval for the cell population to double at the middle of the logarithmic (log) phase.

Cell cycle time or generation time:

The interval from one point in the cell division to the same point in the cycle, one division later. Thus cell cycle time is measured form one point in the cell cycle until the same point is reached again.

Confluence:

It denotes the culture stage wherein all the available substrate (growth area) is utilized, and the cells are in close contact with each other.

Contact inhibition:

Inhibition of cell motility and plasma membrane ruffling when the cells are in complete contact with other adjacent cells. This mostly occurs at confluence state, and results in the ceasation of the cell proliferation.

Cell density:

The number of cells per ml of the medium.

Saturation density:

The density of the cells (cells/ml^2, surface area) in the plateau phase.

MEASUREMENT OF CELL GROWTH IN CULTURES

a. Direct measure of cell number.
b. Determination of DNA/RNA content.
c. Estimation of protein/ATP concentration

MEASUREMENT OF SENESCENCE

a. Loss of metabolic activity
b. Lack of labeled precursor (^{3}H-thymidine) incorporation into DNA.
c. Certain histochemical techniques.

SENESCENCE-ASSOCIATED B-GALACTOSIDASE ACTIVITY ASSAY

There occurs an overexpression of the lysosomal enzyme β-galactosidase at senescence. This enzyme elevation is also associated with an increase in the cell size as the cell enters a permanent non-dividing state. The number of senescent cells in a culture can be measured by senescence-associated β-galactosidase (SA-β) assay.

CELL LINES (FINITE AND CONTINUOUS) SELECTION AND ROUTINE MAINTENANCE

The development and various other aspects of primary culture are described above. The term cell line refers to the propagation of culture after the first subculture.

In other words, once the primary culture is sub-cultured, it becomes a cell line. A given cell line contains several cell lineages of either similar or distinct phenotypes.

It is possible to select a particular cell lineage by cloning or physical cell separation or some other selection method. Such a cell line derived by selection or cloning is referred to as cell strain. Cell strains do not have infinite life, as they die after some divisions.

Finite Cell Lines :

The cells in culture divide only a limited number of times, before their growth rate declines and they eventually die. The cell lines with limited culture life spans are referred to as finite cell lines. The cells normally divide 20 to 100 times (i.e. is 20-100 population doublings) before extinction. The actual number of doublings depends on the species, cell lineage differences, culture conditions etc. The human cells generally divide 50-100 times, while murine cells divide 30-50 times before dying.

Continuous Cell Lines :

A few cells in culture may acquire a different morphology and get altered. Such cells are capable of growing faster resulting in an independent culture. The progeny derived from these altered cells has unlimited life (unlike the cell strains from which they originated).

They are designated as continuous cell lines.
The continuous cell lines are transformed, immortal and tumorigenic. The transformed cells for continuous cell lines may be obtained from normal primary cell cultures (or cells strains) by treating them with chemical carcinogens or by infecting with oncogenic viruses.

Selection of Cell Lines:

Several factors need to be considered while selecting a cell line.

1. Species:

In general, non-human cell lines have less risk of biohazards, hence preferred. However, species differences need to be taken into account while extrapolating the data to humans.

2. Finite or continuous cell lines:

Cultures with continuous cell lines are preferred as they grow faster, easy to clone and maintain, and produce higher yield. But it is doubtful whether the continuous cell lines express the right and appropriate functions of the cells. Therefore, some workers suggest the use of finite cell lines, although it is difficult.

3. Normal or transformed cells:

The transformed cells are preferred as they are immortalized and grow rapidly.

4. Availability:

The ready availability of cell lines is also important. Sometimes, it may be necessary to develop a particular cell line in a laboratory.

5. Growth characteristics:

The following growth parameters need to be considered:
i. Population doubling time
ii. Ability to grow in suspension
iii. Saturation density (yield per flask)
iv. Cloning efficiency.

6. Stability:
The stability of cell line with particular reference to cloning, generation of adequate stock and storage are important.

7. Phenotypic expression:

It is important that the cell lines possess cells with the right phenotypic expression.

Maintenance of Cell Cultures:
For the routine and good maintenance of cell lines in culture (primary culture or subculture) the examination of cell morphology and the periodic change of medium are very important.

Cell Morphology:
The cells in the culture must be examined regularly to check the health status of the cells, the absence of contamination, and any other serious complications (toxins in medium, inadequate nutrients etc.).

Replacement of Medium:
Periodic change of the medium is required for the maintenance of cell lines in culture, whether the cells are proliferating or non-proliferating. For the proliferating cells, the medium need to be changed more frequently when compared to non-proliferating cells. The time interval between medium changes depends on the rate of cell growth and metabolism.
For instance, for rapidly growing transformed cells (e.g. HeLa), the medium needs to be changed twice a week, while for slowly growing non-transformed cells (e.g. IMR-90) the medium may be changed once a week. Further, for rapidly proliferating cells, the sub-culturing has to be done more frequently than for the slowly growing cells.

Maintenance of cells in culture

It is important that after seeding, flasks are clearly labelled with the date, cell type and the number of times the cells have been subcultured or passaged. Moreover, a strict regime of feeding and subculturing should be established that permits cells to be fed at regular intervals without allowing the medium to be depleted of nutrients or the cells to overgrow or become super confluent. This can be achieved by following a standard but routine procedure for maintaining cells in a viable state under optimum growth conditions. In addition, cultures should be examined daily under an inverted microscope, looking particularly for changes in morphology and cell density. Cell shape can be an important guide when determining the status of growing cultures. Round or floating cells in subconfluent cultures are not usually a good sign and may indicate distressed or dying cells. The presence of abnormally large cells can also be useful in determining the well-being of the cells, since the number of such cells increases as a culture ages or becomes less viable. Extremes in pH should be avoided by regularly replacing spent medium with fresh medium. This may be carried out on alternate days until the cultures are approximately 90% confluent, at which point the cells are either used for experimentation or trypsinised and subcultured

The volume of medium added to the cultures will depend on the confluency of the cells and the surface area of the flasks in which the cells are grown. As a guide, cells which are under 25% confluent may be cultured in approximately 1 cm^3 of medium per 5 cm^2 and those between 25% and 40% or $\geqq$ 45% confluency should be supplemented with 1.5 cm^3 or 2 cm^3 culture medium per 5 cm^2, respectively. When changing the medium it is advisable to pipette the latter on to the sides or the opposite surface of the flask from where the cells are attached. This is to avoid making direct contact with the monolayers as this will damage or dislodge the cells.

THE FOLLOWING FACTORS NEED TO BE CONSIDERED FOR THE REPLACEMENT OF THE MEDIUM:

1. **Cell concentration:** The cultures with high cell concentration utilize the nutrients in the medium faster than those with low concentration; hence the medium is required to be changed more frequently for the former.

2. **A decrease in pH:** A fall in the pH of the medium is an indication for change of medium. Most of the cells can grow optimally at pH 7.0, and they almost stop growing when the pH falls to 6.5. A further drop in pH (between 6.5 and 6.0), the cells may lose their viability. The rate of fall in pH is generally estimated for each cell line with a chosen medium. If the fall is less than 0.1 pH units per day, there is no harm even if the medium is not immediately changed. But when the fall is 0.4 pH units per day, medium should be changed immediately.

3. **Cell type:** Embryonic cells, transformed cells and continuous cell lines grow rapidly and require more frequent sub-culturing and change of medium. This is in contrast to normal cells, which grow slowly.

4. **Morphological changes:** Frequent examination of cell morphology is very important in culture techniques. Any deterioration in cell morphology may lead to an irreversible damage to cells. Change of the medium has to be done to completely avoid the risk of cell damage.

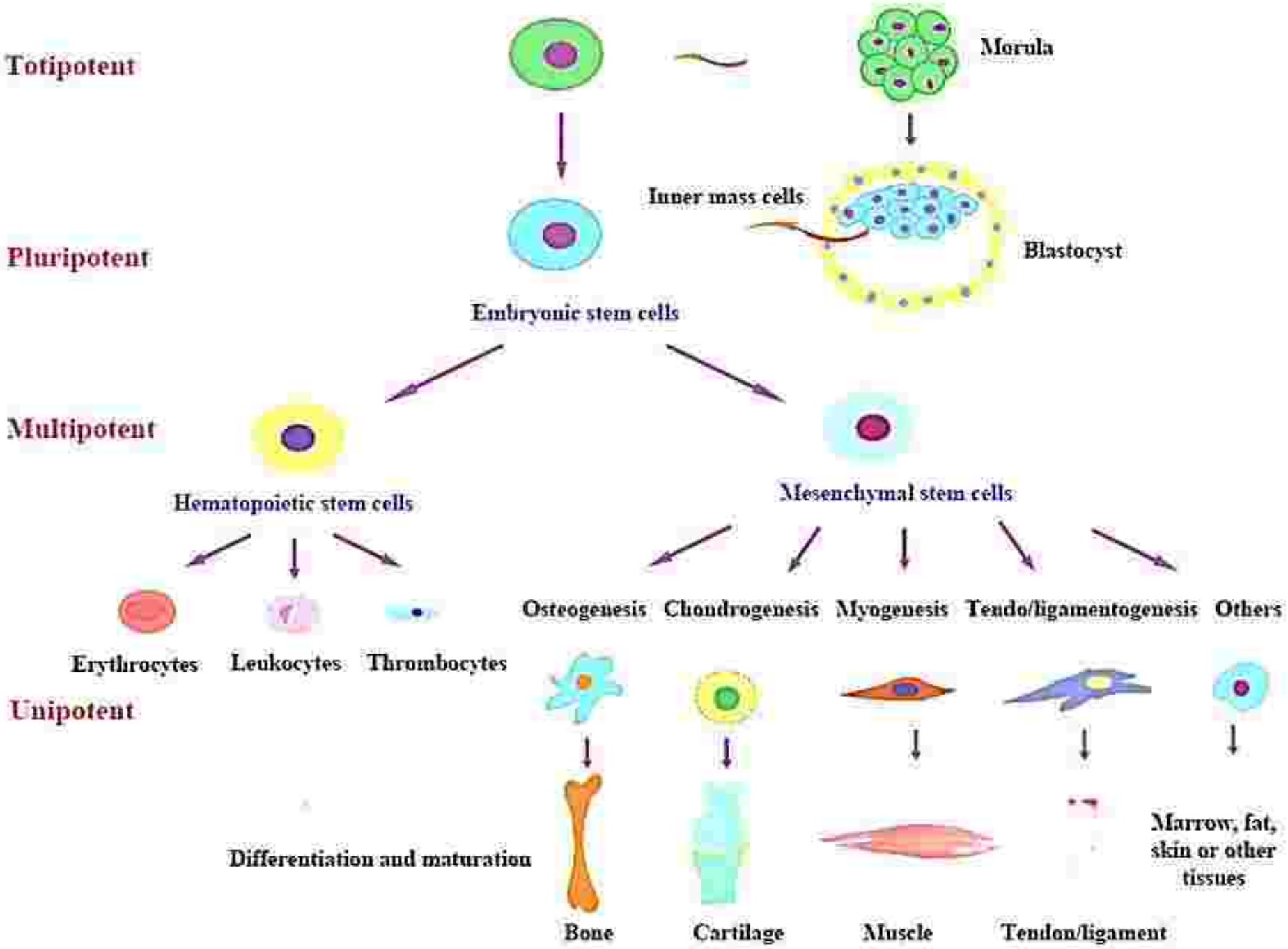
PLURIPOTENT AND TOTIPOTENT STEM CELLS

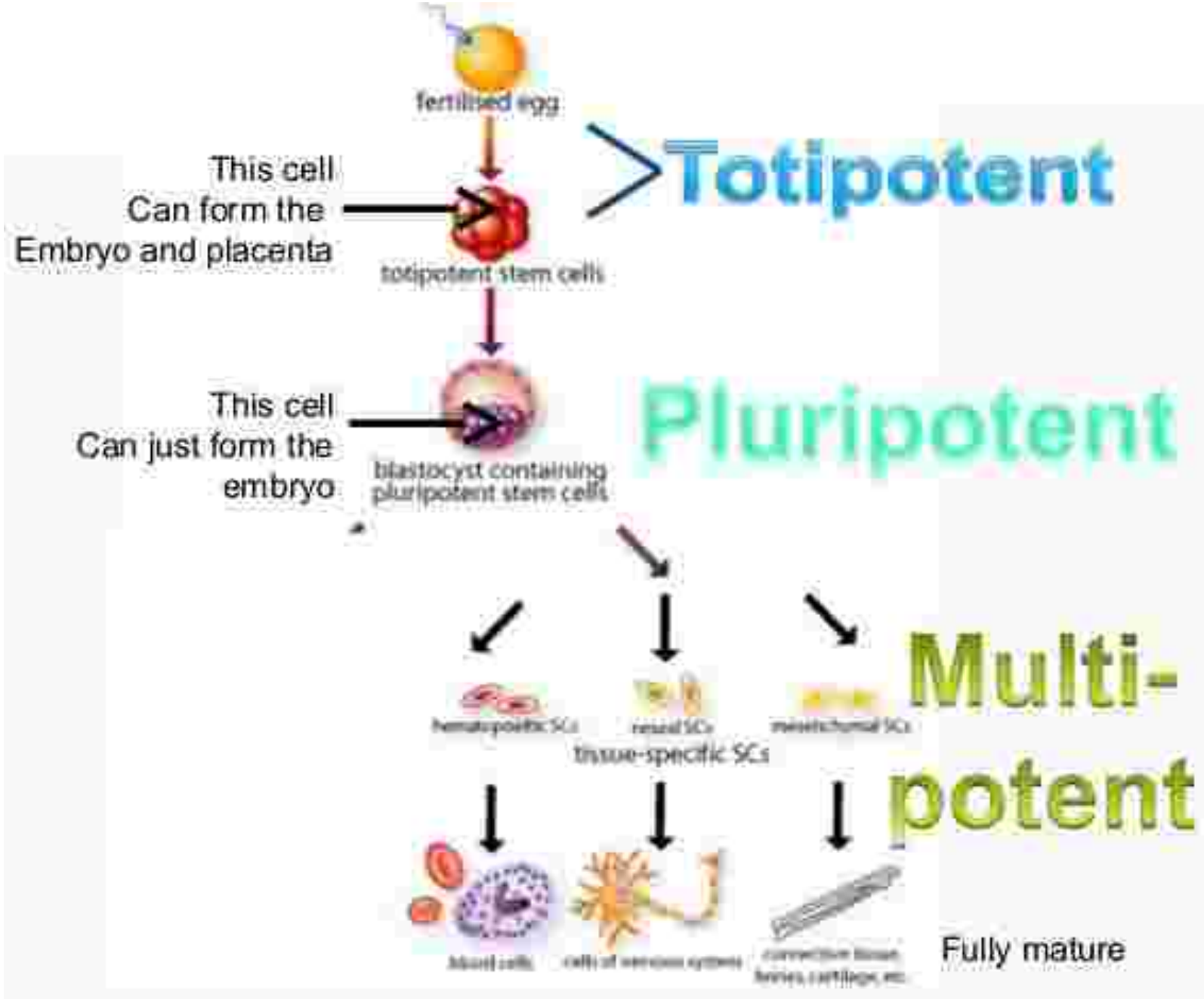
HIERARCHY OF CELL POTENCY

Totipotent Stem Cells: Totipotent (omnipotent) stem cells can give rise to any of the 220 cell types found in an embryo as well as extra-embryonic cells (placenta).

Pluripotent Stem Cells: Pluripotent stem cells can give rise to all cell types of the body (but not the placenta).

Multipotent Stem Cells: Multipotent stem cells can develop into a limited number of cell types in a particular lineage.


Totipotent
Morula
Inner mass cells
Pluripotent
Blastocyst
Embryonic stem cells
Multipotent
Hematopoietic stem cells
Mesenchymal stem cells
Erythrocytes
Leukocytes
Thrombocytes
Osteogenesis
Chondrogenesis
Myogenesis
Tendo/ligamentogenesis
Others
Unipotent
Differentiation and maturation
Marrow, fat, skin or other tissues
Bone
Cartilage
Muscle
Tendon/ligament


fertilised egg
This cell
Can form the
Embryo and placenta
totipotent stem cells
Totipotent
This cell
Can just form the
embryo
blastocyst containing
pluripotent stem cells
Pluripotent
Multi-
potent
tissue-specific SCs
Fully mature

Totipotent vs Pluripotent vs Multi-potent Comparaison Charte

	Totipotent	Pluripotent	Multipotent
Relative potency	High	Medium	Low
Cell types capable of generating	Differentiate into any cell type	Differentiate into cells from any of the three germ layers	Differentiate into a limited range of cell types
Terminology	Toti = Whole	Pluri = Many	Multi = Several
Examples	Zygote, early morula	Embryonic stem cells, Induced pluripotent stem cells	Haematopoietic stem cells, neural stem cells, mesenchymal stem cells
Found	Early cells of fertilised egg	Inner mass cells of the blastocyst	In many tissues
Expression of pluripotency genes	+++	++	+
Expression of lineage-specific genes	+	++	+++
Pros of use in research	Easy to isolate and grow	Easy to isolate and grow	Less ethical issues, less chance of immune rejection if taken from same patient
Cons of use in research	Ethical issues	Ethical issues, teratoma formation	Hard to isolate, limited differentiation, scarce

APPLICATION OF STEM CELLS

STEM CELLS ARE CALLED THE BUILDING BLOCKS OF LIFE, BECAUSE AN ENTIRE HUMAN BEING DEVELOPS FROM THE VERY FIRST STEM CELL. THE POTENTIAL OF STEM CELLS THEREFORE IS ENORMOUS AND ALREADY PROVIDES FOR ENTIRELY NEW THERAPEUTIC OPTIONS IN THE FIELD OF INDIVIDUALIZED, REGENERATIVE MEDICINE

- Stem cells from the umbilical cord are special. They are young, potent, and viable. Numerous clinical studies are being conducted worldwide researching the suitability of stem cells for the regeneration of damaged tissues after accidents, degenerative diseases like e.g. slipped intervertebral discs, or cancer treatment.

- Stem cells have been applied in the treatment of serious diseases for more than 55 years. They are applied especially to treat cancers, which require high-dose chemotherapy within the scope of medical care. The patient's own stem cells are extracted from bone marrow or peripheral blood prior to high-dose chemotherapy, stored temporarily and transplanted after the treatment in order to minimize the side effects of the aggressive chemotherapy and to support the regeneration of destroyed cells.

- Besides cancer, several 100,000 people come down with common diseases like dementia, which belongs to the neurodegenerative diseases, cardiac infarction, stroke, arthritis, or diabetes every year. The lifelong therapy causes enormous costs in the health care system. Stem cell therapy offers great potential for the treatment of such diseases. Experts expect that every seventh person up to the age of 70 will need a therapy based on stem cells in the future to regenerate sick or aged cells and tissues.

Stem cells have already been applied successfully for:

- Hematopoietic disorders
  - Acute and chronic leukemia (AML/ALL or CML/CLL)
  - Myelodysplastic syndrome
  - Lymphomas (Hodgkin lymphoma, non-Hodgkin lymphoma)
  - Aplastic anemia
  - Sickle cell anemia
  - Beta thalassemia

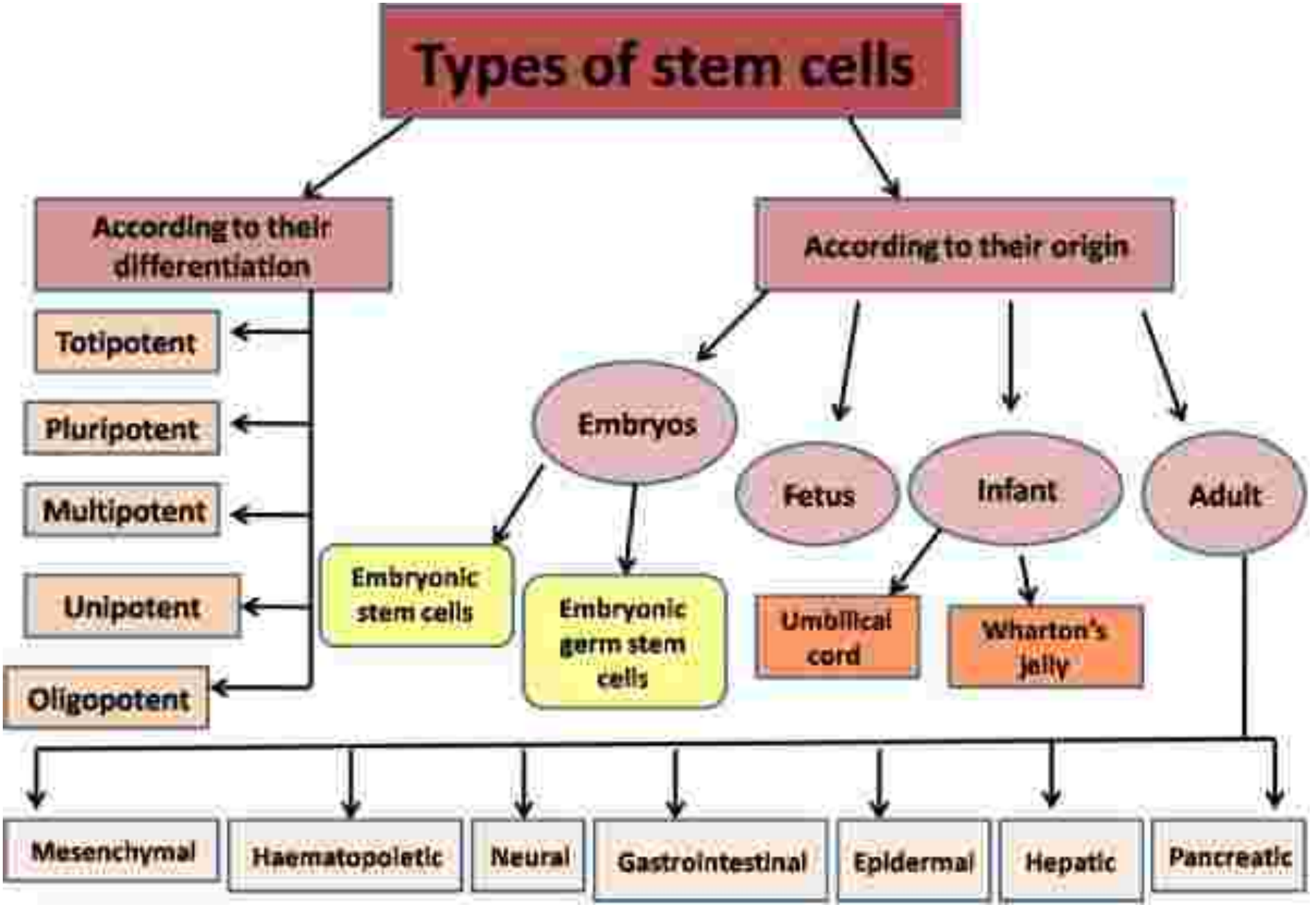
- Immunodeficiency
  - SCID
  - Whiskott Aldrich syndrome

- Metabolic disorders
  - Mucopolysaccharidosis

- Cancer
  - Multiple myeloma
  - Neuroblastoma

In clinical studies and treatment attempts, stem cell therapies are tested with the following indications:

- Autoimmune diseases
  - Diabetes mellitus type 1
  - Rheumatoid arthritis
  - Lupus
  - Crohn's disease
- Graft-versus-host disease (GvHD)
- Impairments of the brain
  - Dementia, in particular Alzheimer's disease
  - Stroke
  - Brain injuries due to accidents or cancer
  - Infantile brain damage (cerebral paresis)
- Cardiovascular diseases
  - Cardiac infarction
- Multiple sclerosis
- Amyotrophic lateral sclerosis
- Autism
- Hearing loss
- HIV
- Cirrhosis of the liver
- Epidermolysis bullosa ("butterfly children")


Types of stem cells
According to their differentiation
Totipotent
Pluripotent
Multipotent
Unipotent
Oligopotent
According to their origin
Embryos
Fetus
Infant
Adult
Embryonic stem cells
Embryonic germ stem cells
Umbilical cord
Wharton's jelly
Mesenchymal
Haematopoietic
Neural
Gastrointestinal
Epidermal
Hepatic
Pancreatic

Promises of stem cells in regenerative medicines

(1)
(i) Improvement of spinal cord injury
(ii) Regeneration of retinal sheet
(iii) Generation of retinal ganglion cells
(iv) Healing of heart defects
(v) Hepatic cell formation
(vi) Formation of insulin secreting β-cells
(vii) Cartilage lesion treatment
(viii) Regeneration of pacemaker
(ix) In vitro gametogenesis

(2)
(i) Treatment of diabetes and retinopathy
(ii) Neurodental therapeutic applications
(iii) Restoration of cognitive functions
(iv) Brain and cancer treatment
(v) Ear acoustic function restoration
(vi) Regeneration of intestinal mucosa
(vii) Treatment of vision defects
(viii) Muscle regeneration
(ix) Regeneration of fallopian tube

(3)
(i) Regeneration of bladder tissue
(ii) Muscle regeneration
(iii) Regeneration of teeth tissue
(iv) Healing of orthopedic injuries
(v) Recovery from muscle injuries
(vi) Hear scar repair after attack

Healthy donor

(1) ESCs, (2) TSPSCs,
(3) MSCs , (4) UCSCs,
(5) BMSCs , (6) IPSCs

Patient

(4)
(i) T1DM and T2DM treatment
(ii) SLE (autoimmune disease) treatment
(iii) Application for HI treatment
(iv) Krabbe's disease treatment
(v) Hematopoiesis in neuroblastoma

(5)
(i) Treatment of anemia and blood cancer
(ii) Retroviral therapy
(iii) Correction of neuronal defects
(iv) Generation of functional platelets
(v) Alveolar bone regeneration
(vi) Regeneration of diaphragm tissue

(6)
(i) Regeneration of kidney tissue
(ii) Vision restoration in AMD
(iii) Treatment of placental defects
(iv) Treatment of brain cortex defects
(v) ASD and autism treatment
(vi) Treatment of liver and lung disease
(vii) Generation of serotonin neurons
(viii) Regeneration of pacemaker

MEASURE OF VIABILITY AND TOXICITY

Determination of cell viability is extremely important, since the survival and growth of the cells may depend on the density at which they are seeded. The degree of viability is most commonly determined by differentiating living from dead cells using the dye exclusion method. Basically, living cells exclude certain dyes that are readily taken up by dead cells. As a result, dead cells stain the colour of the dye used whilst living cells remain refractile owing to the inability of the dye to penetrate into the cytoplasm. One of the most commonly used dyes in such assays is trypan blue. This is incubated at a concentration of 0.4% with cells in suspension and applied to a haemocytometer. The haemocytometer is then viewed under an inverted microscope set at 100 × magnification and the cells counted

The parameters that define cell viability in a particular experiment can be as diverse as the redox potential of the cell population, the integrity of cell membranes, or the activity of cellular enzymes such as esterases. Each assay provides a different snapshot of cell health, and can individually or together form the basis of an assay for cell viability, cytotoxicity, or drug efficacy with several integrated components.

Specially designed cell viability indicators have been developed for sensing the different characteristics and providing a visual readout of cell health using a fluorescence microscope, microplate reader, or flow cytometer. All indicators have positive and negative attributes; however, their sensitivity, reliability, and compatibility with relevant cell lines are important factors in determining utility.

The total number of cells is calculated using the following equation as described previously:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor} \times \text{dilution factor}$$

and the percentage of viable cells determined using the following formula:

$$\%\ \text{viability} = \frac{\text{number of unstained cells counted}}{\text{total number of cells counted}} \times 100$$

To avoid underestimating cell viability it is important that the cells are not exposed to the dye for more than 5 min before counting. This is because uptake of trypan blue is time sensitive and the dye may be taken up by viable cells during prolonged incubation periods. Additionally, trypan blue has a high affinity for serum proteins and as such may produce a high background staining. The cells should therefore be free from serum, which can be achieved by washing the cells with PBS before counting.

Microplate assays for cell viability

Population-based assays for measuring cell viability.

- Cellular reduction potential
- Multi-parameter assays for live and dead cells

Imaging microbial viability

Imaging assays optimized for use with bacteria and yeast.

- Imaging bacteria and yeast
- Imaging biofilms

Imaging mammalian cell viability

Assays for measuring cell viability using imaging.

- High-content, multi-parameter imaging assays
- Scalable from single slides to microplates

Other Imaging Assays

Related assays often used to assess viability and cytotoxicity.

- Cell proliferation
- Mitochondria function assays
- Cellular senescence

CYTOTOXICITY ASSAYS: IN VITRO METHODS TO MEASURE DEAD CELLS

Membrane integrity is the feature most often used to detect whether eukaryotic cells cultured *in vitro* are alive or dead. Cells that have lost membrane integrity and allow movement of otherwise non-permeable molecules are classified as non-viable or dead. Detection of dead cells is accomplished by measuring movement of molecules either into or out of cells across membranes that have become leaky and cannot be repaired. A major class of molecules that serve as indicators of dead cells include markers that exist in the cytoplasm of viable cells, but leak into the surrounding culture medium upon loss of membrane integrity. The marker can exist naturally such as an enzyme, or be introduced artificially, such as loading radioactive [^{51}Cr] or a fluorescent marker into viable cells. Artificially introduced markers enable selective detection of target cell cytotoxicity for experiments using more than one population of cells such as cell mediated cytotoxicity. A second class of molecules that serves as an indicator of dead cells is referred to as "vital dyes". These dyes typically are not permeable to viable cells, but can enter dead cells through damaged membranes. Examples include trypan blue and many fluorogenic DNA binding dyes. Addition of these molecules to cells in culture results in selective staining of the dead cells.

A frequent use of cells in culture is for a commonly used cytotoxicity assay where cells are exposed to a test compound and after some period of incubation, a marker is measured to reflect the number of viable cells present compared to positive (toxin) and negative (vehicle) control treatments. In addition to estimating the number of live cells, it can be of great value to measure the number of dead cells that have accumulated over the course of the experiment and to be able to distinguish between cytotoxicity and cytostasis or growth arrest. In some cases, estimating the number of accumulated dead cells may be more sensitive than measuring a decrease in a marker used to estimate viable cell number.

Dyes That Selectively Penetrate Dead Cells

Trypan blue

The selective staining of dead cells with trypan blue and microscopic examination on a hemocytometer is one of the most frequently used routine methods to determine the cell number and percent viability in a population of cells. The general concept is that trypan blue is excluded from live cells, but penetrates dead cells with a damaged plasma membrane. Longer incubations with solutions of trypan blue may result in faint staining of the viable cells in the population, possibly due to slow uptake of dye molecules. The mechanism of selective staining of dead cells may actually involve impermeability of aggregates of trypan blue (1). There are several sources for published protocols or instructional videos describing the use of trypan blue and the many details associated with correctly using a hemocytometer (2).

Counting cells using a hemocytometer

Cell Counting Using the Trypan Blue Exclusion Method

Counting of cells using Trypan Blue and a haemocytometer

The trypan blue staining technique is usually performed on a single sample (such as when passaging a stock culture flask of cells) or relatively small numbers of samples from simple experiments. The main disadvantages of this technique are: the error involved with measuring a single sample, the subjective judgement of the user to determine what is a dead cell or stained debris, inconsistency among operators, and the time and manual labor involved with measuring multiple samples (3). There are benchtop instruments designed to automate imaging and improve the biased analysis steps of this basic staining technique using trypan blue or other fluorescent vital dyes (Bio Rad TC10 / TC20 Automated Cell Counter; Olympus Cell Counter model R1; ThermoFisher Scientific Countess II Automated Cell Counter (fluorescent); Roche Cedex HiRes Analyzer; Nexcelom Bioscience Cellometer Auto T4); however, the primary intent of these automated instruments is to establish reliable and reproducible counting of live and dead cells prior to seeding into microwell plates and not for high-throughput

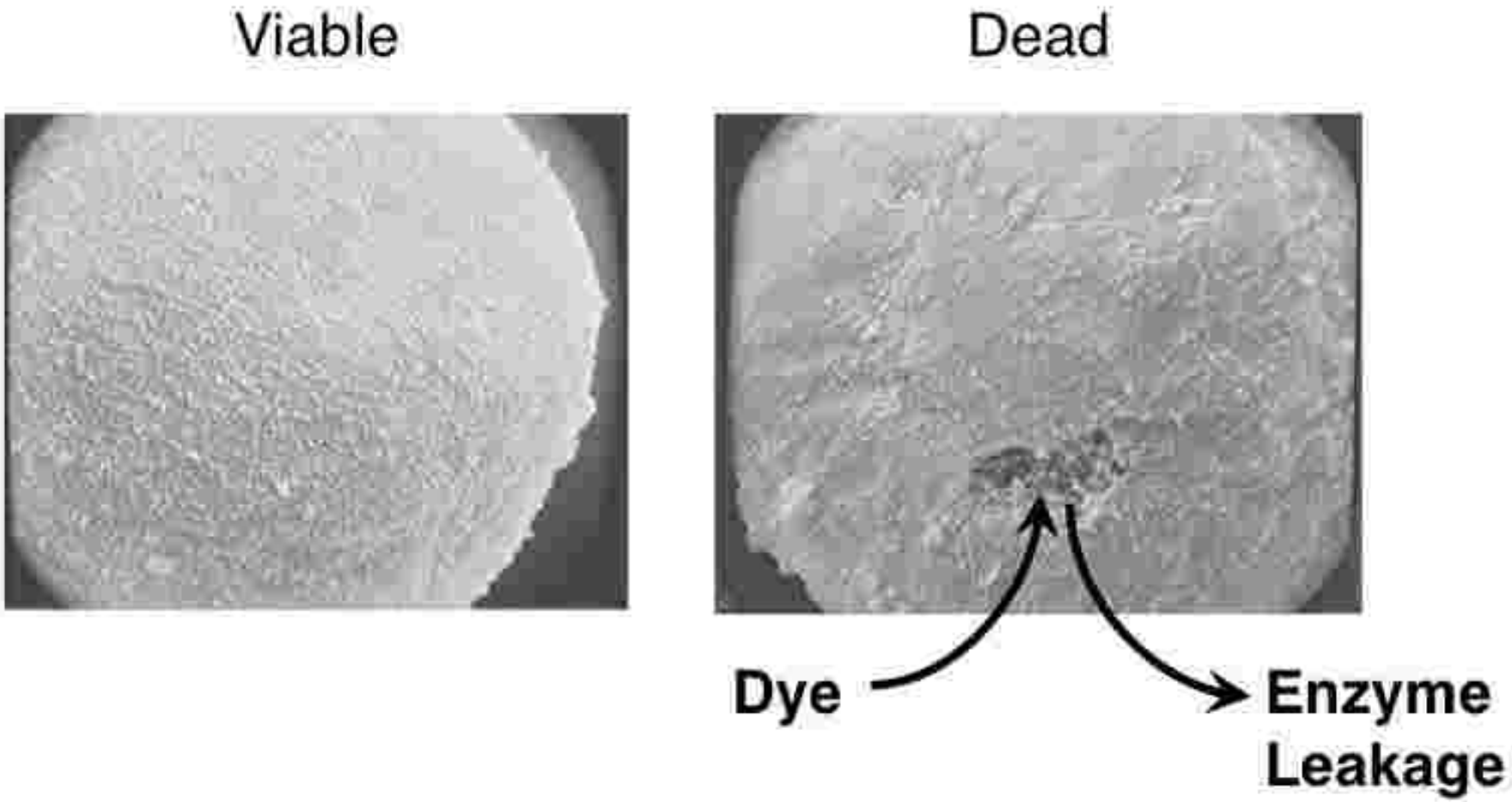


Figure 1. Illustrates scanning electron micrographs of isolated rat hepatocytes. The left image is meant to depict an intact live cell and the image on the right depicts a dead cell with a damaged membrane. The loss of membrane integrity enables leakage of dead cell markers such as enzymes from the cytoplasm into the surrounding culture medium and penetration of materials (vital dyes) that normally are non-permeable to live cells.

Fluorescent DNA Binding Dyes That Penetrate Dead Cells

There is a large number of nucleic acid binding dyes that can be used to stain cells for microscopy or flow cytometry but have limitations for use with assays using plate readers to detect signal. Many dyes have somewhat similar properties which make it difficult to choose the most appropriate probe for a particular purpose.

There are many fluorescent DNA binding dyes to select from which are generally considered to be non-permeable to viable cells and can be used for detection of the accumulation of dead cells in culture using a multi-well plate format; however, there are a variety of factors to consider when selecting the most appropriate dye for assay development. The most important and practical factors to consider when choosing a dye include: the emission wavelength, selectivity for staining DNA, cell permeability, solubility at the vendor-recommended concentration, detection sensitivity and cytotoxicity. Fluorogenic DNA dyes that readily pass through the intact cell membrane and stain the nucleus of live cells should be avoided for consideration for measuring dead cells.

An important factor to consider when choosing any fluorescent dye is the emission wavelength spectrum. Knowledge of the excitation and emission spectra and the extent of any spectral overlap can be used to predict compatibility of two fluorescent assays and to select an appropriate filter set to avoid overlap of emission of different fluorophores. For example, a green-emitting DNA binding dye would be a logical candidate to multiplex with an assay detecting a red fluorescent protein.

The DNA binding dyes can be considered to be environmental sensors, meaning they change fluorescent properties after binding to various molecules. The various nucleic acid binding dyes may exhibit between a 20- to 1000-fold increase in fluorescence upon binding to double stranded DNA. That fold-increase can contribute to the relative sensitivity of detection of the number of dead cells. In most experimental conditions using a growing population of cultured cells *in vitro*, the quantity of DNA is proportional to the total number of cells present; however, changing culture conditions to induce rapid cell growth, to starve cells of nutrients, or induce differentiation to result in multinucleation may have a greater influence to change the amount of nucleic acid present in cells. The ideal situation for quantitatively detecting dead cells is for the dye to selectively bind to double stranded DNA. If the dye binds to double stranded RNA which may change under stimulatory or stressful culture conditions, using dyes that also bind to RNA can lead to artifacts and misinterpretation of results.

Even slight adverse effects of DNA binding dyes can limit their usefulness for real-time assay protocols where the dye is exposed to cells for extended periods of time. Dyes that cause cytotoxicity upon long term exposure to cells may be the result of partial permeability. Membrane permeability may depend on the cell type, the overall health of the cells or whether the dyes are substrates for efflux pumps that result in expulsion from the cytoplasm even if the dye does enter the cell. Reagent toxicity is not a problem if the dye is going to be used to stain cells for an endpoint assay protocol; however, cytotoxicity is critical to consider if cells will be cultured in the presence of the dye for an extended period to perform a real-time assay.

The use of DNA binding dyes for long term real-time detection of the accumulation of dead cells must consider if there is any influence of the assay reagent on the health or responsiveness of the cell model system. For example, for some cell viability assays, it is well known that reagents to estimate viable cell number (e.g. MTT and resazurin) can be toxic to the population of cells, even during a few hours of exposure . Similar reagent cytotoxicity effects are known for the DNA binding dyes. **Figure 2** shows the effects of three different DNA binding dyes continuously exposed to four different cell types for 72 hours before measuring cell viability using an ATP assay.

The data suggest similar nucleic acid binding dyes may have different effects on cell viability and those effects can be cell type specific. Understanding the mechanism and use of these DNA dyes is therefore important to determine the best probe for the desired assay design. Like all potential toxins, the cytotoxic effect of assay reagent components can be expected to depend on the concentration, the duration of exposure, and the susceptibility of individual cell types. Appropriate controls (vehicle only without dye) are recommended to validate each dye and cell type combination to determine if there is a cytotoxic effect of the assay reagent. It is suggested to use the vendor recommended concentration as a starting point and test a range of concentrations of dyes with each cell model system to confirm there is not an artefactual cytotoxic or cytostatic effect.

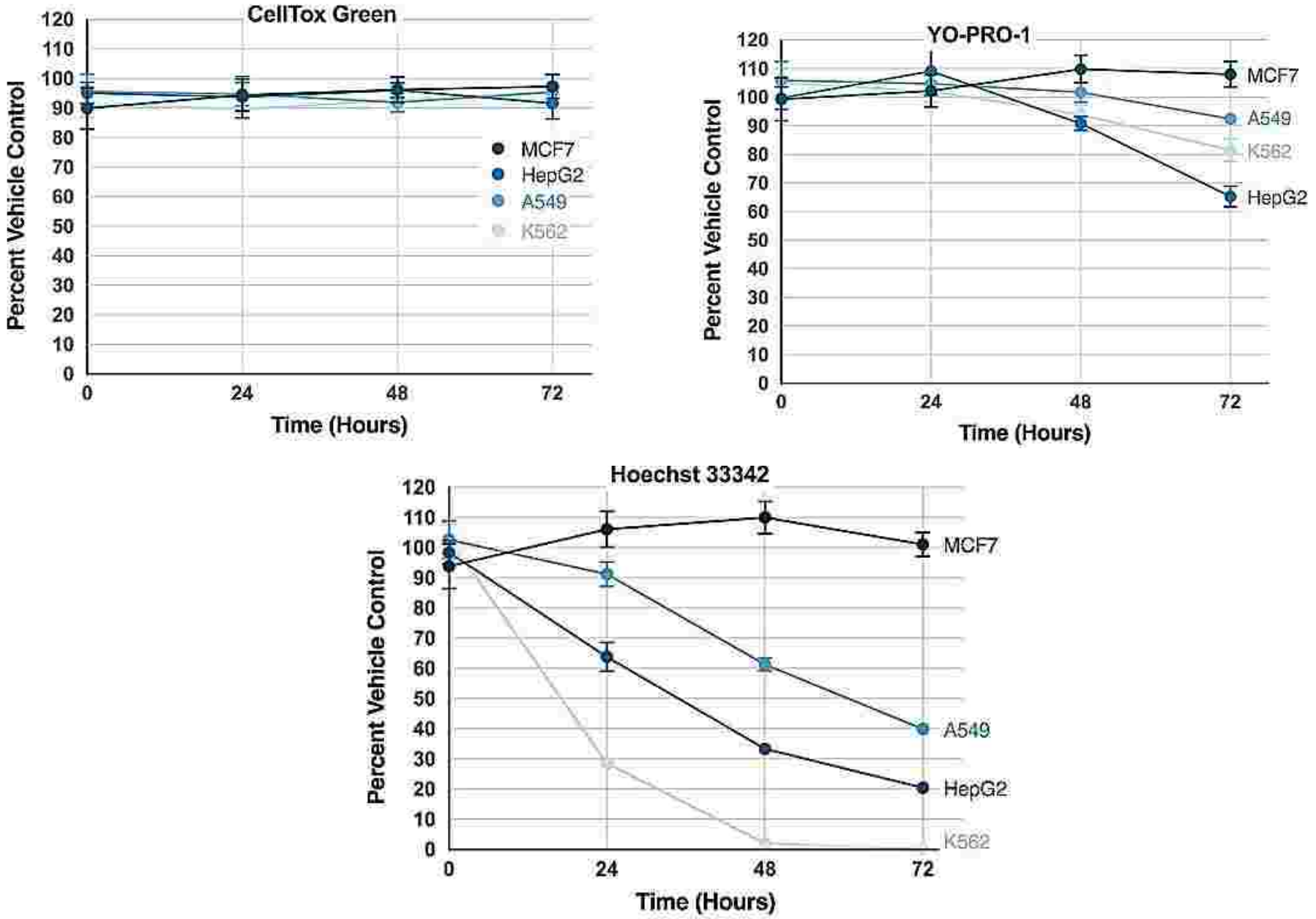


Figure 2. Effect of DNA binding dyes on cell viability. Four different cell types were treated with the vendor recommended concentration of DNA binding dye and cell viability assayed at various times up to three days using ATP content as the marker.

Markers That Leak Out of the Cytoplasm of Dead Cells into Culture Medium

The presence of dead cells that have lost membrane integrity can be detected by measuring markers that leak from the cytoplasm into the culture medium. The most common marker used for this type of assay is lactate dehydrogenase Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactate and in the process, converts NAD^+ to NADH. The reducing capacity of NADH can be used to reduce a variety of substrate molecules into products that are either colored, fluorescent, or luminogenic. **Figure 5** illustrates the general scheme and assay chemistry used to detect LDH-release from the cytoplasm of dead cells. An excess amount of lactate and NAD+ as substrates are delivered in a reagent mixture to drive LDH to generate pyruvate and NADH. The reducing power of NADH is used to convert the substrate (resazurin) into the fluorogenic product (resorufin). Colorimetric versions of this assay chemistry have used a tetrazolium compound as the diaphorase substrate which is converted into an intensely colored formazan product that can be measured using a spectrophotometer. Similarly, a luminometric assay can use a "pro-luciferin" substrate which is converted into a luciferin product that is linked to a firefly luciferase reaction to generate a luminescent signal. The colorimetric version of the assay was developed decades ago and lacks detection sensitivity. In addition, because of buffer incompatibility with live cells, it requires removal of culture supernatant to a different container to perform the assay. The fluorescent assay protocol is homogeneous and more sensitive than the colorimetric version. Advances in formulating the reagent to be compatible with viable cells enabled a homogeneous fluorescent protocol to be developed. The luminogenic version of the assay is far more sensitive than the fluorogenic version that enables sampling of 2-5 µl of culture supernatant at various times which can be stored frozen for future analysis of the trends of LDH release over time.

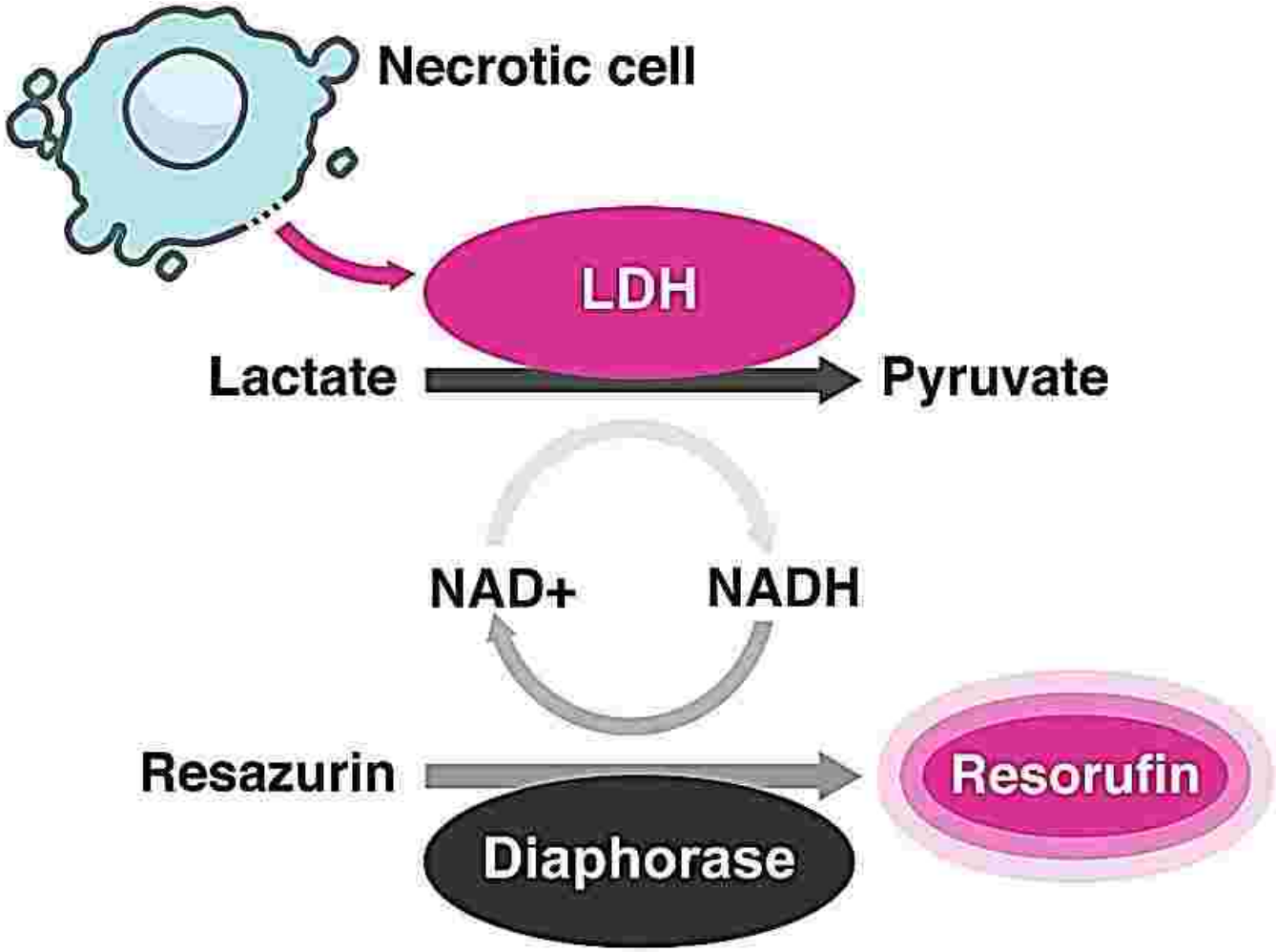


Figure 5. A schematic representation of the fluorogenic LDH-release assay chemistry. LDH from dead cells that leaked into the culture medium catalyzes the conversion of lactate to pyruvate and in the process generates NADH. In the presence of the diaphorase (reductase) enzyme, the NADH can reduce resazurin into the fluorogenic resorufin product that can be measured using a plate reading fluorometer.

Other enzymes that do not use the NADH cycling assay chemistry also have been used as markers of dead cells. Examples include enzymes such as adenylate kinase (AK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that can produce ATP by providing a reaction cocktail containing the necessary ingredients to generate a cycling assay chemistry however, those enzymes may be less stable than LDH and lose enzymatic activity sooner after release from dead cells.

Another option is to measure protease activity as a marker that is released from dead cells with damaged membranes . Aminopeptidase activity can be measured using substrates containing a short sequence of amino acids (alanine-alanine-phenylalanine) conjugated via a peptide bond to either rhodamine 110 or aminoluciferin. Enzymatic removal of the amino acids can generate free rhodamine-110 for a fluorescent assay or free aminoluciferin which can be used by firefly luciferase to generate light

Another optional assay approach is to load cells to contain a measurable marker. Loading target cells with an artificial measurable marker such as pro-fluorescent Calcein-AM or radioactive ^{51}Cr has been used to measure cytotoxicity, mostly for assays that involve mixtures of more than one cell type (e.g. effector and target cells in antibody dependent cell mediated cytotoxicity assays). Target cells incubated with ^{51}Cr will take up the radioactive marker which becomes bound as protein complexes in the cytoplasm of live cells. Similarly, calcein-AM is taken up by live cells where cytoplasmic esterase activity removes the AM group to generate fluorescent calcein which is retained in live cells. Upon cell death and loss of membrane integrity, the fluorescent calcein or the radioactive ^{51}Cr is released from the cytoplasm into the culture medium where they can be quantified relative to the background spontaneous release from the viable cell population. The disadvantages of this approach include: the extra handling step to label the target cells prior to performing an assay, spontaneous release from the live cell population, and the safety and cost issues associated with using and disposing of radioactivity.

Still another cytotoxicity assay option is to genetically engineer cells to express luciferase as a marker . When cells die, the luminescence declines because the luciferase activity diminishes once cytoplasmic components are released into the culture medium. An advantage of this approach is the ability to selectively measure death of one type of cell in a mixed cell culture model such as antibody dependent cell mediated cytotoxicity assays. Disadvantages include the need to engineer cells to express luciferase and the assay measures a decrease in luminescent signal with increased cell killing which may make it difficult to detect small changes in the number of dead cells.

References

1. Satyanarayana, U. “Biochemistry” 5th Edition, Elsevier India 2017, ISBN: 9788131249406
2. Voet D, Voet J. .G “Biochemistry”, 4th Edition, John Wiley & Sons, Inc.,2010. ISBN: 978-0-470-57095-1
3. David L. Nelson; Michael M. Cox “Lehninger Principles of Biochemistry” Seventh Edition, Macmillan 2017, ISBN:9781464187957
4. Wilson, K., Walker, J. (eds.); Cambridge University Press, Cambridge, 2000, 784 pp., ISBN 0-521-65873-X (paperback)

All the original contributors of the concept and findings published are gratefully acknowledged while preparing the e-content for the students of Biotechnology and allied sciences