DEPARTMENT OF BIOTECHNOLOGY

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Subject Name	Cell culture techniques
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Cell Culture

Techniques

Cell culture is the multifaceted process through which cells are isolated from animal or plant and their subsequent growth under controlled artificial conditions, generally outside their natural environment. In this procedure cells are directly isolated from body or disaggregated by enzymatic or mechanical procedure or they may be derived from cell lines or cell strains. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. The in vitro propagation of cells has become a common practice in many laboratories for a huge numbers of applications. The ranges of cell types grown are vast. Generally the cells are sensitive to a wide range of compounds and it is therefore necessary to ensure that they come into contact onlywith those under study and not with extraneous materials. Adherent mammalian cells require a suitable surface for attachment.

Primary cell culture: Primary cell culture is the primary step of cell culturing in which the cell is first isolated from tissue and then proliferated under the appropriate conditions until they consume all available contents for their growth. Now the cell is ready for subculturing by transferring them to new growth medium that furnish more opportunity for continued growth.

Cell lines: A cell line is a permanently established cell culture that will proliferate indefinitely in appropriate fresh medium and space. Cell lines differ from cell strains in that they have absconded the Hayflick limit and become immortalised. The Hayflick limit (or Hayflick Phenomenon) is the number of times a normal cell population will divide before it stops, presumably because the telomeres reach a critical length. A cell line arises from a primary culture at the time of the first successful subculture. The terms finite or continuous are used as prefixes if the status of the culture is known.

Cell Strain: By applying cloning, the positive population of cell lines are selected, therefore this cell lines now becomes a cell strain. A cell strain often acquires additional genetic changes resulting to the initiation of the parent line.

There are two types of cell culture:

Continuous cell culture: A continuous cell culture is one that is apparently capable of an unlimited number of population doublings, often referred to as an immortal cell culture. Such cells may or may not express the characteristics of in vitro neoplastic or malignant transformation. Continuous cell lines are usually aneuploid and often have a chromosome number between the diploid and tetraploid values. There is also considerable variation in chromosome number and constitution among cells in the population (heteroploidy).

Some important properties of Continuous cell lines:

- Reduced serum requirement
- Reduced density limitation of growth
- Growth in semisolid media
- Aneuploidy

Several normal cells do not give rise to continuous cell lines. The classical example are normal human fibroblasts that remain euploid throughout their life span and at crisis (usually around 50 generations) will stop dividing, although they may remain viable for up to 18 months thereafter. Human glia and chick fibroblasts behave similarly. Epidermal cells, on the other hand, have shown gradually increasing life spans with improvements in culture techniques.

Finite cell culture: A finite cell culture is capable of only a limited number of population doublings after which the culture ceases proliferation.

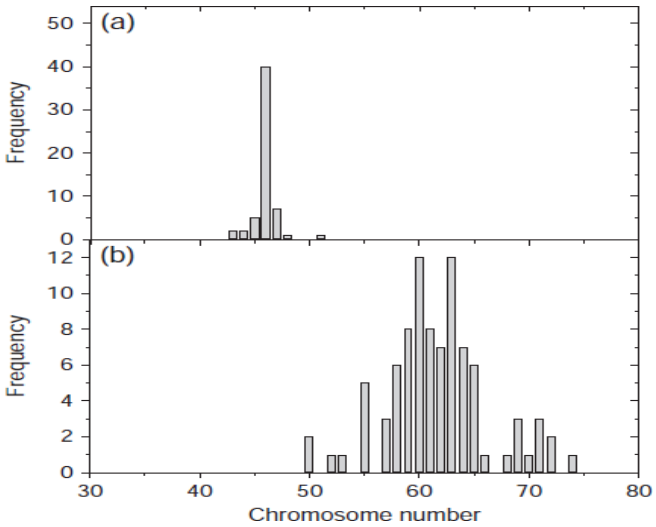


Figure 1: Chromosome Numbers of Finite and Continuous Cell Lines.

(a) A normal human glial cell line. (b) A continuous cell line from human metastatic melanoma.

Table 1: Properties of finite and continuous cell lines

Properties	Finite	Continuous(transformed)
Ploidy	Euploid, Diploid	Aneuploid, Hetroploid
Transformation	Normal	Immortal growth control altered and tumerigenic
Anchorage dependence	Yes	No
Contact inhibition	Yes	No
Density limitation of cell proliferation	Yes	Reduced or lost
Mode of growth	Monolayer	Monolayer or suspension
Maintainance	Cyclic	Steady state possible
Serum Requirement	High	Low
Cloning efficiency	Low	High
Markers	Tissue specific	Chromosomal, enzymatic, antigenic
Special function (e.g. virus susceptibility and differentiation)	May be retained	Often lost
Growth rate	Slow(T_D of 24-96 h)	Rapid(T_D of 12-24 h)
Yield	Low	High
Control parameter	Generation time, tissue specific marker	Stain characteristics

Features of cell line: To use any cell line for the production of biological product, one should have knowledge of following things related to cell lines:

- Age, sex and species of the donor tissue.
- For human cell lines, the donor's medical history and if available, the results of tests performed on the donor for the detection of adventitious agents
- Culture history of the cell line including methods used for the isolation of the tissues from which the line was derived, passage history, media used and history of passage in animals, etc.
- Previous identity testing and the results of all available adventitious agents testing

Characteristics of Cell lines: Each cell line is distinguished by characteristic features which render these cells unique and biomedically or biotechnologically useful.

- The growth pattern and morphological appearance of the cell line should be determined and should be stable from the master cell bank to the end-of-production cells.
- If there are specific markers that may be useful in characterizing the cell line (such as marker chromosomes, specific surface markers), these should be characterized for stability.
- Mostly cultured cell lines are allowed to generate their own ECM (extra cellular matrix), but primary culture and propagation of some specialized cells, exogenous provision of ECM.
- Many transformed cell lines have provided the best model for the induction of differentiation.
- Since normal cells has limited dividing capacity, therefore after a fixed number of population doublings cell lines derived from normal tissue will die out. This is a genetically determined event involving several different genes and this phenomenon is known as senescence. If the cells have an identified finite life expectancy, the total number of population doubling levels through senescence should be determined.

- Some cell lines may avoid senescence and give rise to continuous cell lines. The ability of a cell line to grow continuously probably reflects its capacity for genetic variation, allowing subsequent selection.
- A common feature of many human continuous cell lines is the development of a subtetraploid chromosome number. The alteration in a culture that gives rise to a continuous cell line is commonly called in vitro transformation and may occur spontaneously or be chemically or virally induced.

Table 2: Selection in cell line Development

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

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

1. **pH:** Most cell lines grow well at pH 7.4. Although the optimum pH for cell growth varies relatively little among different cell strains, some normal fibroblast lines perform best at pH 7.4 to pH 7.7, and transformed cells may do better at pH 7.0 to pH 7.4.
2. **Buffering:** Culture media must be buffered under two sets of conditions:
   a) Open dishes, where the evolution of CO_2 causes the pH to rise
   b) Overproduction of CO_2 and lactic acid in transformed cell lines at high cell concentrations, when the pH will fall.
3. **Temperature:** The temperature recommended for most human and warm-blooded animal cell lines is 37°C, closely to body heat, but generally set a little lower for safety, because overheating may become major problem than under heating.

4. **Media:** Although many cell lines are still propagated in medium supplemented with serum, in many instances cultures may now be propagated in serum-free media. Media that have been produced commercially will have been tested for their capability of sustaining the growth of one or more cell lines. However under certain circumstance we can use our own media.
5. **Growth curve:** A growth curve gives three parameters of measurement: (1) the lag phase before cell proliferation is initiated after subculture, indicating whether the cells are having to adapt to different conditions; (2) the doubling time in the middle of the exponential growth phase, indicating the growth promoting capacity of the medium; and (3) the maximum cell concentration attainable indicating whether there are limiting concentrations of certain nutrients. In cell lines whose growth is not sensitive to density (e.g., continuous cell lines), the terminal cell density indicates the total yieldpossible and usually reflects the total amino acid or glucose concentration.

Generation of cell lines: Stably transfected cell lines are used extensively in drug discovery. Cell lines expresses a target of interest, such as a G-protein coupled receptor (GPCR) or a reporter gene, form the basis for most cell-based compound screening campaigns. In establishing new assays for high throughput screening, creation of the appropriate cell line is a bottleneck. Typically, a stable cell line is created by transfection with a plasmid encoding the target of interest or reporter gene construct, and an additional gene which allows for chemical selection of successfully transfected cells (usually an antibiotic resistance gene). Through a lengthy selection process and subsequent limiting dilution to obtain clones, the desired stable cell line is generated. This process takes approximately 2-3 months, usually yielding 5-10 usable clones and allowing little control over the end result throughout the process.

Technique for cell line generation:

LEAP (Laser-Enabled Analysis and Processing) has been developed for high-throughput laser-mediated cell elimination for cell purification. It is a precise laser-based cell ablation enables cell purification based on fluorescent and morphological criteria. It has whole well imaging system i.e all cells in the well can be analysed. It has F-theta scanning optics i.e Image up to 40X faster than typical HCS (High-content screening) systems. Image magnifications of 3X, 5X, 10X, or 20X can be obtained. Combinations of

8 excitation and 8 emission wavelengths is possible. LEAP images all cells within a well, selects a specific population of cells by gating, and eliminates selected cells at $>10^3$ per second. LEAP can select cells of interest based on fluorescent properties, morphological properties, or a combination of both. By replacing the antibiotic resistance gene used for chemical selection with a gene encoding a fluorescent protein, transfected cells can be selected based on fluorescence. These cells can then be purified using LEAP by specifically eliminating non-fluorescent cells using laser elimination. By selecting cells that remain fluorescent and proliferate over a period of time, stable cell lines are isolated. In addition, fluorescence level may be used to identify cell lines with a specific desired expression level of the transfected construct. The fluorescent reporter gene may also be replaced by a variety of fluorescent cell physiology read outs, enabling the selection of cells based on functional responses.

Applications:

The generation of stably-transfected cell lines is essential for a wide range of applications:

- Cell line can be used for gene function studies
- Drug discovery assays or the production of recombinant proteins can be carried out by cell lines.
- In contrast to transient expression, stable expression of cell line allows long term, as well as defined and reproducible expression of the gene of interest.

Table 3: Types of culture system for cell lines

Culture System	Advantage	Application
Batch culture - Polyclonal	Fast, useful for cells which do not grow in single cell culture	Over expressions, protein expression system (e.g. for Basic research)
Limiting Dilution-monoclonal	Defined cell clone	Study of gene function, protein production(e.g. for therapeutic applications)

In a batch culture system, a mixed population of drug resistant cells is selected on plates or in flasks and can be used directly for experimental analysis. During a limiting dilution procedure, cells are usually diluted and selected e.g., in a 96-well plate for outgrowth of cell clones or single colony growth. Subsequently, colonies can be picked and used to generate monoclonal cell lines.

Culture conditions for generation of stable cell lines:

As for transient transfection experiments, culture conditions (passage number, split rhythm, etc.) of selected cell type are very important for the generation of stably-transfected cell lines. The American Type Culture Collection (ATCC®; www.atcc.org) is a reliable source for various cell types. Generally, the cell line should be passaged two days before the experiment to promote good proliferation and cell physiology. Cellpassage should not be higher than 30. Interference of higher passage numbers with integration efficiency is possible and may be cell-type dependent. Depending on the scope of experiment, cells can be cultivated as polyclonal batches or monoclonal single cell clones post transfection.

Transfection Method: Stable expression can be influenced by the transfection method used. The choice of transfection method determines which cell type can be targeted for stable integration. While biochemical transfection reagents can be used to transfer DNA into standard cell lines, efficient delivery of DNA into difficult-to-transfect suspension cell lines or even primary cells is only possible with viral methods or Nucleofection. Unfortunately, viral methods suffer from several limitations, such as time consuming production of vectors and safety concerns.

Table 4: Experimental outlines for the Generation of cell lines

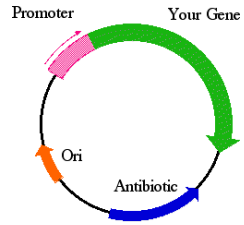
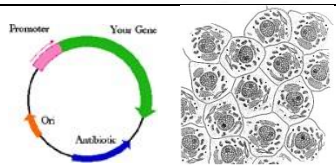
	Procedure Outline	Important Information
 Expression Plasmid	Design experiment and choose cell type, expression vector and transaction method	Make sure that transfection method and expression vector are suitable for the cell type
	Determine the appropriate cell number per plate (only for limiting dilution) and G418 concentration	Cells differ in their susceptibility to G418. The activated concentration of stock G418 can vary from batch to batch
 Expression plasmid + cells	Transfect expression vector into cells.	Amount of expression vector per expression is dependent on transfection method and cell type.
	Plant transfect cells and cultivate cells into medium without G418	Do not add G418 to culture medium immediately after transfection as this may drastically increase mortallity
	Dilute cell into culture plate and start selection 24-48 hour post transfection. Feed every 2-3 days (for batch culture) or 10 days (for limiting dilution)	Choose culture condition [batch culture limiting dilution] depending upon the experimental design. Refresh selection medium is important to avoid false positive cells.
	Analyze stably transfected cell	Make sure the chosen cell is suitable for your application.

Table 5: Commonly used cell lines

Type of cell line	Cell lines	Morphology	Origin	Species	Age	Ploidy	Characteristics
Finite from normal tissue	IMR-90	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection contact inhibited
	MRC-5	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection, contact inhibited
	MRC-9	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection, contact inhibited
	WI-38	Fibroblast	Lung	Human	Embryonic	Diploid	Susceptible to human viral infection, contact inhibited
Continuous from normal tissue	3T3-A31	Fibroblast		Mouse BALB/c	Embryonic	Aneuploid	contact inhibited, radily transformed
	BEAS-2B	Epithelial	Lung	Human	Adult		
	BHK21-C13	Fibroblast	Kidney	Syrian Hamster	Adult	Aneuploid	Transformable by polyoma
	BRL3A	Epithelial	Liver	Rat	New born		Produce IGF-2
	C2	Fibroblastoid	Skeletal muscle	Mouse	Embryonic		Myotubes
	C7	Epitheloid	Hypothalamous	Mouse			Neurophysin, Vasopressin
	COS-1, COS-7	Epitheloid	Kidney	Pig	Adult		Good hosts for DNA transfection
	CHO-K1	Fibroblast	Overy	Chinise Hamster	Adult	Diploid	FactorVIII, Angiotensin II converting enzyme

	HaCaT	Epithelial	Keratinocytes	Human	Adult	Diploid	Comification
	LLC-PKI	Epithelial	Kidney	Pig	Adult	Diploid	Na^+ dependent glucose uptake
	NRK49F	Fibroblast	Kidney	Rat	Adult	Aneuploid	Induction of suspension growth by TGF-α, β
Continuous from neuroplastic tissues	A2780	Epithelial	Overy	Human	Adult	Aneuploid	Chemosensitive with resistant variant
	A549	Epithelial	Lung	Human	Adult	Aneuploid	Synthesize surfactant
	B16	Fibroblast	Melanoma	Mouse	Adult	Aneuploid	Melanin
	HeLa	Epithelial	Cervix	Human	Adult	Aneuploid	G6PD Type A
	HeLa-S3	Epithelial	Cervix	Human	Adult	Aneuploid	High plating efficiency, will grow well in suspension
	HEP-G2	Epitheloid	Heptoma	Human	Adult	Aneuploid	Retain some microsomal metabolizing enzyme
	K-562	Suspention	Myeloid leukemia	Human	Adult	Aneuploid	Hemoglobin
	SK-HEP-1	Endothelial	Hepatoma, Endothelium	Human	Adult	Aneuploid	Factor VIII
	MCF-10	Epithelial	Fibrocytic mammary tissue	Human	Adult	Near Diploid	Dome formation
	HT-29	Epithelial	Colon	Human	Adult	Aneuploid	Differentiation inducible with NaBt

Interesting facts:

- There are several different types of cell lines that can be finite or continuous, prepared from normal tissue as well as neuroplastic tissues.
- Cell bank system is generally used for maintenance of cell line with constant supply of starting material as well as it perform cell lines characterization and detection of cell line cross contamination.
- In drug discovery, stably transfected cell lines are generally used.

Questions:

1. What are the basic types of cell lines and which type of tissue involve in formation of cell line?
2. What are the conditions required for cell line growth?
3. What are the criteria for choosing cell lines?
4. Which type of precaution one should take during cell line maintenance?
5. Give some example of human cell lines and what are the characteristic of those cell lines?

References:

1. Fresheny, I. "Culture of animal cell – A manual of basic technique and specialized application" by; chaptor-2: Biology of cultured cells; page-23
2. http://www.biology online.org/dictionary/Cell_line
3. http://atcc.custhelp.com/app/answers/detail/a_id/355/~/definition-of-%22cell-line%22
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5. http://bio.lonza.com/uploads/tx_mwaxmarketingmaterial/Lonza_TechREF_Generation_of_Stable_Cell_Lines_low_res.pdf

Characterization of Cells

Characterization of a cell line is vital for determining its functionality and in proving its authenticity as pure cell line. Special attention must be paid to the possibility that the cell line has become cross-contaminated with an existing continuous cell line or misidentified because of mislabeling or confusion in handling DNA profiling. This has now become the major standard procedure for cell line identification, and a standard procedure with universal application.

The various important factors for cell line characterization are:

(1) It leads to authentication or confirmation that the cell line is not cross-contaminated or misidentified

(2) It is confirmation of the species of origin

(3) It is used for correlation with the tissue of origin, which comprises the following characteristics:

a) Identification of the lineage to which the cell belongs
b) Position of the cells within that lineage (i.e., the stem, precursor, or differentiated status)

(4) For determination whether the cell line is transformed or not:

a) Whether the cell line is finite or continuous?
b) Whether the cell line expresses properties associated with malignancy?

(5) It indicates whether the cell line is prone to genetic instability and phenotypic variation

(6) Identification of specific cell lines within a group from the same origin, selected cell strains, or hybrid cell lines, all of which require demonstration of features unique to that cell line or cell strain

Table 1: Decisive factors for characterization of cell lines and corresponding methods

Decisive factor	**Method**
DNA profile	PCR of microsatellite repeats
Karyotype	Chromosome spread with banding
Isoenzyme analysis	Agar gel electrophoresis
Genome analysis	Microarray
Gene expression analysis	Microarray
Proteomics	Microarray
Cell surface antigen	Immunohistochemistry
Cytoskeleton	Immunocytochemistry with antibodies specific cytokeratins

Parameters of Characterization

The nature of the technique used for characterization depends on the type of work being carried out. Some of the parameters are:

1. In case molecular technology, DNA profiling or analysis of gene expression are most useful.
2. A cytology laboratory may prefer to use chromosome analysis coupled with FISH (fluorescence in situ hybridization) and chromosome painting. Chromosomal analysis also known as karyotyping, is one of the best traditional methods for distinguishing among species. Chromosome banding patterns can be used to distinguish individual chromosomes. Chromosome painting, explicitly using combinations of specific molecular probes that hybridize to individual chromosomes, adds further resolution and specificity to this technique. These probes identify individual chromosome pairs and are species specific. Chromosome painting is a good method for distinguishing between human and mouse chromosomes in potential cross-contaminations.

3. A laboratory with immunological capability may prefer to use MHC (Major Histo compatibility complex) analysis (e.g., HLA typing) coupled with lineage specific markers.

   Combined with a functional assay related to our own interests, these procedures should provide sufficient data to authenticate a cell line as well as confirm that it is suited to the concerned.

4. **Lineage or Tissue markers:** The progression of cells down a particular differentiation pathway towards a specific differentiated cell type and can be considered as a lineage, and as cells progress down this path they acquire lineage markers specific to the lineage and distinct from markers expressed by the stem cells. These markers often reflect the embryological origin of the cells from a particular germ layer. Lineage markers are helpful in establishing the relationship of a particular cell line to its tissue of origin. There are some lineage markers which are described as follows:

a) **Cell surface antigen:** These markers are particularly useful in sorting hematopoietic cells and have also been effective in discriminating epithelium from mesenchymally derived stroma with antibodies such as anti- and anti-HMFG 1 and, distinguishing among epithelial lineages, and identifying neuroectodermally derived cells (e.g., with anti-A2B5).

b) **Intermediate filament proteins**: These are among the most widely used lineage or tissue markers. Glial fibrillary acidic protein (GFAP) for astrocytes and desmin for muscle are the most specific, whereas cytokeratin marks epithelial cells and mesothelium.

c) **Differentiated products and functions**: Haemoglobin for erythroid cells, myosin or tropomyosin for muscle, melanin for melanocytes, and serum albumin for hepatocytes are examples of specific cell type markers, but like all differentiation markers, they depend on the complete expression of the differentiated phenotype.

Transport of inorganic ions, and the resultant transfer of water, is characteristic of absorptive and secretary epithelia. Polarized transport can also be demonstrated in epithelial and endothelial cells using Boyden chambers or filter well inserts. Other tissue-specific functions that can be expressed in vitro include muscle contraction and depolarization of nerve cell membrane.

d) **Enzymes:** Three parameters are available in enzymatic characterization:

- The constitutive level (in the absence of inducers or repressors)
- The induced or adaptive level (the response to inducers and repressors)
- Isoenzyme polymorphisms

Table 1: Enzymatic markers used for cell line

Enzyme	Cell types	Inducer	Repressor
Alkaline ephosphatase	Type II pneumocyte (in lung alveolus)	Dexamethasone, Oncostain, IL-6	TGF-β
Alkaline Phosphatase	Enterocytes	Dexamethanose, NaBt collagen, Matrigel	-
Angiotensin-converting enzyme	Endothelium	Collagen, Matrigel	-
Creatine Kinase BB	Neurons, neuroendocrine cells, SCLC	-	-
Creatine Kinase MM	Muscle cells	IGF-II	FGF-1,2,7
DOPA-decarboxylase	Neuron, SCLC	-	-
Glutamyl synthetase	Astroglia (brain)	Hydrocortisone	Glutamine
Neuron specific enolase	Neuron, neuroendocrine cell	-	-
Non-specific esterase	Macrophage	PMA, Vitamin D3	-
Proline hydrolase	Fibroblasts	Vitamin C	-
Sucrase	Enterocytes	NaBt	-

e) **Regulation:** The level of expression of many differentiated products is under the regulatory control of environmental influences, such as nutrients, hormones, the matrix, and adjacent cell. Hence the measurement of specific lineage markers may require preincubation of the cells in, for example, a hormone such as hydrocortisone, specific growth factors, or growth of the cells on extracellular matrix of the correct type.

f) **Lineage fidelity:** Lineage markers are more properly regarded as tissue or cell type markers, as they are often more characteristic of the function of the cell than its embryonic origin.

5. **Unique Markers:** Unique markers include specific chromosomal aberrations (e.g., deletions, translocations, polysomy), major histocompatibility (MHC) group antigens (e.g., HLA in humans), which are highly polymorphic, and DNA fingerprinting or SLTR DNA profiling. Enzymic deficiencies, such as thymidine kinase deficiency (TK−) and drug resistance such as vinblastine resistance (usually coupled to the expression of the P-glycoprotein by one of the *mdr* genes that code for the efflux protein) are not truly unique, but they may be used to distinguish among cell lines from the same tissues but different donors.

6. **Transformation:** The transformation status forms a major element in cell line characterization and is dealt with separately.

a) **Cell Morphology:** Observation of morphology is the simplest and most direct technique used to identify cells. Most of these are related to the plasticity of cellular morphology in response to different culture conditions. For example, epithelial cells growing in the center of a confluent sheet are usually regular, polygonal, and with a clearly defined birefringent edge, whereas the same cells growing at the edge of a patchmay be more irregular and distended and, if transformed, may break away from the patch and become fibroblast-like in shape.

b) **Microscopy:** The inverted microscope is one of the most important tools in the tissue culture laboratory, but it is often used incorrectly. As the thickness of the closed culture vessel makes observation difficult from above, because of the long working distance, the culture vessel is placed on the stage, illuminated from above, and observed from below. As the thickness of the wall of the culture vessel still limits the

working distance, the maximum objective magnification is usually limited to 40X. The use of phase-contrast optics, where an annular light path is masked by a corresponding dark ring in the objective and only diffracted light is visible, enables unstained cells to be viewed with higher contrast than is available by normal illumination. Because this means that the intensity of the light is increased, an infrared filter should be incorporated for prolonged observation of cells.

It is useful to keep a set of photographs at different cell densities for each cell line, prepared shortly after acquisition and at intervals thereafter, as a record in case a morphological change is subsequently suspected. Photographs of cell lines in regular use should be displayed above the inverted microscope. Photographic records can be supplemented with photographs of stained preparations and digital output from DNA profiling and stored with the cell line record in a database or stored separately and linked to the cell line database.

c) **Staining:** A polychromatic blood stain, such as Giemsa, provides a convenient method of preparing a stained culture. Giemsa stain is usually combined with May–Grunwald stain when staining blood, but not when staining cultured cells. Alone, it stains the nucleus pink or magenta, the nucleoli dark blue, and the cytoplasm pale gray-blue. It stains cells fixed in alcohol or formaldehyde but will not work correctly unless the preparation is completely anhydrous.

Chromosome Content: Chromosome content or karyotype is one of the most characteristic and best-defined criteria for identifying cell lines and relating them to the species and sex from which they were derived. Chromosome analysis can also distinguish between normal and transformed cells because the chromosome number is more stable in normal cells (except in mice, where the chromosome complement of normal cells can change quite rapidly after explantation into culture).

Chromosome Banding: This group of techniques was devised to enable individual chromosome pairs to be identified when there is little morphological difference between them. For Giemsa banding, the chromosomal proteins are partially digested by crude trypsin, producing a banded appearance on subsequent staining. Trypsinization is not required for quinacrine banding. The banding pattern is characteristic for each chromosome pair.

Other methods for banding are:

a) Using trypsin and EDTA rather than trypsin alone
b) Q-banding, which stains the cells in 5% (w/v) quinacrine dihydrochloride in 45% acetic acid, followed by rinsing Giemsa banding the slide, and mounting it in deionized water at pH 4.5
c) C-banding, which emphasizes the centromeric regions

Techniques have been developed for discriminating between human and mouse chromosomes, principally to aid the karyotypic analysis of human-mouse hybrids. These methods include fluorescent staining with Hoechst 33258, which causesmouse centromeres to fluoresce more brightly than human centromeres.

Chromosome painting: Chromosome paints are available commercially from a number of sources. The hybridization and detection protocols vary with each commercial source, but a general scheme is available. Karyotypic analysis is carried out classically by chromosome banding, using dyes that differentially stain the chromosomes. Thus each chromosome is identified by its banding pattern. However, traditional banding techniques cannot characterize many complex chromosomal aberrations. New karyotyping methods based on chromosome painting techniques—namely spectral karyotyping (SKY) and multicolour fluorescence in situ hybridization (M-FISH)—have been developed. These techniques allow the simultaneous visualization of all 23 human chromosomes in different colours.

Chromosome Analysis

The following are methods by which the chromosome complement may be analyzed:

(1) Chromosome count: Count the chromosome number per spread for between 50 and 100 spreads. (The chromosomes need not be banded.)

(2) Karyotype: Digitally photograph about 10 or 20 good spreads of banded chromosomes. Image analysis can be used to sort chromosome images automatically to generate karyotypes.

Chromosome counting and karyotyping allow species identification of the cells and, when banding is used, distinguish individual cell line variations and marker chromosomes. However, karyotyping is time-consuming, and chromosome counting with a quick check on gross chromosome morphology may be sufficient toconfirm or exclude a suspected cross-contamination.

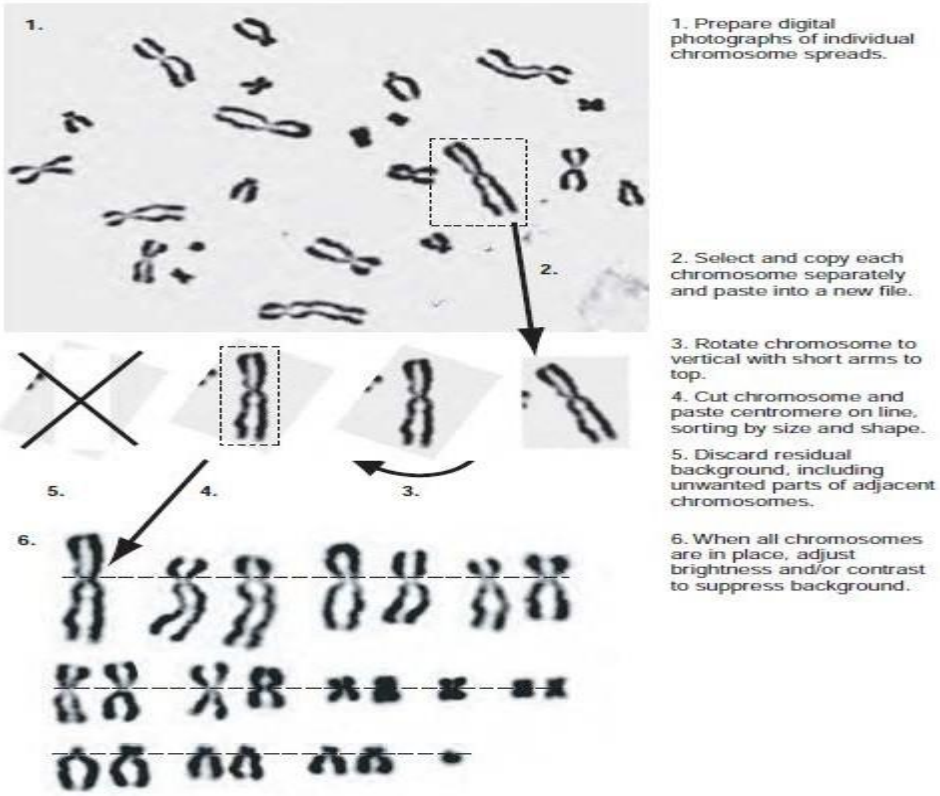

Figure 1: Karyotype Preparation Steps in the preparation of a karyotype from digital microphotographs of metaphase spread. Chinese hamster cells recloned from the Y-5 strain.

DNA Analysis: DNA content can be measured by propidium iodide fluorescence with a CCD camera or flow cytometry, although the generation of the necessary single-cell suspension will, of course, destroy the topography of the specimen. DNA can be estimated in homogenates with Hoechst 33258 and other DNA fluorochromes such as DAPI, propidium iodide, or Pico Green (Molecular Probes). Analysis of DNA content is particularly useful in the characterization of transformed cells that are often aneuploid and heteroploid.

DNA Hybridization: Hybridization of specific molecular probes to unique DNA sequences (Southern blotting) can provide information about species specific regions, amplified regions of the DNA, or altered base sequences that are characteristic to that cell line. Thus strain-specific gene amplifications, such as amplification of the dihydrofolate reductase (DHFR) gene, may be detected in cell lines selected for resistance to methotrexate; amplification of the MDR gene in vinblastine-resistant cells overexpression of a specific oncogene, or oncogenes in transformed cell lines or deletion, or loss, of heterozygosity in suppressor genes. Although DNA aberrations can be detected in restriction digests of extracts ofwhole DNA, this is limited by the amount of DNA required.

It is more common to use the polymerase chain reaction (PCR) with a primer specific to the sequence of interest, enabling detection in relatively small numbersof cells. Alternatively, specific probes can be used to detect specific DNA sequences by in situ hybridization having the advantage of displaying topographicaldifferences and heterogeneity within a cell population.

DNA fingerprinting: DNA fingerprints appear to be quite stable in culture, and cell lines from the same origin, but maintained separately in different laboratories for many years, still retain the same or very similar DNA fingerprints. DNA fingerprinting is a very powerful tool in determining the origin of a cell line, if the original cell line, or DNA from it or from the donor individual, has been retained. This emphasizes the need to retain a blood, tissue, or DNA sample when tissue is isolated for primary culture. Furthermore, if a cross-contamination or misidentification is suspected, this can be investigated by fingerprinting the cells and all potential contaminant.

Antigenic Markers: Immunostaining and ELISA assays are among the most useful techniques available for cell line characterization facilitated by the abundance of antibodies and kits which is commercially available. Antibody is essential to be certain of its specificity by using appropriate control material. This is true for monoclonal antibodies and polyclonal antisera alike; a monoclonal antibody ishighly specific for a particular epitope.

Immunostaining: Antibody localization is determined by fluorescence, wherein the antibody is conjugated to a fluorochrome, such as fluorescein or rhodamine, or by the deposition of a precipitated product from the activity of horseradish peroxidase or alkaline phosphatase conjugated to the antibody. Various methods have been used to enhance the sensitivity of detection of these methods, particularly theperoxidase linked methods. In the peroxidase–anti-peroxidase (PAP) technique, a further amplifying tier is added by reaction with peroxidase conjugated to anti- peroxidase antibody from the same species as the primary antibody. Even greater sensitivity has been obtained by using a biotin-conjugated second antibody with streptavidin conjugated to peroxidase or alkaline phosphatase or gold-conjugated second antibody with subsequent silver intensification.

Differentiation: Many of the characteristics described under antigenic markers or enzyme activities may also be regarded as markers of differentiation, and as such they can help to correlate cell lines with their tissue of origin as well as define their phenotypic status. Although sometimes constitutively expressed (e.g., melanin in B16 melanoma or Factor VIII in endothelial cells), expression of differentiated lineage markers may need to be induced before detection is possible.

Interesting facts:

- EDTA, a chelator of divalent cations, is added to trypsin solutions to enhance activity.
- The calcium and magnesium in the extracellular matrix, which aids in cell-cell adhesion, also obliterates the peptide bonds that trypsin acts on.
- The EDTA is added to remove the calcium and magnesium from the cell surface which allows trypsin to hydrolyze specific peptide bonds. This activity can be arrested by adding a serum media mixture or a trypsin inhibitor (from soybean, for example) in serum-free systems.

Questions:

1. Why characterization of cell line is necessary?
2. What are the parameters on which characterization depends?
3. What is the role of trypsin and EDTA in cell culture? How EDTA help trypsin in cell detachment?

References:

1. Fresheny, I.; "Culture of animal cell – A manual of basic technique and specialized application" by; chaptor-15: Characterization

Contamination in cell culture

Contamination: Contamination is the presence of a minor and unwanted constituent (contaminant) in material, physical body, natural environment, at a workplace, etc. In biological sciences accidental introduction of foreign material (contamination) can seriously distort the results of experiments where small samples are used. In cases where the contaminant is a living microorganism, it can often multiply and take over the experiment, especially cultures, and render them useless.

Source of Contamination: Maintaining asepsis is one of the most difficult challenges to work with living cells. There are several potential routes to contamination including failure in the sterilization procedures for solutions, glassware and pipettes, turbulence and particulates (dust and spores) in the air in the room, poorly maintained incubators and refrigerators, faulty laminar-flow hoods, the importation of contaminated cell lines or biopsies, and lapses in sterile technique.

Table 1: Route of Contamination

Technique	Route or cause	Prevention
(Manipulations, pipetting, dispensing, etc.)	Nonsterile surfaces and equipment	Work area of items not in immediate use should be clear.
	Spillage on necks and outside of bottles and on work surface	Swab regularly with 70% alcohol. Do not pour liquids. Dispense or transfer by pipette, auto dispenser or transfer device. If pouring is unavoidable: (1) do so in one smooth movement, (2) discard the bottle that you pour from, and (3) wipe up any spillage.
	Touching or holding pipettes too low down, touching necks of bottles, inside screw caps.	Hold pipettes above graduations.
	Splash-back from waste beaker	Discard waste into a beaker with a funnel or, preferably, by drawing off the waste into a reservoir by means of a vacuum pump.
	Sedimentary dust or particles of skin settling on the culture or bottle; hands or apparatus held over an open dish or bottle	Do not work over (vertical laminar flow and open bench) or behind and over (horizontal laminar flow) an open bottle or dish.
Work surface	Dust and spillage	Swab the surface with 70% alcohol before during, and after work. Mop up spillage immediately.

Operator hair, hands, breath, clothing	Dust from skin, hair, or clothing dropped or blown into the culture	Wash hands thoroughly or wear gloves. Wear a lint-free lab coat with tight cuffs and gloves overlapping them.
	Aerosols from talking, coughing, sneezing, etc.	Keep talking to be minimum, Avoid working with a cold or throat infection, or wear a mask. Long hair should be tie back or wear a cap. Generally wear a lab coat different from the one which wear in the general lab area or animal house.
Materials and reagents		
Solutions	Non-sterile reagents and media	Filter or autoclave solutions before using them
	Dirty storage conditions	Clean up storage areas and disinfect regularly.
	Inadequate sterilization procedures	Monitor the performance of the autoclave with a recording thermometer or sterility indicator. Check the integrity of filters with a bubble-point or microbial assay after using them. Test all solutions after sterilization.
	Poor commercial supplier	Test solutions; change suppliers.
Glassware and screw caps	Dust and spores from storage	Shroud caps with foil. Wipe bottles with 70% alcohol before taking them into the hood. Replace stocks from the back of the shelf. Do not store anything unsealed for more than 24 h.
	Ineffective sterilization (e.g., an overfilled oven or sealed bottles, preventing the ingress of steam)	Check the temperature of the load throughout the cycle. In the autoclave; keep caps slack on empty bottles. Stack oven and autoclave correctly.
Instruments, pipettes	Ineffective sterilization	Sterilize items by dry heat before using them. Monitor the performance of the oven.
	Contact with a nonsterile surface or some other material	Do not grasp any part of an instrument or pipette that will pass into a culture vessel.

Culture flasks and media bottles in use	Dust and spores from incubator or refrigerator	Use screw caps instead of stoppers. Swab bottles before placing in hood. Box plates and dishes.
	Dirty storage or incubation conditions.	Cover caps and necks of bottles with aluminum foil during storage or incubation. Wipe flasks and bottles with 70% alcohol before using them. Clean out stores and incubators regularly.
	Media under the cap and spreading to the outside of the bottle.	Discard all bottles that show spillage on the outside of the neck. Do not pour.
Equipment and facilities	Room air Drafts, eddies, turbulence, dust, aerosols	Clean filtered air. Reduce traffic and extraneous activity. Wipe the floor and work surfaces regularly.
Laminar-flow hoods	Perforated filter	Check filters regularly for holes and leaks.
	Change of filter needed	Check the pressure drop across the filter.
	Spillages, particularly in crevices or below a work surface	Keep around and below the work surface clear regularly. Let alcohol run into crevices.
Equipment and facilities		
Room air	Drafts, eddies, turbulence, dust, aerosols	Clean filtered air. Reduce traffic and extraneous activity. Wipe the floor and work surfaces regularly.
Laminar-flow hoods	Perforated filter	Check filters regularly for holes and leaks.
	Change of filter needed	Check the pressure drop across the filter.
	Spillages, particularly in crevices or below a work surface	Clear around and below the work surface regularly. Let alcohol run into crevices.
Dry incubators	Growth of molds and bacteria on spillages	Wipe up any spillage with 70% alcohol on a swab. Clean out incubators regularly.
CO_2, humidified incubators	Growth of molds and bacteria on walls and shelves in a humid atmosphere.	Clean out with detergent followed by 70% alcohol

	Spores, etc., carried on forced-air circulation	Enclose open dishes in plastic boxes with close-fitting lids (but do not seal the lids). Swab incubators with 70% alcohol before opening them. Put a fungicide or bacteriocide in humidifying water (but check firstfor toxicity).
Other equipment	Dust on cylinders, pumps, etc	Wipe with 70% alcohol before bring in
Mites, insects, and other infestations in wooden furniture, or benches, in incubators, and on mice, etc., taken from the animal house	Entry of mites, etc., into sterile packages	Seal all sterile packs. Avoid wooden furniture if possible; use plastic laminate, one-piece, or stainless steel bench tops. If wooden furniture is used, seal it with polyurethane varnish or wax polish and wash it regularly with disinfectant. Keep animals out of the tissue culture lab.
Importation of biological materials		
Tissue samples	Infected at source or during dissection	Do not bring animals into the tissue culture lab. Incorporate antibiotics into the dissection fluid. Dip all potentially infected large-tissue samples in 70% alcohol for 30 s.
Incoming cell lines	Contaminated at the source or during transit	Handle these cell lines alone, preferably in quarantine, after all other sterile work is finished. Swab down the bench or hood after usewith 2% phenolic disinfectant in 70% alcohol, and do not use it until the next morning. Check for contamination by growing a culture for two weeks without antibiotics. (Keep a duplicate culture in antibiotics at the first subculture.) Check for contamination visually, by phase-contrast microscopy and Hoechst stain for mycoplasma. Using indicator cells allows screening before first subculture.

Monitoring For Contamination:

Potential sources of contamination are enumerated along with the precautions that should be taken to avoid them. Even in the best laboratories contaminations do arise, so the following procedure is generally recommended:

(1) Contamination by eye and with a microscope at each handling of a culture should be checked properly.

(2) If it is suspected that a culture is contaminated and the fact cannot be confirmed in situ, the hood or bench should be kept clear except suspected culture and Pasteur pipettes. Because of the potential risk to other cultures, this should be better to do after all your other culture work is finished. A sample should be removed from the culture and placed on a microscope slide. Slide should be checked with a microscope, preferably by phase contrast. If it is confirmed that the culture is contaminated, pipettes should be discarded, hood or bench should be swabbed with 70% alcohol containing a phenolic disinfectant. The hood or bench should not be used until the next day.

(3) Nature of the contamination should be recorded.

(4) If the contamination is new and is not widespread, the culture, the medium bottle used to feed it, and any other reagent (e.g., trypsin) that has been used in conjunction with the culture should be discarded properly into disinfectant, preferably in a fume hood and outside the tissue culture area.

(5) If the contamination is new and widespread all media, stock solutions, trypsin, and so forth in current use should be discarded immediately.

(6) If the same kind of contamination has occurred before check stock solutions for contamination (a) by incubation alone or in nutrient broth (b) by plating out the solution on nutrient agar. If (a) and (b) prove negative, but contamination is still suspected, 100 mL of solution should be incubated, filtered it through a 0.2-μm filter, and plated out filter on nutrient agar with an uninoculated control.

(7) If the contamination is widespread, multispecific, and repeated then one should check (a) the laboratory's sterilization procedures (e.g., the temperatures of ovens and autoclaves, particularly in the center of the load, the duration of the sterilization cycle), (b) the packaging and storage practices, (e.g., unsealed glassware should be resterilized every 24 h), and (c) the integrity of the aseptic room and laminar-flow hood filters.

(8) One should not be attempting to decontaminate cultures unless they are irreplaceable.

Visible Microbial Contamination: Characteristic features of microbial contamination are as follows:

(1) A sudden change in pH, usually a decrease with most bacterial infections, very little change with yeast until the contamination is heavy, and sometimes an increase in pH with fungal contamination.

(2) Cloudiness in the medium, sometimes with a slight film or scum on the surface or spots on the growth surface that dissipate when the flask is moved

(3) Under a 10X objective, spaces between cells will appear granular and may shimmer with bacterial contamination. Yeasts appear as separate round or ovoid particles that may bud off smaller particles. Fungi produce thin filamentous mycelia and, sometimes, denser clumps of spores which may be blue or green. With toxic infection, some deterioration of the cells will be apparent.

(4) Under a 100X objective, it may be possible to resolve individual bacteria and distinguish between rods and cocci. At this magnification, the shimmering that is visible in some infections will be seen to be caused by mobility of bacteria. Some bacteria form clumps or associate with the cultured cells.

(5) With a slide preparation, the morphology of the bacteria can be resolved with a 100× objective, but this is not usually necessary. Microbial infection may be confused with precipitates of media constituents (particularly protein) or with cell debris, but can be distinguished by their regular morphology. Precipitates may be crystalline or globular and irregular and are not usually as uniform in size. Clumps of bacteria may be confused with precipitated protein, but, particularly if shaken, many single or strings of bacteria will be seen. If you are in doubt, plate out a sample of medium on nutrient agar.

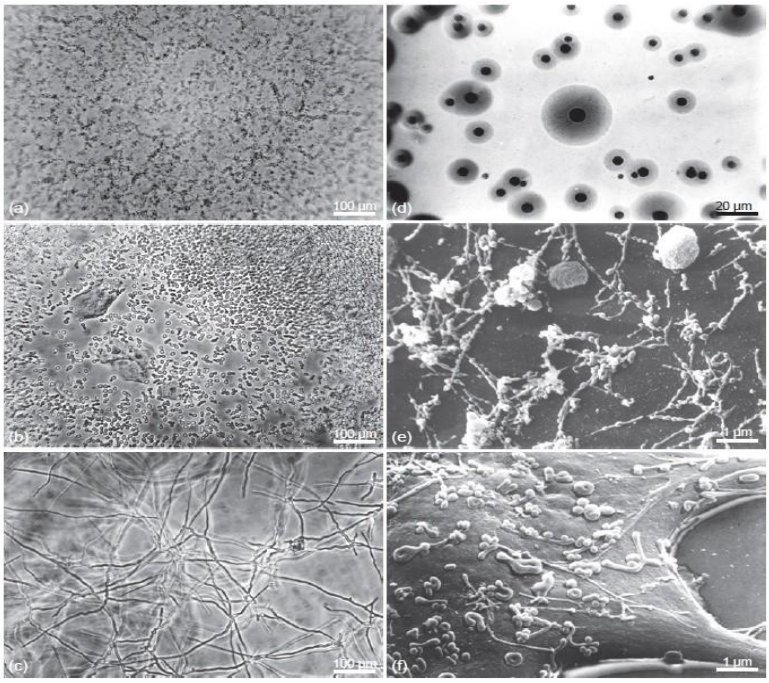

Figure-1: Types of Contamination. Examples of microorganisms found to contaminate cell cultures. (a) Bacteria. (b) Yeast. (c) Mold. (d) Mycoplasma colonies growing on special nutrient agar (e, f). Scanning electron micrograph of mycoplasma growing on the surface of cultured cells. From where has this figure been taken

Ways of Disposal of Contaminated Cultures: The following procedures are generally used for disposal of contaminated culture:

- It is important to ensure that all contaminated material is disposed of correctly. Culture vessels should be removed from the culture area, unopened if possible, and autoclaved.
- Open items, such as Petri dishes with the lids in place, and pipettes or other items that have come in contact with a contaminated culture should be immersed in hypochlorite disinfectant (Petri dishes can be opened while submerged).
- If only one of a series of similar cultures is contaminated, it is necessary to discard the bottle of medium that was used with it, but if the contamination is widespread, then all medium as well as all other stock solutions and reagents, used with these cells, should be discarded into hypochlorite.

Eradication of Contamination: Eradication of Contamination in cell culture is a challenging job during safe culturing. There are different way for different organism, some example are given as follows:

Case-I: Bacteria, Fungi, and Yeasts: The most reliable method of eliminating a microbial contamination is to discard the culture and the medium and reagents used with it as treating a culture may be unsuccessful or lead to the development of an antibiotic- resistant microorganism. This procedure is optimal; however, the majority of cell lines donot form spheroids in this manner. Alternatively, aggregates may be formed from cell suspensions in stationary flasks, previously base-coated with agar. Aggregates may beleft in the original flasks or transferred individually (by pipette) to multi well plates, where continued growth over weeks will yield spheroids of maximum size, about 1000 μm.

Decontamination should be attempted only in extreme situations, under quarantine, and with expert supervision. If unsuccessful, the culture and associated reagents should be autoclaved as soon as failure becomes obvious. The general rule remains that contaminated cultures are discarded and that decontamination is not attempted unless it isabsolutely vital to retain the cell strain. In any event, complete decontamination is difficult to achieve, particularly with yeast, and attempts to do so may produce hardier, antibiotic-resistant strains.

Case-II: Eradication of Viral Contamination: There are no reliable methods for eliminating viruses from a culture at present; disposal or tolerances are the only options.

Cross Contamination: During the development of tissue culture, a number of cell strains have evolved with very short doubling times and high plating efficiencies. Although these properties make such cell lines valuable experimental material, they also make them potentially hazardous for cross-infecting other cell lines.

Interesting facts:

1. Once cell lines are infected, they may undergo spontaneous differentiation or altered function due to adaptation, which may have a profound impact on experimental results.

Questions:

1. What are the possible routes of contamination in cell culture?
2. What are possible methods for monitoring contamination?
3. What are the general procedures for disposal of contaminated culture?

References:

1. Fresheny**,** I.; "Culture of animal cell – A manual of basic technique and specialized application"
2. http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Stem-Cell-Research/Stem-Cell-Analysis/Cell-Line-Contamination.html.

Cell line differentiation

Differentiation: Differentiation in cell line is the process which leads to the expression of phenotypic properties and characteristic of the functionally mature cell in vivo. It is the phenomenon in which less specialized cell develops or matures to become more distinct in form and function. This may be irreversible when there is cessation of DNA synthesis in the erythroblast nucleus, neuron, or mature keratinocyte. The process may be reversible, when the dedifferentiation of mature hepatocytes into precursors happens during liver regeneration. Some of the properties of the differentiated cells are adaptive, such as albumin synthesis in differentiated hepatocytes, which is often lost in culture but can be reinduced. Differentiation is the combination of constitutive (stably expressed without induction) and adaptive (subject to positive and negative regulation of expression) properties found in the mature cell.

Terminal differentiation: Terminal differentiation is another type of differentiation in which a cell has progressed down a particular lineage to a point at which the mature phenotype is fully expressed and beyond which the cell cannot progress. This stage may be reversible in some cells, such as fibrocytes, that can revert to a less differentiated phenotype, or even a stem cell, and resume proliferation. It may be irreversible in cells like erythrocytes, neurons, skeletal muscle, or keratinized squamous cells. The growth of cells on floating collagen has been used to improve the survival of epithelial cells and promote terminal differentiation

Pluripotent cell: A cell that is able to differentiate into all cell types of the adultorganism is known as pluripotent. Such cells are called embryonic stem cells in animals and meristematic cells in higher plants.

Totipotent cell: A cell that is able to differentiate into all cell types, including the placental tissue, is known as totipotent. In mammals, only the zygote and subsequent blastomeres are totipotent.

Control of Differentiation: Differentiation is controlled by various parameters. There are five major parameters that control differentiation. Figure 1 shows the diagrammatic representation of the figures.

1. Cell–cell interaction
2. Cell–matrix interaction
3. Cell shape and polarity
4. Oxygen tension
5. Soluble systemic factors

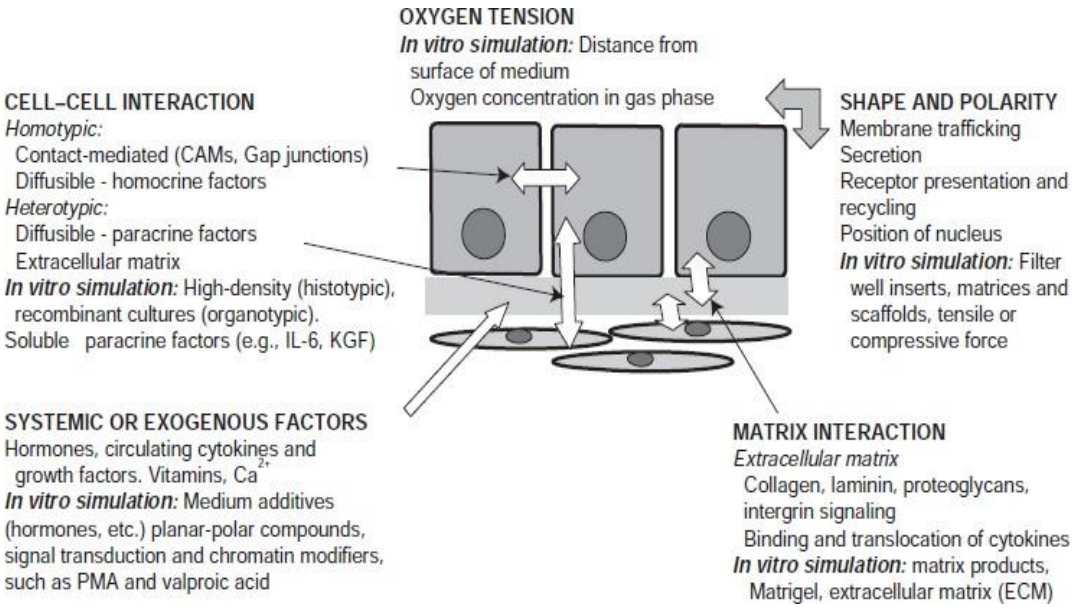

Figure 1: Parameter factors for cell differentiation.

1. Cell- cell Interaction: Cell-cell interactions are of two types, Homotypic and heterotypic. They can be detailed as follows:

Homotypic cell-cell interaction: Homologous cell interaction occurs at high cell density. It may involve gap junctional communication in which metabolites, second messengers such as cyclic AMP, diacylglycerol (DAG), Ca^{2+}, or electrical charge may beinvolved. This interaction harmonizes the expression of differentiation within a population of similar cells, rather than initiating its expression. Homotypic cell–cell adhesion molecules, (CAMs) or cadherins, which are calcium-dependent, provide another mechanism by which contacting cells may interact. These adhesion molecules promote interaction primarily between like cells via identical, reciprocally acting,

extracellular domains, and they appear to have signal transduction potential via phosphorylation of the intracellular domains.

Heterotypic cell-cell interaction: Heterologous cell interaction such as between mesodermally and endodermally or ectodermally derived cells is responsible for initiating and promoting differentiation. During and immediately after gastrulation in the embryo, and later during organogenesis, mutual interaction between cells originating in different germ layers promotes differentiation.

2. Cell–Matrix Interactions

Animal cells are not surrounded by cell walls. Animal cells are surrounded by a plasma membrane which is complex mixture of glycoproteins and proteoglycans surface that is highly specific for each tissue, and even for parts of a tissue. Recreation of this complex microenvironment, involving cell–cell and cell–matrix interactions has been shown to be vital in the expression of the mature keratinocyte phenotype in the reconstruction of skin equivalents and the maintenance of the stem cell compartment. Collagen has been found to be essential for the functional expression of many epithelial cells and for endothelium to mature into capillaries. Small polypeptides containing this sequence effectively block matrix-induced differentiation, implying that the intact matrix molecule is required. Defined matrices are required; although fibronectin, laminin, collagen, and numerous other matrix constituents are commercially available, the specificity probably lies largely in the proteoglycan moiety, within which there is the potential for wide variability, particularly in the number, type, and distribution of the sulfated glycosaminoglycan, such as heparan sulfate. The extracellular matrix may also play important role in the modulation of growth factor activity. One type of extracellular matrix is exemplified by the thin, sheet-like basal laminae, previously called basement membranes, upon which layers of epithelial cells rest. In addition to supporting sheets of epithelial cells, basal laminae surround muscle cells, adipose cells, and peripheral nerves. Extracellular matrix is most abundant in connective tissues.

3. Polarity and cell shape: Various studies shows that growth of the cells on collagen gel and the subsequent release of the gel from the bottom of the dish with a spatula or bent Pasteur pipette are required for full maturation of cell. This process allows shrinkage of the gel and modification in the shape of the cell from flattened to cuboidal or even columnar shape. Following the shape change and also possibly due to contact to medium through the gel, the cells develop polarity which is visible by electron microscopy. When the nucleus becomes asymmetrically distributed nearer to the bottom of the cell an active Golgi complex is formed and secretion is observed towards the apical surface.

4. Oxygen Tension: Gas exchange enhances when positioning the cells at the air–liquid interface, particularly facilitating oxygen uptake without raising the partial pressure and risking free radical toxicity. It is also possible that the thin film above mimics the physicochemical conditions in vivo (surface tension, lack of nutrients) as well as oxygenation.

Table 1: Common Physiological Inducers of Differentiation

Type of Inducer	Common Inducers	Cell type
Steroid and related	Hydrocorticosone	Glia, glioma Lung alveolar typeII cells Hepatocytes Mammary epithelium Myloid leukemia
	Retinoid	Tracheobranchial epithelium Endothelium Enterocytes (Caco-2) Embryonal carcinoma Malanoma Myeloid leukemia Neuroblastoma
Peptide Hormones	Melanotropin	Melanocytes
	Thyotropin	Thyroid
	Erythropoietin	Erythroblasts
	Prolactin	Mammary epithelium
	Insulin	Mammary epithelium
Cytokines	Nerve growth factor	Neuron
	Glia maturation factor, CNTF, PDGF, BMP2	Glial cell
	Epimorphin	Kidney epithelium
	Fibrocyte-pneumocyte factor	Type II pneumocyte
	Interferon-α, β	A549 cells

		HL60, myeloid leukemia
	Interferon-γ	Neuroblastoma
	CNTF	Type 2 astrocytes
	IL-6, OSM	AS49
	BMP	1 OT 1/2
	KGF	Keratinocytes Prostatic epithelium
	HGF	Kidney (MDCK) Hepatocyte
	TGF-β	Bronchial epithelium Melanocyte
	Endothelium	Melanocyte
Vitamins	Vitamin E	Neuroblastoma
	Vitamin D3	Monocytes (U937) Myeloma Enterocyte (IEC-6)
	Vitamin K	Hepatoma Kidney epithelium
Minerals	Ca++	Keranocytes

Table 2: Common Non-Physiological Inducers of Differentiation

Type of Inducer	Common Inducers	Cell type	Fate
Planar- polar compound	DMSO	Murin erytholeukemia	Immature erythrocytes
		Myeloma	Granulocyte
		Neuroblastoma	Neurons
		Mammary epithelium	Secretory epithelium
		Hepatocyte precursors, HepaRG hepatoma	Hepatocyte
	Sodium butyrate	Erythroleukemia	Immature erythrocytes
		Colon cancer	Absorptive epithelium
	N- methyl acetamide	Glioma	Astrocyte
	N- methyl formamide, Dimethyl formamide	Colon cancer	Absorptive epithelium
	HMBA	Erythroleukemia	Immature erythrocytes
	Butylated hydroxyanisole	Adipose derived stem cells	Neuron
	Benzodiazepines	Erythroleukemia	Immature erythrocytes
Cytotoxic drugs	Genistein	Erythroleukemia	Immature erythrocytes
	Cytosin arabinoside	Myeloid leukemia	Granulocyte
	Mitocin C, anthracyclines	Melanoma	Melanocyte
	Metotrexate	Colorectal carcinoma	Adsorptive & mucin-secreting epithelium
Chromatin modifiers	Valproic acid	Adipose derived stem cells	Neuron Neurite extension

		PC12 cells	
	Azacytidine	NG108-15 neuronal cells	Cholinergic neuron
Signal transduction modifiers	Isobutylmethyl xanthine	Adipose derived stem cells	Adipocytes
	Forkoline	Adipose derived stem cells	Neuron
	PMA	Bronchial epithelium	Squamous epithelium
		Mammary epithelium	
		Colon(HT29, Caco-2)	Ductal morphogenesis
		Monocyte leukemia(U937)	Monocytes
		Erythroleukemia(K562)	Immature erythrocytes
		Neuroblastoma	Neurite growth

Relationship between Differentiation and Malignancy:

With increasing progression of cancer, histology of a tumor indicates poorer differentiation, and from a prognostic standpoint, patients with poorly differentiatedtumors generally have a lower survival rate than patients with differentiated tumors. Cancer is principally a malfunction of cells to differentiate normally. It is therefore surprising to find that many tumors grown in tissue culture can be induced to differentiate. Indeed much of the fundamental data on cellular differentiation has been derived from murine leukemia, mouse and human myeloma, hepatoma, and neuroblastoma. Nevertheless, there appears to be an inverse relationship between the expression of differentiated properties and the expression of malignancy associated properties, even to the extent that the induction of differentiation has often been proposedas a mode of therapy. In correct environmental conditions, and assuming that the appropriate cells are present, partial, or even complete, differentiation is achievable in cell culture. As a general approach to promoting differentiation, as opposed to cellproliferation and propagation, the following aspects need to be taken care.

i. Selection of the correct cell type by use of appropriate isolation conditions and a selective medium.
ii. Growing the cells to a high cell density (>1×10^5 cells/cm^2) on the appropriate matrix. The matrix may be collagen of a type that is appropriate to the site of origin of the cells, with or without fibronectin or laminin, or it may be more complex, tissue derived or cell derived, such as Matrigel or a synthetic matrix (e.g., poly-D-lysine for neurons).
iii. Changing the cells to a differentiating medium rather than a propagation medium for other cell types this step may require defining the growth factors appropriate to maintaining cell proliferation and those responsible for inducing differentiation.
iv. Addition of differentiation-inducing agents, such as glucocorticoids, retinoids, vitamin D_3, DMSO, HMBA, prostaglandins and cytokines, such as bFGF, EGF, KGF, HGF, IL-6, OSM, TGF-β, interferons, NGF, and melanocyte-stimulating hormone (MSH), as appropriate for the type of cell.
v. Addition of the interacting cell type during the growth phase, the induction phase or both phases. Selection of the correct cell type is not always clear, but lung fibroblasts for lung epithelial maturation, glial cells for neuronal maturation, and bone marrow stromal cells for hematopoietic cells are some of the better-characterized examples.

Equilibrium between cell proliferation and differentiation:

Cells in culture can be thought to be in a state of equilibrium between cell proliferation and differentiation. Normal culture conditions i.e. in low cell density, mitogens in the medium will favor cell proliferation, whereas high cell density and addition of differentiation factors will induce differentiation. The position of the equilibrium will depend on culture conditions. Dedifferentiation of the culture may be due to the effect of growth factors or cytokines inducing a more proliferative phenotype, reprogramming of gene expression, or overgrowth of a precursor cell type. The relationship between differentiation and cell proliferation may become relaxed but it is not lost. For example, B16 melanoma cells still produce more pigment at a high cell density and at a low rate of cell proliferation than at a low cell density and a high rate of cell proliferation.

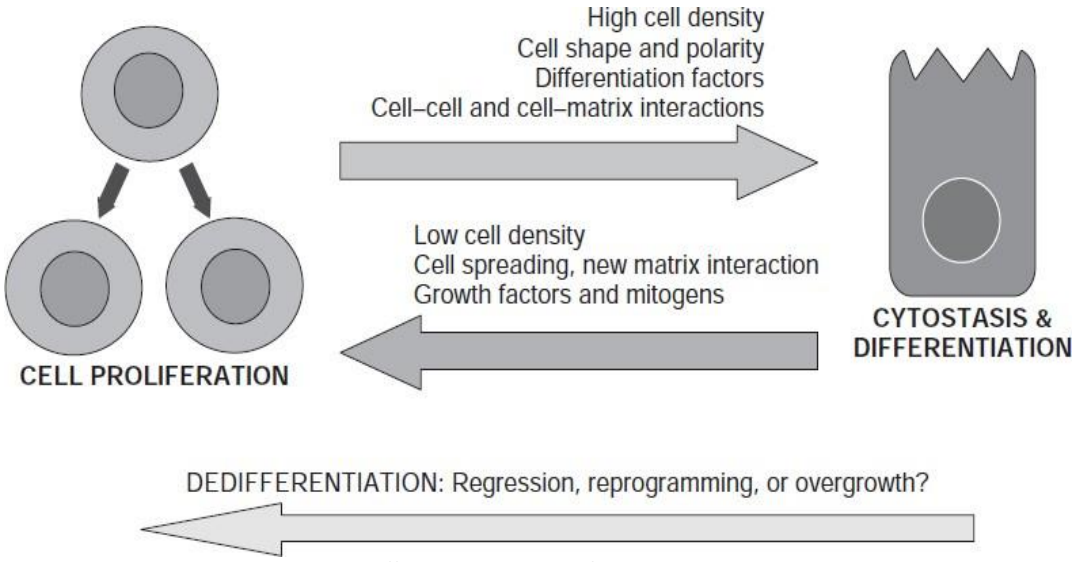

Figure 2: Differentiation and Proliferation

As differentiation progresses, cell division is reduced and ultimately ceases. In most cell systems, cell proliferation is incompatible with the expression of differentiated properties. Tumor cells can sometimes break this restriction, and in melanoma, for example, melanin continues to be synthesized while the cells are proliferating. Even in these situation, synthesis of the differentiated product increases when division stops.

Differentiation from stem cells:

It may be useful to think of a cell culture as being an equilibrium between stem cells, undifferentiated precursor cells, and mature differentiated cells and to suppose that the equilibrium may shift according to the environmental conditions.

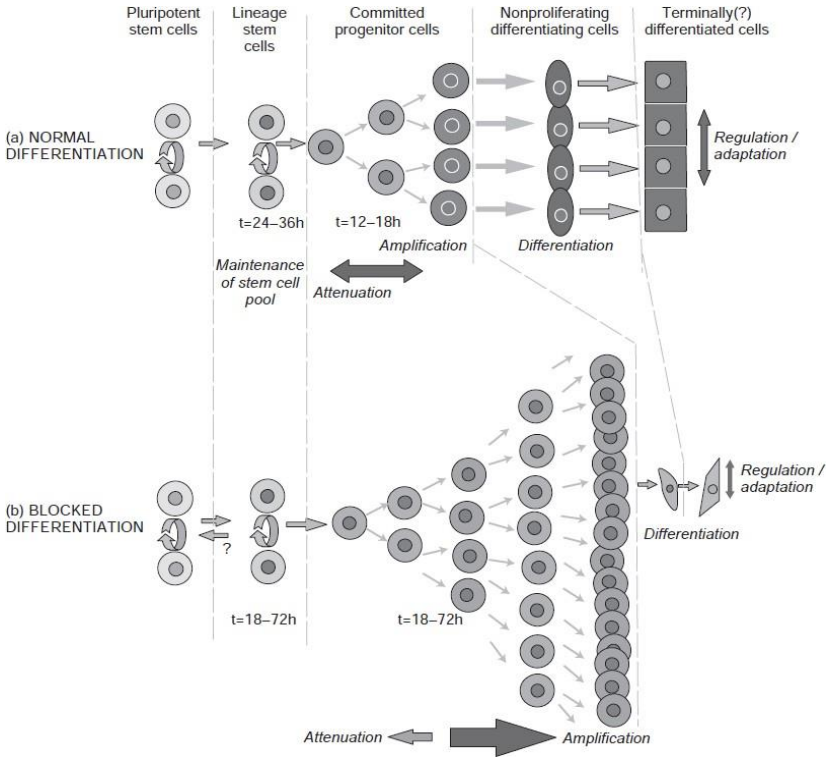

Figure 3: (a) In vivo, a small stem cell pool gives rise to a proliferating progenitor compartment that produces the differentiated cell pool. (b) In vitro, differentiation is limited proliferation, and the population becomes predominantly of progenitor cells, though stem cells may also be present.

Diamond nanoparticles have also been used to modify the substrate for the proliferation and differentiation of neural stem cells and the configuration of the growth surface can also be altered by photoetching. Treatment of the substrate with denatured collagen improves the attachment of many types of cells, such as epithelial cells, and the nondenatured gel may be necessary for the expression of differentiated functions.

Interesting facts:

1. Cells in culture can be thought to be in a state of equilibrium between cell proliferation and differentiation. Normal culture conditions i.e. in low cell density, mitogens in the medium will favor cell proliferation, whereas high cell density and addition of differentiation factors will induce differentiation.

2. Patients with poorly differentiated tumors generally have a lower survival rate than patients with differentiated tumors.

Questions:

1. What is differentiation? What are the major pathways of differentiation?
2. What are the parameters that are required for control of differentiations?
3. What are the relationship between differentiation and malignancy?

References:

1. Fresheny, I.; "Culture of animal cell – A manual of basic technique and specialized application.

Three dimensional cell culture

Three Dimentional Culture: Three-Dimensional (3-D) cell cultures have been widely used in biomedical research since the early decades of this century. The potential of 3-D cell cultures is currently being exploited in various areas of biomedical research. One reason for the recent progress in research on multi cell systems may be the increasing interaction between researchers working in different fields of biomedical science and using similar 3-D culture techniques. Such a research effort mirrors the common need for improved and more refined in vitro models as a link between cell-free systems or single cells and organs or whole organisms in vivo. One major advantage of 3-D cell cultures is their well-defined geometry, which makes it possible to directly relate structure to function and which enables theoretical analyses such as diffusion fields. Subsequently,the most promising data on these cultures may be obtained by using techniques allowing for spatial resolution. Combining such approaches with molecular analysis has clearly confirmed that, in comparison with conventional cultures, cells in 3-D cultures more closely resemble the in vivo situation with regard to cell shape and cellular environment. These parameters (shape and environment) can determine gene expression and the biological behaviour of the cells. In contrast to 2D monolayer, 3D cell culture models are modular, adaptable biomedical systems consisting range in complexity from a single cell type (monotypic), representing the minimum unit of the differentiated tissue *in vivo* to complex co-culture models that recapitulate both the 3D architecture and the multicellular complexity of the parental tissue. There will always be a number of questions that can only be answered by investigations using single cells or cell-free systems. At the same time, 3-D cultures cannot completely replace the testing of biological mechanisms for their relevance in vivo, e.g., in knockout animals

Effect of Cell Density:

Cell–cell interaction is manifested at the simplest level when a cell culture reaches confluence and the constituent cells begin to interact more strongly with each other because of contact mediated signaling, formation of junctional complexes and increased potential for exchange of homocrine factors. The first noticeable effect is cessation of cellmotility (contact inhibition) and withdrawal from cell cycle (density limitation of cell proliferation) in normal cells and reduced cell proliferation and increased apoptosis in transformed cells.

Reciprocal Interaction:

When different cells interact in their population, they have tendency to show reciprocal effect on their respective phenotypes, and the resultant phenotypic changes lead to new interactions. Cell interaction is therefore not just a single event, but a continuing cascade of events. Similarly exogenous signals do not initiate a single event, as may be the case with homogeneous populations, but initiate a new cascade, as a result of the exogenously modified phenotype of one or both partners.

Choice of Model for Three Dimensional Cultures:

There are two major way to approach these goals.

- One is to accept the cellular distribution within the tissue, explant it and maintain it as an organ culture.
- The second is to purify and propagate individual cell lineages, study them alone under conditions of homologous cell interaction, recombine them, and study their mutual interactions.

Types of Three Dimensional cultures: There are three main types of three dimensional cultures:

- Organ culture
- Histotypic culture
- Organotypic culture

1) **Organ culture:** Organ culture in which whole organs or representative parts are maintained as small fragments in culture and retain their intrinsic distribution, numerical and spatial, of participating cells. Organ culture seeks to retain the original structural relationship of cells of the same or different types, and hence their interactive function in order to study the effect of exogenous stimuli on further development. Organ culture seeks to retain the original structural relationship of cells of the same or different types and hence their interactive function, in order to study theeffect of exogenous stimuli on further development.
2) **Histotypic culture:** Histotypic culture in which propagated cells are grown alone to high density in a three-dimensional matrix or are allowed to form three-dimensional aggregates in suspension
3) **Organotypic culture:** Organotypic culture in which cells of different lineages are recombined in experimentally determined ratios and spatial relationships to recreate a component of the organ under study

Organ Culture:

Gas and Nutrient Exchange: A major deficiency in tissue architecture in organ culture is the absence of a vascular system, limiting the size (by diffusion) and potentially the polarity of the cells within the organ culture. When cells are cultured as a solid mass of tissue, gaseous diffusion and the exchange of nutrients and metabolites is from the periphery, and the rate of this diffusion limits the size of the tissue. The dimensions of individual cells cultured in suspension or as a monolayer are such that diffusion is not limiting, but survival of cells in aggregates beyond about 250 μm in diameter (~5000 cell diameters) starts to become limited by diffusion, and at or above 1.0 mm in diameter (~2.5 × 10^5 cell diameters) central necrosis is often apparent. To alleviate this problem, organ cultures are usually placed at the interface between the liquid and gaseous phases, to facilitate gas exchange while retaining access to nutrients. This is achieved by most

system by positioning the explant in a filter well insert on a raft or gel exposed to the air, but explants anchored to a solid substrate can also be aerated by rocking the culture, exposing it alternately to a liquid medium and a gas phase or by using a roller bottle or rotating tube rack. Anchorage to a solid substrate can lead to the development of an outgrowth of cells from the explant and resultant alterations in geometry even though this effect can be minimized by using a hydrophobic surface. One of the advantages of culture at the gas–liquid interface is that the explant retains a spherical geometry if the liquid is maintained at the correct level. If the liquid is too deep, gas exchange is impairedwhereas if it is too shallow, surface tension will tend to flatten the explants and promote outgrowth. Permeation of oxygen increases by using increasing O_2 concentrations up to pure oxygen or by using hyperbaric oxygen. As increasing the O_2 tension will not facilitate CO_2 release or nutrient metabolite exchange, the benefits of increased oxygen may be overridden by other limiting factors.

Structural Integrity: The maintenance of structural integrity is the main reason for adopting organ culture as an in vitro technique in preference to cell culture. Whereas cell culture utilizes cells dissociated by mechanical or enzymatic techniques or spontaneous migration, organ culture deliberately maintains the cellular associations found in the tissue.

Growth and Differentiation: There is a relationship between growth and differentiation such that differentiated cells no longer proliferate. It is also possible that cessation of growth, regardless of cell density, may contribute to the induction of differentiation, if only by providing a permissive phenotypic state that is receptive to exogenous inducersof differentiation. Because of density limitation of cell proliferation and the physical restrictions imposed by organ culture geometry, most organ cultures do not grow or if they do proliferation is limited to the outer cell layers. Hence the status of the culture is permissive to differentiation and the appropriate cellular interactions and soluble inducers are provided as an ideal environment for differentiation to occur.

Limitations of Organ Culture:

- Organ cultures depend largely on histological techniques and they do not impart themselves readily into biochemical and molecular analyses.
- Biochemical monitoring requires reproducibility between samples, which is less easily achieved in organ culture than in propagated cell lines, because of sampling variation in preparing an organ culture, minor differences in handling and geometry, and variations in the ratios of cell types among cultures.
- Organ cultures are also more difficult to prepare than replicate cultures from a propagated cell line and do not have the advantage of a characterized reference stock to which they may be related.
- Organ culture is essentially a technique for studying the behaviour of integrated tissues rather than isolated cells.
- Organ culture has contributed a great deal to our understanding of developmental biology and tissue interactions and that it will continue to do so in the absence of adequate synthetic systems.

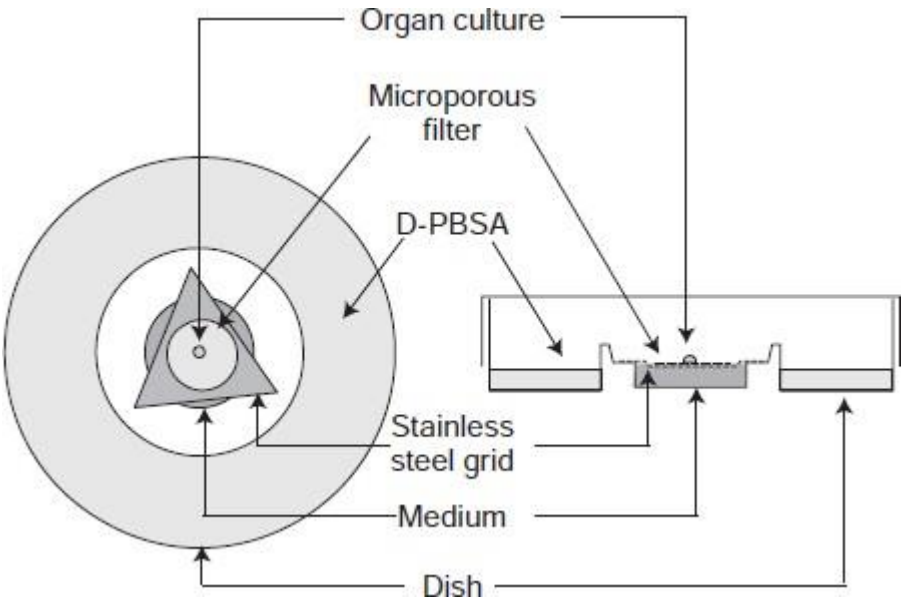

Figure 1: Organ Culture Small fragment of tissue on a filter laid on top of a stainless steel grid over the central well of an organ culture dish

Histotypic culture:

Histotypic culture is defined as high-density cell culture with the cell density approaching that of the tissue in vivo. Various attempts have been made to regenerate tissue-like architecture from dispersed monolayer cultures. As cells reach a high density, medium nutrients will become limiting. To avoid this, the ratio of medium volume to cell number should remain approximately as it was in low density culture. This can be achieved by seeding cells on a small coverslip in the center of a large non–tissue-culture grade dish or by use of filter well inserts, which give the opportunity for the formation of both high-density polarized cultures and heterotypic combinations of cell types to create organotypic cultures. A high medium-to-cell ratio can also be maintained by perfusion.

Gel and Sponge Techniques: Use of three-dimensional sponges and gels has increased extensively with the development of tissue engineering. Two commonly used gel in this technique.

- **Collagen gel:** Collagen gel (native collagen, as distinct from denatured collagen coating) provides a matrix for the morphogenesis of primitive epithelial structures. Many different types of cell can be shown to penetrate such matrices and establish a tissue-like histology.
- **Matrigel:** Matrigel is a commercial product derived from the extracellular matrix of the Engelbreth–Holm–Swarm (EHS) mouse sarcoma which has been used for coating plastic but can also be used in gel form. It is composed of laminin, collagen, fibronectin, and proteoglycans with a number of bound growth factors, although it can be obtained in a growth factor-depleted form. It has been used as a substrate for epithelial morphogenesis formation of capillaries from endothelial cells and in the study of malignant invasion.

Organotypic Culture:

High density three-dimensional culture involving the recombination of different cell lineages may be referred to as organotypic culture, a term that used to distinguish these reconstruction techniques from organ culture where the original cells are not dissociated. The key event that distinguishes these constructs from histotypic culture is the introduction of heterotypic cell interaction including diffusible paracrine effects and signaling implicating the extracellular matrix. The relationship of the cells allows the

generation of a structured microenvironment, cell polarity and enhanced differentiation. Creation of organotypic culture by mixing cells randomly and allowing them to interact and sort, as can happen spontaneously particularly with embryonic cells or the construct may be design to keep the interacting cells separate so that their interactions may be studied.

Tissue Equivalents: The advent of filter well technology boosted by its commercial availability, has produced a rapid expansion in the study of organotypic culture methods.

Tissue Engineering: Just as organotypic culture needs cell interaction, constructs for tissue engineering often require similar interactions, as in the interaction between endothelium and smooth muscle in blood vessel reconstruction. In addition to biological interactions, some constructs require physical forces; skeletal muscle needs tensile stress, bone and cartilage needs compressive stress, and vascular endothelium in a blood vessel construct needs pulsatile flow.

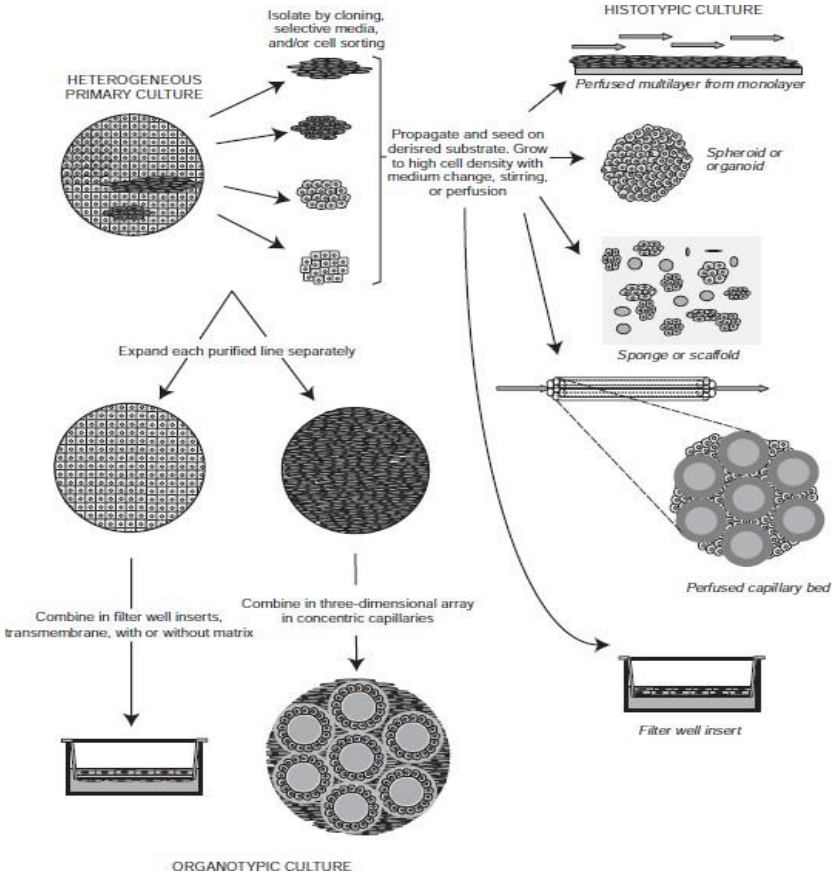

Figure 2: Histotypic and Organotypic Culture

Imaging Cells in 3-D Constructs: As we know that microscopic observation becomes difficult when cells are incorporated into a scaffold in a three-dimensional organotypic construct, alternative methods must be used to visualize the status of the cells within the construct. This can be done by NMR if the bioreactor housing the constructs is placed within an NMR detector and the output displayed as an MRI, and the emission spectrum being analysed.

Interesting facts:

1. Organ culture is a technique for studying the behaviour of integrated tissues.
2. Organ culture has contributed a great deal to our understanding of developmental biology and tissue interactions.

Questions:

1. What are the types of three dimensional cultures?
2. What are the advantages of three dimensional cultures?
3. What are Gel and Sponge Techniques and how its use increase with increasing genetic engineering?

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Role of matrix in cell growth

Role of matrix in cell growth: Matrix is an insoluble, dynamic gel in the cytoplasm, believed to be involved in cell shape determination and locomotive mechanism, across a solid substrate. It consists of polymeric microtubules, actin microfilaments and intermediate filaments interacting with a number of other proteins.

Extracellular matrix (ECM): The extracellular matrix (ECM) is a part of three connective tissue layers (endomysium, perimysium, and epimysium) surrounding muscle fibres. Extracellular matrix is composed of proteins including collagens and proteoglycans.

Component of extra cellular matrix: ECM is comprised variously of collagen, laminin, fibronectin, hyaluronan and proteoglycans such as beta glycan, decorin, perlecan, and syndecan-1, some of which bind to growth factors or cytokines.

- Proteoglycans in extracellular matrix form a cross-linked meshwork with fibrous proteins
- Some proteins bind multiple other proteins and glycosaminoglycans (fibronectin).
- Integrin is a family of proteins that mediate signalling between cell interior and extracellular matrix
- Mass of interactions between cells and matrix not only anchors cells to matrix but also provides paths that direct migration of cells in developing tissue and (through integrin) conveys information in both directions across plasma membrane. Figure 1 depicts structure of cell matrix.

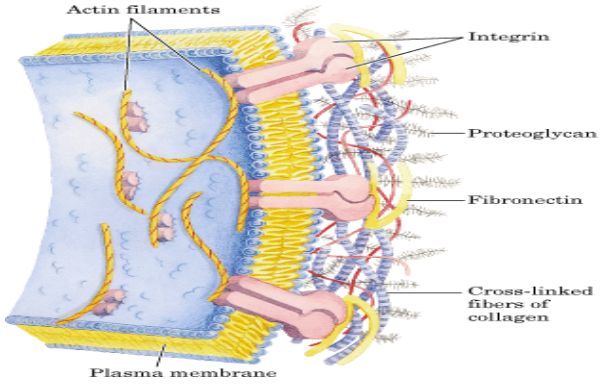

Figure 1: Cell matrix

ECMs are composed of proteins such as collagen and elastin that serve as scaffolds for cells as well as networks of various adhesion ligands and growth factors, which promote cell signalling. ECM is complex in both structure and composition.

Role of matrix: There are some important roles that matrix play in biological system which is described as follows.

- Matrixes are generally used for providing support
- It involve in segregating tissues from one another
- It takes part in regulation of intercellular communication
- Extracellular Matrix cells have been found to cause regrowth and healing of tissue.
- Some time it acts as fibrosis
- The use of ECM constituents can be highly beneficial in enhancing cell survival, proliferation, or differentiation, but unless recombinant molecules are used
- In human foetuses, the extracellular matrix works with stem cells to grow and regrow all parts of the human body and foetus can regrow anything that gets damaged in the womb
- In case of injury repair and tissue engineering, the extracellular matrix serves two main functions

a) It prevents the immune system by triggering from the injury and responding with inflammation and scar tissue

b) It facilitates the surrounding cells to repair the tissue instead of forming scar tissue

Molecular components: Components of the ECM are produced intracellularly by resident cells and secreted into the ECM through exocytosis. Once secreted, they then aggregate with the existing matrix. The ECM is composed of an interlocking mesh of fibrous proteins and glycosaminoglycans (GAGs).

Proteoglycans: Since we know that, GAGs are carbohydrate polymers and are usually attached to extracellular matrix proteins to form proteoglycans (exception-hyaluronic acid). Proteoglycans have a net negative charge that attracts positively charged sodium ions (Na^+) which attracts water molecules via osmosis, keeping the ECM and resident cells hydrated. Proteoglycans may also help to trap and store growth factors within the ECM. There are the different types of proteoglycan found within the extracellular matrix.

1. Heparin sulphate: Heparin sulphate (HS) is a linear polysaccharide found in all animal tissues. It occurs as a proteoglycan (PG) in which two or three HS chains are attached in close proximity to cell surface or extracellular matrix proteins. HS binds to a variety of protein ligands and involve in regulation of a wide variety of biological activities, including developmental processes, angiogenesis, blood coagulation and tumour metastasis. In the extracellular matrix, particularly basement membranes, the multi-domain proteins perlecan, agrin and collagen XVIII are the main proteins to which heparin sulphate is attached.

2. Chondroitin sulphate: Chondroitin sulfates help to provide the tensile strength of cartilage, tendons, ligaments and walls of the aorta. They have also been known to affect neuroplasticity.

3. Keratan sulphate: Keratan sulfates have variable sulfate content and unlike many other GAGs, do not contain uronic acid. They are present in the cornea, cartilage, bones and the horns of animals.

Non-proteoglycan polysaccharide: There are various non-proteoglycan polysaccharides.

1. **Hyaluronic acid:** Hyaluronic acid (or hyaluronan at physiological pH) is a polysaccharide containing alternating residues of D-glucuronic acid and *N*-acetyl glucosamine. Unlike other glycosaminoglycan (GAGs) it is not found as a proteoglycan. Like cellulose and chitin, it is synthesized at the plasma membrane by a transmembrane hyaluronan synthase. Hyaluronan is the only GAG that occurs as a single long polysaccharide chain. Hyaluronate is also an essential component of the extracellular matrix of cartilage and tendons, to which it contributes tensile strength and elasticity as a result of its strong interactions with other components of the matrix. A number of proteoglycans interact with hyaluronan to form large complexes in the extracellular

matrix. A well-characterized example is aggrecan, the major proteoglycan of cartilage. Hyaluronic acid acts as an environmental sign that regulates cell behaviour during embryonic development, healing processes, inflammation and tumour development. It interacts with a specific trans-membrane receptor, CD44.

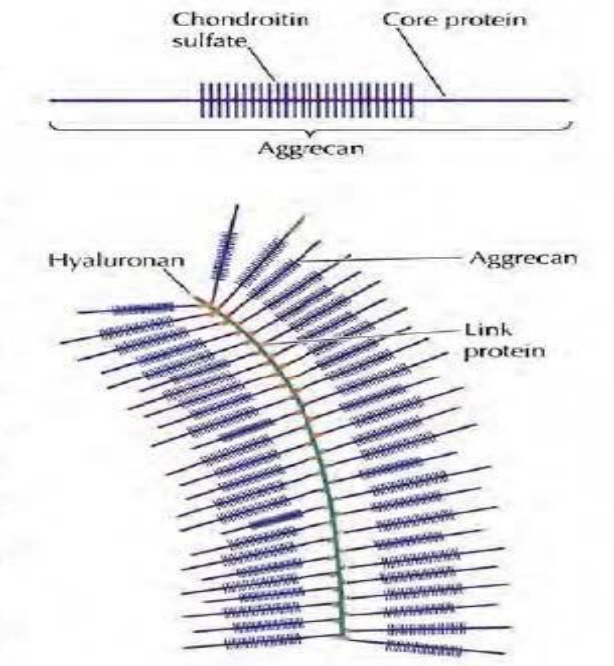

Figure 2: Structure of hyaluronan and aggrecan

2. **Matrix Structural Proteins:** Extracellular matrices are composed of tough fibrous proteins embedded in a gel-like polysaccharide ground substance-a design basically similar to that of plant cell walls.

Collagen: In ECM of most animals, collagens are the abundantly found structural protein. In fact, collagen is the most abundant protein in the human body and accounts for90% of bone matrix protein content. Collagens are present in the ECM as fibrillarproteins and give structural support to resident cells. Collagens are a large family of proteins containing at least 27 different members. They are characterized by the formation of triple helices in which three polypeptide chains are wound tightly around one another in a rope-like structure. The different collagen polypeptides can assemble into 42 different trimers. The triple helix domains of the collagens consist of repeats of the amino acid sequence Gly-X-Y. A glycine (the smallest amino acid, with a side chain consisting only of hydrogen) is required in every third position, so that the polypeptide chains can pack together close enough to form the collagen triple helix. Proline is

frequently found in the X position and hydroxyproline in the γ position; because of their ring structure these amino acids stabilize the helical conformations of the polypeptide chains. The unusual amino acid hydroxyproline is formed within the endoplasmic reticulum by modification of proline residues that have already been incorporated into collagen polypeptide chains. Lysine residues in collagen are also frequently converted to hydroxylysines. The hydroxyl groups of these modified amino acids are thought tostabilize the collagen triple helix by forming hydrogen bonds between polypeptide chains. These amino acids are rarely found in other proteins although hydroxyproline is also common in some of the glycoproteins of plant cell walls.

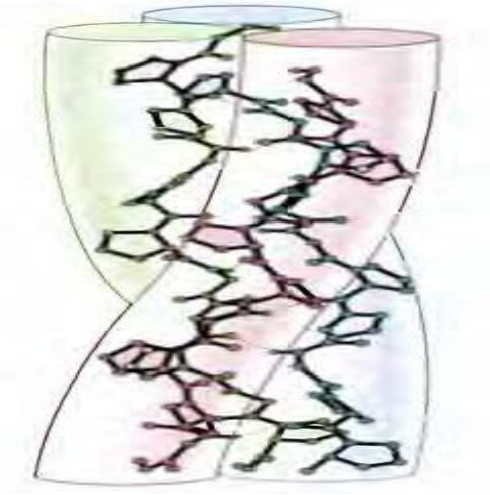

Figure 3: Structure of triple helix collagen

The collagen can be divided into several families according to the types of structure they form:

Table 1: Classification of collagens

Collagen class	Type	Tissue Distribution
Fibril- forming	I	Most connective tissue
	II	Cartilage and vitreous humors (e.g. skin and lung)
	III	Tissue containing collagen I
	V	Cartilage
	XI	Bone and cornea
	XXIV	Eye, ear and lung
	XXVII	Cartilage
Fibril-associated	IX	Cartilase
	XII	Tissue containing collagen I
	XIV	Tissue containing collagen I
	XVI	Many tissue
	XIX	Many tissue
	XX	Cornea
	XXI	Many tissue
	XXII	Cell junctions
	XXVI	Testis and overy
Network forming	IV	Basal laminae
	VIII	Many tissues
	X	Cartilase
Anchoring fibrils	VII	Attachement of basal laminae to underlying connective tissue
Transmembrane	XVII	Skin hemidesmosomes
	XXV	Nerve cells

The most abundant type of collagen (type I collagen) is one of the fibril forming collagens that are the basic structural components of connective tissues. The polypeptide chains of these collagens consist of approximately a thousand amino acids or 330 Gly-X- Y repeats. After being secreted from the cell these collagens assemble into collagenfibrils in which the triple helical molecules are associated in regular staggered arrays. These fibrils do not form within the cell because the fibril forming collagens are synthesized as soluble precursors (procollagens) that contain nonhelical segments at both ends of the polypeptide chain. Procollagen is cleaved to collagen after its secretion, so theassembly of collagen into fibrils take place only outside the cell. The association of collagen molecules in fibrils is further strengthened by the formation of covalent

crosslinks between the side chains of lysine and hydroxylysine residues. Frequently, the fibrils further associate with one another to form collagen fibers, which can be several micrometers in diameter.

Elastin: In contrast to collagens, Elastins give elasticity to tissues, allowing them to stretch when needed and then return to their original state. This is useful in blood vessels,the lungs, in skin, and the ligaments. Elastins are synthesized by fibroblasts and smooth muscle cells. Elastins are highly insoluble, and tropoelastins are secreted inside a chaperone molecule, which releases the precursor molecule upon contact with a fiber of mature elastin. Tropoelastins are then deaminated to become incorporated into the elastin strand. Diseases such as cutis laxa and Williams syndrome are associated with deficientor absence of elastin fibers in the ECM.

Matrix adhesion proteins: Matrix adhesion proteins, the final class of extracellular matrix constituents are responsible for linking the components of the matrix to one another and to the surfaces of cells. They interact with collagen and proteoglycans to specify matrix organization and are the major binding sites for integrins.

1. Fibronectin: Fibronectin is the principal adhesion protein of connective tissues. Fibronectin is a dimeric glycoprotein consisting of two polypeptide chains, each containing nearly 2500 amino acids. Fibronectin are proteins that connect cells with collagen fibers in the ECM, allowing cells to move through the ECM. Within the extracellular matrix, fibronectin is often cross-linked into fibrils. Fibronectin has binding sites for both collagen and GAGs so it cross-links these matrix components. Fibronectins bind collagen and cell surface integrins, causing a reorganization of the cell's cytoskeleton and facilitating cell movement. Fibronectin are secreted by cells in an unfolded, inactive form. Binding to integrins unfolds fibronectin molecules, allowing them to form dimers so that they can function properly. Fibronectins also help at the site of tissue injury by binding to platelets during blood clotting and facilitating cell movement to the affected areas during wound healing.

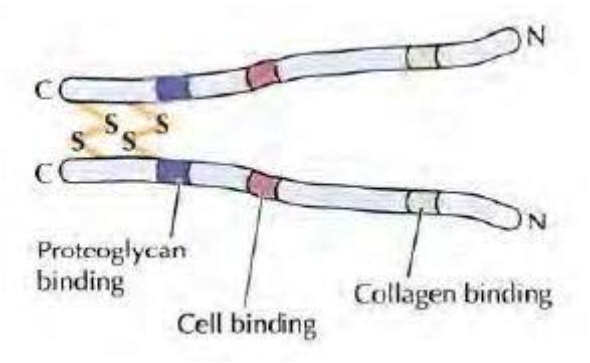

Figure 4: Structure of Fibronectin

2. Laminin: In almost all animals, Basal laminae contain distinct adhesion proteins of the laminin family. Laminins are heterotrimers of α, β and γ subunits which are the products of five α genes, four β genes, and three γ genes. Like type IV collagen, laminins can self-assemble into meshlike polymers. Such laminin networks are the major structural components of the basal laminae synthesized in very early embryos, which do not contain collagen. The laminins also have binding sites for cell surface receptors such as integrins, type IV collagen, and the heparan sulfate proteoglycan, perlecan. In addition, laminins are tightly associated with another adhesion protein, called entactin, which also binds to type IV collagen. As a result of these multiple interactions, laminin, entactin, type IV collagen, and perlecan form cross-linked networks in the basal lamina. They also support in cell adhesion.

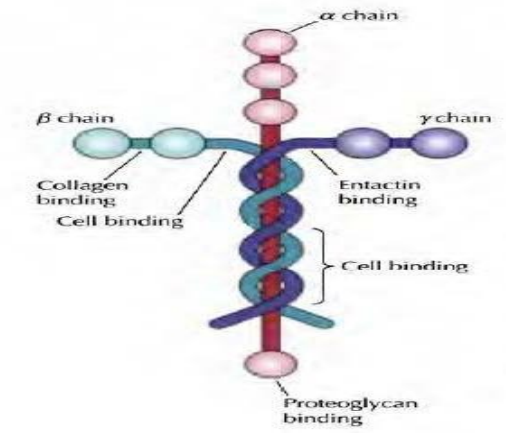

Figure 5: Structure of laminin

Cell adhesion to the ECM: Many cells bind to components of the extracellular matrix. Cell adhesion can occur in two ways by focal adhesions, connecting the ECM to actin filaments of the cell, and hemi-desmosomes, connecting the ECM to intermediate filaments such as keratin. This cell-to-ECM adhesion is regulated by specific cell surface cellular adhesion molecules (CAM) known as integrins. The integrins are a family of transmembrane proteins consisting of two subunits, designated α and β. Integrins are cell surface proteins that bind cells to ECM structures such as fibronectin and laminin, and also to integrin proteins on the surface of other cells.

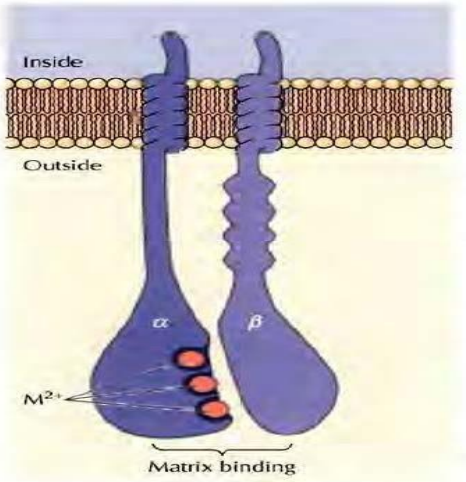

Figure 6: Structure of Integrin

Fibronectins bind to ECM macromolecules and facilitate their binding to transmembrane integrins. The attachment of fibronectin to the extracellular domain initiates intracellular signaling pathways as well as association with the cellular cytoskeleton via a set of adaptor molecules such as actin.

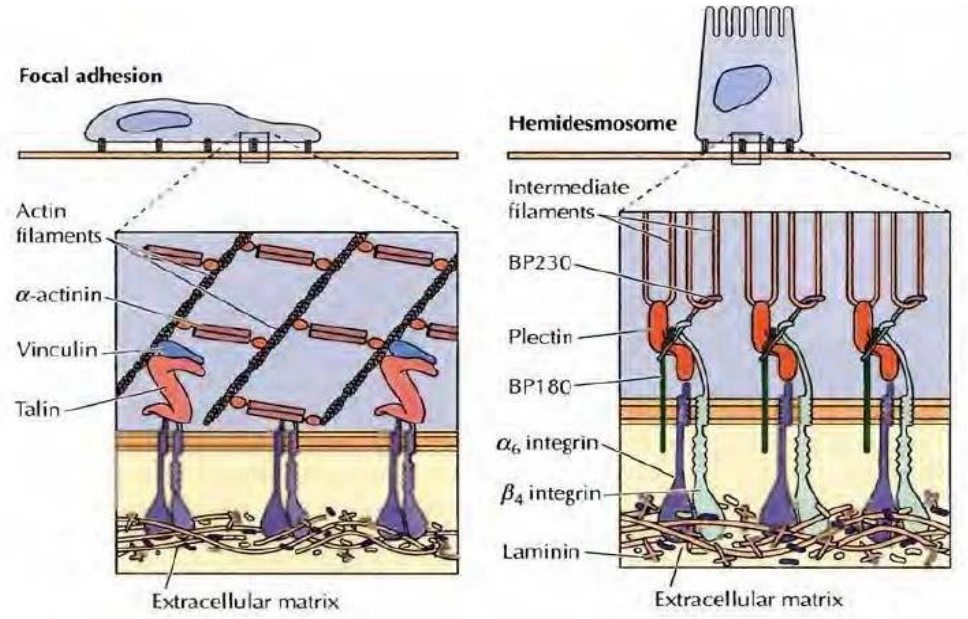

Figure 7: Focal adhesion and Hemidesmosome

Commercially available matrices:

Commercially available matrices such as Matrigel™ (Becton Dickinson) from the Engel breth–Holm–Swarm (EHS) sarcoma, contain laminin, fibronectin, and proteoglycans,with laminin predominating. Other matrix products include Pronectin F (Protein Polymer Technologies), laminin, fibronectin, vitronectin entactin (UBI), heparan sulfate, EHS Natrix (BD Biosciences), ECL (US Biological), and Cell-tak (BD Biosciences). Some of these products are purified, whereas others are a mixture of matrix products that have been poorly characterized and may also contain bound growth factors. If cell adhesion for survival is the main objective, and defined substrates are inadequate, the use of these matrices is acceptable, but if mechanistic studies are being carried out, they can only be an intermediate stage on the road to a completely defined substrate.

Table 2: Examples of some matrix

Material	Composition	Source
Matrigel	Laminin, fibronectin, collagen IV, proteoglycans, growth factors (growth factor depleted available)	EHS sarcoma
EHS Natrix	Laminin, fibronectin, collagen IV, proteoglycan, growth factors	Cell line from EHS sarcoma
Cell-Tak	Polyphenolic protein	Mytilus edulis
Collagens(various)	Collagen I, II, III, IV	Human, bovine, rat tail
ProNectin F	Protein polymer with multiple copies of RGD containing epitops	Recombinant
Laminin	Attachement protein from basement membrane	Natural
Laminin	Attachement protein from from basement membrane	Recombinant
Fibronectin	Attachement protein from from extracellular matrix	Natural
Fibronectin	Attachement protein from from extracellular matrix	Recombinant
Heparan sulfate	Matrix proteoglycan	Natural
ECL	Enactin-collagen IV-laminin	Natural
Vitronectin	Attachement protein from extracellular matrix	Natural
ECM	Extracellular matrix	natural

Interesting facts:

1. Collagens are abundantly found structural protein in the human body and accounts for 90% of bone matrix protein content.

2. Elastins give elasticity to tissues, allowing them to stretch when needed and then return to their original state.

3. Fibronectins bind to ECM macromolecules and facilitate their binding to transmembrane integrins.

4. Basal laminae contain distinct adhesion proteins of the laminin family in almost all animals.

Questions:

1. Explain the role of matrix in cell culture?
2. Give the detail about matrix material that are generally used?
3. What is the difference between proteoglycan and non-proteoglycan polysaccharide and how can they involve in formation of matrix?

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4. What are the types of matrixes? What are the advantages of matrix adhetion protein?