M.Sc., Biochemistry **2019-2021**

Semester III

19BCP205B COREELECTIVE –II 4H-4C

ANIMAL TISSUE CULTURE

Instruction hours/week:L:4 T:0P:0Marks: Internal: **40** External: **60**Total: **100**

End Semester Exam: 3 Hours

Course objectives

- To impart knowledge on basic tissue culture techniques, chemically defined and serum free media preparation
- To perform the animal cell cultures, maintenance of cell lines and thier preservation.
- To convey knowledge on various types of cultures- suspension cultures, continuous flow cultures, immobilized cultures; somatic cell fusion and organ cultures.
- To use cell cultures as a source of valuable products through tissue engineering

Course outcomes (CO's)

1. Learn the foundationals of animal cell culture techniques and competence in laboratory techniques.
2. Student can set up a tissue culture lab to carry out research based on cell lines.
3. Demonstrate different types of cell lines, their maintenance and tissue engineering provcess

UNIT I: Introduction to cell culture

Introduction, importance, history of cell culture development, different tissue culture techniques including primary and secondary culture, continuous cell lines, suspension culture, organ culture, advantages and limitations medical/pharmaceutical products of animal cell culture-genetic engineering of animal cells and their applications. Risks in a tissue culture laboratory and safety - biohazards.

UNIT II: Different types of cell culture media

Different types of cell culture media, growth supplements, serum free media, balanced salt solution, other cell culture reagents, culture of different tissues and its application. Facilities for animal cell culture-infrastructure, equipment, culture vessels. Biology and characterization of cultured cells-cell adhesion, proliferation, differentiation, morphology of cells and identification.

UNIT III: Types of cell culture techniques

Primary cell culture techniques - mechanical disaggregation, enzymatic disaggregation, separation of viable and non-viable cells. Mass culture of cells - manipulation of cell line selection - types of cell lines -maintenance of cell lines - immobilization of cells and its application - synchronization of cell cultures and cell division - production of secondary metabolites - biotransformation - Induction of cell line mutants and mutations - cryopreservation – germplasm conservation and establishment of gene banks.

UNIT IV: Animal cell culture scale up

Animal cell culture scale up: Scale up in suspension - stirrer culture, continuous flow culture, air-lift fermentor culture; Scale up in monolayer - Roller bottle culture, multisurface culture, multiarray disks, spirals and tubes - monitoring of cell growth. Organ culture - whole embryo culture - specialized culture techniques - measurement of cell death.

UNIT V: Tissue engineering

Tissue engineering: Design and engineering of tissues - tissue modeling. Embryonic stem cell engineering - ES cell culture to produce differential cells - Human embryonic stem cell research. Transgenic animals-transgenic animals in xenotransplantation

SUGGESTED READINGS

1. Darling, D.C., and Morgan, S.J., (1994). Animal Cells Culture and Media, BIOS Scientific Publishers Limited.

2. Ranga, M.M., (2000). Animal Biotechnology,. Agrobios, India.

3. Satyanarayana, U., (2006). Biotechnology, Books and Allied (P) Ltd. India.

4. Harris, A., (1996). Epithelial Cell Culture, Cambridge University Press, London.

5. Mathur, J.P., and David Barnes, D., (1998). Methods in Cell Biology, Volume 57, Animal Cell Culture Methods Academic Press.

KARPAGAM ACADEMY OF HIGHER EDUCATION

(Deemed to be University)
(Established Under Section 3 of UGC Act 1956)
Coimbatore - 641021.
(For the candidates admitted from 2018 onwards)
DEPARTMENT OF BIOCHEMISTRTY

SUBJECT : MOLECULAR BIOLOGY

SEMESTER : II

SUBJECT CODE: 19BCP202 **CLASS : I M. Sc. BC**

LECTURE PLAN
DEPARTMENT OF BIOCHEMISTRTY

S.No	Lecture Duration Hour	Topics to be Covered	Support Material/Page Nos
		UNIT-I	
1	1	Molecular definition of gene, chromosomal organization of genes and non-coding DNA,	T1: 406-408
2	1	Protein coding genes	T2:409-411
3	1	Tandomly repeated genes,	T1: 411-412
4	1	Single sequence DNA.	T1: 413
5	1	Structural organization of eukaryotic chromosomes- histone proteins,	T1:424-425
6	1	Chromatin, functional elements.	T1:425-428
7	1	Mobile DNA elements- bacterial IS elements, Transposons, viral transposons and non- viral transposons.	T1:414-423
8	1	Mutation- types.	T2: 91-94
	Total No Of Hours Planned For Unit 1=8		
		UNIT-II	
1	1	General features of chromosomal replication.	T2:73-75
2	1	Enzymology of DNA replication.	T2:75-78
3	1	DNA replication machinery.	T3:78-82
4	1	Replication in prokaryotes - Initiation, elongation and termination.	T1:82-86

5	1	Replication in eukaryotes- Initiation, elongation and termination.	T2:86-90
6	1	DNA damage-types. Repair mechanism of DNA damage-all types.	T2:95-100
	Total No Of Hours Planned For Unit II=06		
		UNIT-III	
1	1	Prokaryotic gene transcription- Initiation, elongation and termination.	T2:185-193
2	1	Eukaryotic gene transcription- transcription unit	T3:241-244
3	1	RNA polymerases- types, Transcription and processing of mRNA, tRNA and rRNA.	T1:211-217
4	1	Regulatory sequences in protein coding genes- TATA box, initiators, CpG island	T2:217-221
5	1	Promoter-proximal element, activators and repressors of transcription,	T2:221-223
6	1	Multiple transcription control elements.	T1:486-489
7	1	Regulation of transcription factor activity by lipid-soluble hormones.	T1:481-484
	Total No Of Hours Planned For Unit III=07		
		UNIT-IV	
1	1	Deciphering genetic code, features.	T4:1065-1066
2	1	Wobble hypothesis.	T4:1066-1072
3	1	Initiation, elongation and termination of prokaryotic translation.	T2:273-278
4	1	Initiation, elongation and termination of eukaryotic translation.	T2:279-282
5	1	Fidelity of translation.	T4:1095-1098
6	1	Post translational modifications-all types	T3:283-286
7	1	Protein targeting-Targeting protein to nucleus.	T2:286-287
8	1	Protein targeting- ER.	T4:1100-1101
9	1	Protein degradation- Golgi complex.	T4:1101-
10	1	Protein degradation- ubiquitin mediated degradation.	T1: 71-72
	Total No Of Hours Planned For Unit IV=10		
		UNIT-V	

1	1	Operon model – Lac operon	T5:1294-1296
2		Operon model – trp and ara operons.	T5:1291-1301
3	1	Regulatory proteins-DNA binding domain, protein- protein interaction domain.	T5:1288-1290
4	1	Recombination- holiday model, Rec BCD enzymes, Rec A protein, Messelson Radding model, site- specific recombination.	T5:1226-1252
5	1	Antisense RNA technology	T5:1323-1325
6	1	Transcriptionally active chromatin, chromatin remodeling,	T1:476-478
7	1	DNA binding transactivators and coactivators.	T1:478-481
8	1	Regulation of gene expression by intracellular and intercellular signal,	T1:481-486
9	1	RNAi.	T1:518-520
	Total no of Hours Planned for unit V=09		
Total Planned Hours		**40**	

References:

T1: Lodish, H., Berk, A., Kaiser, C.A., and Krieger, M., (2012). Molecular Cell Biology, 7th edition. W.H. Freeman & Company,

T2: Turner, P., McLennan, A., Bates, A., and White, M., (2006) Instant Notes in Molecular Biology 3nd Edition. Publisher: Taylor and Francis Group.

T3: Lewin, B., (2008) Genes IX, Oxford University Press, 9th Edition, Oxford, London.

T4: Lehninger, L., Nelson, D.L., and Cox, M.M., (2012). Principles of Biochemistry, WH Freeman and Company, 6th Edition, New York.

T5: Donald Voet and Judith Voet; 2012, Biochemistry, 4th Edition, John Wiley and Sons. Inc

Signature of the Staff

1.What are the techniques of tissue culture?

The basic **techniques** of some important **tissue cultures** are: (1) Meristem **Culture** (2) Embryo **Culture** (3) Anther **Culture** and Haploid Production (4) Protoplast **Culture** and Somatic Hybridization (5) Ovule **Culture** and (6)Micro Propagation.

2.What are the different types of tissue culture?

There are two main **types** of **cultures**: primary (mortal) **cultures** and **cultures** of established (immortal) cell lines. Primary **cultures** consist of normal cells, **tissues**, or organs that are excised directly from **tissue** collected by biopsy from a living organism.

3. What is animal cell culture technology?

Cell culture refers to the process by which **cells** are grown in a controlled artificial environment. ... In a **cell culture** technique, **cells** are removed from an **animal** or a plant, and grown subsequently in a favorable environment. For **animal cell culture** the **cells** are taken from the organ of an experimental **animal**.

4. What are the applications of animal tissue culture?

Animal cells can grow as anchorage dependant or suspension **culture**. **Cell culture** techniques are used in **cell** and molecular biology research and studies. Some of the important areas where **cell culture** plays an important role are toxicity testing, cancer research, virology, gene therapy, drug discovery and many more.

5.What are the four main stages of tissue culture?

Tissue culture can be broadly divided into **four stages**:

(i) During the first **stage**, suitable plant parts (called explants) are cut into small pieces, surface sterilized with specific anti-microbial chemicals and then inoculated on semi-solid **culture** media.

(ii)

6. What are the applications of animal tissue culture?

Animal cells can grow as anchorage dependant or suspension **culture**. **Cell culture** techniques are used in **cell** and molecular biology research and studies. Some of the important areas where **cell culture** plays an important role are toxicity testing, cancer research, virology, gene therapy, drug discovery and many more.

7. Who is the father of animal tissue culture?

There is not specific name for **father of animal tissue**. Marie François Xavier Bichat, a French is **father** of histology (the study of the microscopic structure of **tissues**.)

8. What is a primary cell culture?

Primary culture refers to the stage of the **culture** after the **cells** are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (i.e.,

reach confluence). ... After the first subculture, the **primary culture** becomes known as a **cell** line or subclone.

9. How do you establish a primary cell culture?

Primary culture is the **cell culture** system that is formed by **culture cells** directly obtained from tissue. A **primary culture** starts with the biopsy (~1 cm^3) from tissue or organ via dissection.

10. What is animal tissue culture used for?

They successfully showed that **animal** cells can be grown indefinitely in **culture** medium just like microorganisms. Later **tissues** from warm blooded **animals** like chick and mammals were **used** as material for **tissue culture** purpose.

11. What is an example of a primary cell?

Primary batteries are "single use" and cannot be recharged. Dry **cells** and (most) alkaline batteries **are examples** of **primary** batteries. The second type is rechargeable and is called a secondary **battery**. **Examples** of secondary batteries include nickel-cadmium (NiCd), lead acid, and lithium ion batteries.

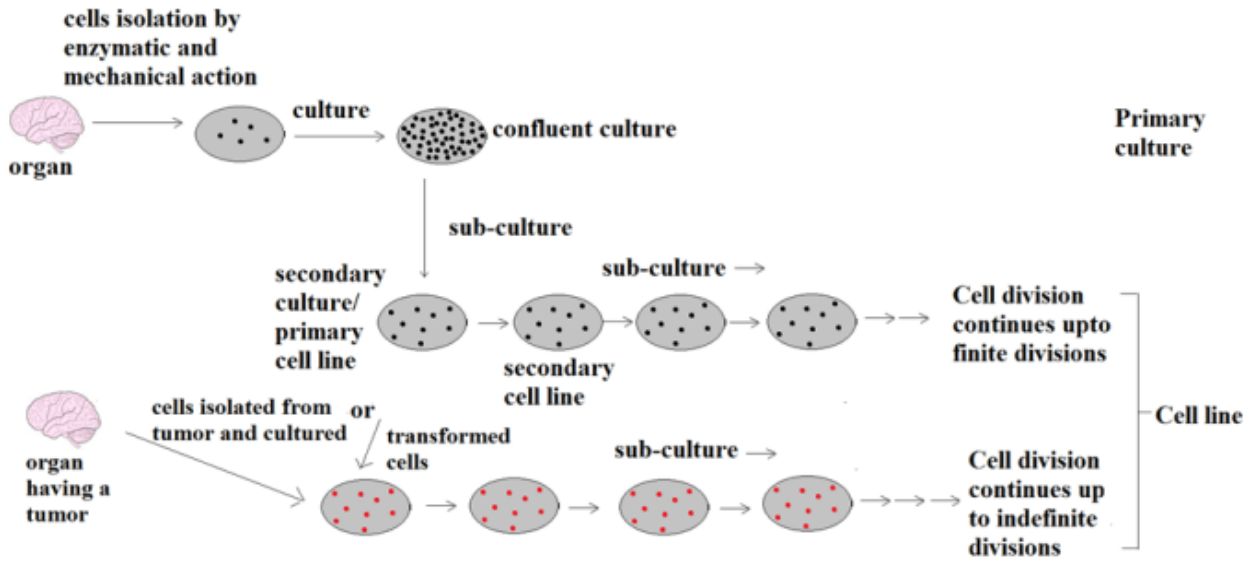


fig: animal cell culture

Cell culture refers to the process by which cells are grown in a controlled artificial environment. Cells can be maintained in vitro outside of their original body by this process which is quite simple compared to organ and tissue culture.

In a cell culture technique, cells are removed from an animal or a plant, and grown subsequently in a favorable environment. For animal cell culture the cells are taken from the organ of an

experimental animal. The cells may be removed directly or by mechanical or enzymatic action. The cells can also be obtained by previously made cell line or cell strain. Examples of cells used to culture are fibroblast, lymphocytes, cells from cardiac and skeletal tissues, cells from liver,

Types of animal cell culture

Based on the number of cell division, cell culture can be classified as primary cell culture and cell lines. Cell lines can undergo finite or infinite cell divisions.

Animal cell culture

A. Primary cell culture

This is the cell culture obtained straight from the cells of a host tissue. The cells dissociated from the parental tissue are grown on a suitable container and the culture thus obtained is called primary cell culture. Such culture comprises mostly heterogeneous cells and most of the cells divide only for a limited time. However, these cells are much similar to their parents.breast, skin, and kidney and different types of tumor cells.

Depending on their origin, primary cells grow either as an adherent monolayer or in a suspension.

Adherent cells

These cells are anchorage dependent and propagate as a monolayer. These cells need to be attached to a solid or semi-solid substrate for proliferation. These adhere to the culture vessel with the use of an extracellular matrix which is generally derived from tissues of organs that are immobile and embedded in a network of connective tissue. Fibroblasts and epithelial cells are of such types.

When the bottom of the culture vessel is covered with a continuous layer of cells, usually one cell in thickness, these are known as monolayer cultures. Majority of continuous cell lines grow as monolayers. As being single layers, such cells can be transferred directly to a cover slip to examine under microscope.

Suspension cells

Suspension cells do not attach to the surface of the culture vessels. These cells are also called anchorage independent or non-adherent cells which can be grown floating in the culture medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells can be grown in suspension. These cells grow much faster which do not require the frequent replacement of the medium and can be easily maintained. These are of homogeneous types and enzyme treatment is not required for the dissociation of cells; similarly these cultures have short lag period.

Confluent culture and the necessity of sub-culture

After the cells are isolated from the tissue and proliferated under the appropriate conditions, they occupy all of the available substrate i.e. reach confluence. For a few days it can become too crowded for their container and this can be detrimental to their growth, generally leading to cell death if left for long time. The cells thus have to be subculture i.e. a portion of cells is transferred to a new vessel with fresh growth medium which provides more space and nutrients for continual growth of both portions of cells. Hence subculture keeps cells in healthy and in growing state.

A passage number refers specifically to how many times a cell line has been sub-cultured. In contrast with the population doubling level in that the specific number of cells involved is not relevant. It B. Secondary cell culture and cell line

When a primary culture is sub-cultured, it is known as secondary culture or cell line or sub-clone. The process involves removing the growth media and disassociating the adhered cells (usually enzymatically).

Sub-culturing of primary cells to different divisions leads to the generation of cell lines. During the passage, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population. However, as they are sub-cultured serially, they become different from the original cell.

On the basis of the life span of culture, the cell lines are categorized into two types:

Finite cell lines

The cell lines which go through a limited number of cell division having a limited life span are known as finite cell lines. The cells passage several times and then lose their ability to proliferate, which is a genetically determined event known as **senescence**. Cell lines derived from primary cultures of normal cells are finite cell lines.

Continuous cell lines

When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a continuous cell line. Such transformation/mutation can occur spontaneously or can be chemically or virally induced or from the establishment of cell cultures from malignant tissue. Cell cultures prepared in this way can be sub-cultured and grown indefinitely as permanent cell lines and are immortal.

These cells are less adherent, fast growing, less fastidious in their nutritional requirements, able to grow up to higher cell density and different in phenotypes from the original tissue. Such cells grow more in suspension. They also have a tendency to grow on top of each other in multilayers on culture-vessel surfaces.

Common cell lines

Human cell lines:

1. MCF-7 (breast cancer)
2. HL 60 (Leukemia)
3. HeLa (Human cervical cancer cells)

Primates cell lines: Vero (African green monkey kidney epithelial cells)

Cell strain

Lineage of cells originated from the primary culture is called strain. These are either derived from a primary culture or a cell line by the positive selection or cloning of cells having specific properties or characteristics. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

simply gives a general indication of how old the cells may be for various assays.

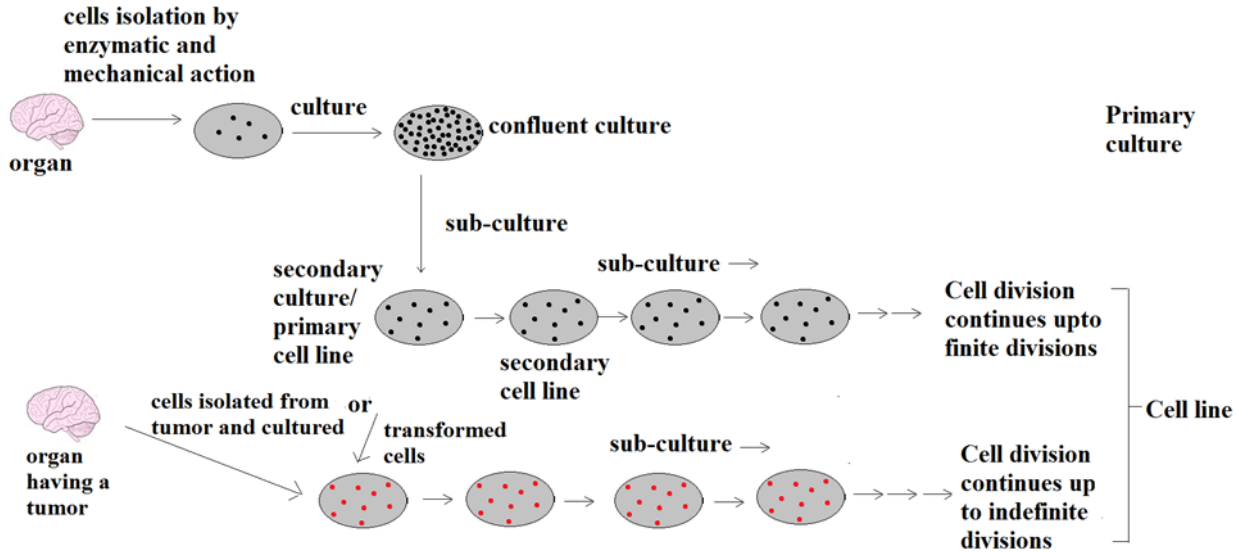


fig: animal cell culture

Methods

The culture media used for cell cultures are generally quite complex, and culture condition widely varies for each cell type. However, media generally include amino acids, vitamins, salts (maintain osmotic pressure), glucose, a bicarbonate buffer system (maintains a pH between 7.2 and 7.4), growth factors, hormones, O_2 and CO_2. To obtain best growth, addition of a small amount of blood erum is usually necessary, and several antibiotics, like penicillin and streptomycin are added to prevent bacterial contamination.

Temperature varies on the type of host cell. Most mammalian cells are maintained at 37^{o}C for optimal growth, while cells derived from cold- blooded animals tolerate a wider temperature

range (i.e. 15^{o}C to 26^{o}C). Actively growing cells of log phage should be used which divide rapidly during culture.

Process to obtain primary cell culture

Primary cell cultures are prepared from fresh tissues. Pieces of tissues from the organ are removed aseptically; which are usually minced with a sharp sterile razor and dissociated by proteolytic enzymes (such as trypsin) that break apart the intercellular cement. The obtained cell suspension is then washed with a physiological buffer (to remove the proteolytic enzymes used). The cell suspension is spread out on the bottom of a flat surface, such as a bottle or a Petri dish. This thin layer of cells adhering to the glass or plastic dish is overlaid with a suitable culture medium and is incubated at a suitable temperature.

Aseptic techniques

Bacterial infections, like Mycoplasma and fungal infections commonly occur in cell culture creating a problem to identify and eliminate. Thus, all cell culture work is done in a sterile environment with proper aseptic techniques. Work should be done in laminar flow with constant unidirectional flow of HEPA filtered air over the work area. All the material, solutions and the whole atmosphere should be of contamination-free.

Cryopreservation

If a surplus of cells is available from sub-culturing, they should be treated with the appropriate protective agent (e.g., DMSO or glycerol) and stored at temperatures below –130°C until they are needed. This stores cell stocks and prevents original cell from being lost due to unexpected equipment failure or biological contaminations. It also prevents finite cells from reaching senescense and minimizes risks of changes in long term cultures.

When thawing the cells, the frozen tube of cells is warmed quickly in warm water, rinsed with medium and serum and then added into culture containers once suspended in the appropriate media.

Applications of Cell Line

A. Vaccines Production

One of the most important uses of cell culture is in research and production of vaccines. The ability to grow large amounts of virus in cell culture eventually led to the creation of the polio vaccine, and cells are still used today on a large scale to produce vaccines for many other diseases, like rabies, chicken pox, hepatitis B, and measles. In early times, researchers had to use live animals to grow poliovirus, but due to the development of cell culture technique they were able to achieve much greater control over virus production and on a much larger scale which eventually develop vaccines and various treatments. However, continuous cell lines are not used in virus production for human vaccines as these are derived from malignant tissue or possess malignant characteristics.

B. Virus cultivation and study

Cell culture is widely used for the propagation of viruses as it is convenient, economic, easy to handle compared to other animals. It is easy to observe cytopathic effects and easy to select particular cells on which virus grow as well as to study the infectious cycle. Cell lines are convenient for virus research because cell material is continuously available. Continuous cell lines have been extremely useful in cultivating many viruses previously difficult or impossible to grow.

C. Cellular and molecular biology

Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying the normal physiology and biochemistry of cells (e.g., metabolic studies, aging), the effects of different toxic compounds on the cells, and mutagenesis and carcinogenesis. The major advantage of using cell culture for any of these applications is the consistency and reproducibility of results that can be obtained from using a batch of clonal cells.

D. In Cancer Research

Normal cells can be transformed into cancer cells by methods including radiation, chemicals and viruses. These cells can then be used to study cancer more closely and to test potential new treatments.

E. Gene therapy

Cells having a functional gene can be replaced to cells which are having non-functional gene, and for which the cell culture technique is used.

F. Immunological studies

Cell culture techniques are used to know the working of various immune cells, cytokines, lymphoid cells, and interaction between disease causing agent and host cell.

G. Others

Cell lines are also used in in-vitro fertilization (IVF) technology, recombinant protein and drug selection and improvement.

Continuous immortalized **cell lines**

Continuous immortalized **cell lines** are comprised of a single **cell** type that can be serially propagated in culture either for a limited number of **cell** divisions (approximately thirty) or otherwise indefinitely. **Cell lines** of a finite life are usually diploid and maintain some degree of differentiation.

What are continuous cell cultures?
Also known as heteroploid **cultures**, **continuous cells** lines are termed so as they can be serially cultivated indefinitely. ... **Cells** of a single type capable of infinite growth In Vitro. Grow faster and their chromosomes are haploid.
What is a cell line in cell culture?
Cell culture refers to the removal of **cells** from an animal or plant and their subsequent growth in a favorable artificial environment. ... If a subpopulation of a **cell line** is positively selected from the **culture** by cloning or some other method, this **cell line** becomes a **cell** strain.
What is the difference between primary cells and cell lines?
Primary Cells Versus **Cell Lines**. ... In contrast to **cell lines**, **primary cells** which are isolated directly from tissues, have a finite lifespan and limited expansion capacity. On the positive side, **primary cells** have normal **cell** morphology and maintain many of the important markers and functions seen in vivo
How many cell lines are there?
Cell line popularity can be estimated by the numerous publications using **cell lines** and American Type Culture Collection (ATCC) **Cell** Biology Collection which consists of over 3,600 **cell lines** from over 150 different species.
What is meant by continuous culture?
continuous culture. Type:Term. Definitions. 1. a technique for production of microbes or microbial products in which nutrients are **continuously** supplied to the fermenter.
What are the 3 cell lines?
Eighteen human and **three** animal **cell lines** that express some megakaryocytic features have been described in the literature. Many of these **cell lines** have primitive multiphenotypic properties of erythroid, myeloid and megakaryocytic **cells**, while some show more restricted megakaryocyte-specific markers.

Suspension cells do not attach to the surface of the **culture** vessels. These cells are also called anchorage independent or non-adherent cells which can be grown floating in the **culture** medium. Hematopoietic stem cells (derived from blood, spleen and bone marrow) and tumor cells can be grown in **suspension**.

Animal cell cultures

Depending on their origin, animal cells grow either as an adherent monolayer or in suspension.

Adherent cells are anchorage-dependent and propagate as a monolayer attached to the cell culture vessel. This attachment is essential for proliferation — many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in this confluent state for too long. Most cells derived from tissues are anchorage-dependent.

Suspension cells can survive and proliferate without being attached to a substratum. Hematopoietic cells (derived from blood, spleen, or bone marrow) as well as some transformed cell lines and cells derived from malignant tumors can be grown in suspension.

Primary cells, finite cultures, and continuous cell lines differ in their proliferative potential (see below). Different cell types vary greatly with respect to their growth behavior and nutritional requirements. Optimization of cell culture conditions is necessary to ensure that cells are healthy and in optimal condition for downstream applications.

Extensive information on culturing cells can be found in reference 1.

Primary cell cultures

Primary cell cultures come from the outgrowth of migrating cells from a piece of tissue or from tissue that is disaggregated by enzymatic, chemical, or mechanical methods. Primary cultures are formed from cells that survive the disaggregation process, attach to the cell culture vessel (or survive in suspension), and proliferate.

Primary cells are morphologically similar to the parent tissue. These cultures are capable of only a limited number of cell divisions, after which they enter a non-proliferative state called senescence and eventually die out. Adherent primary cells are particularly susceptible to contact inhibition, that is, they will stop growing when they have reached confluency. At lower cell densities, however, the normal phenotype can be maintained. Primary cell culture is generally more difficult than culture of continuous cell lines.

Primary cell cultures are sometimes preferred over continuous cell lines in experimental systems. Primary cells are considered by many researchers to be more physiologically similar to in vivo cells. In addition, cell lines cultured for extended periods of time can undergo phenotypic and genotypic changes that can lead to discrepancies when comparing results from different laboratories using the same cell line. Furthermore, many cell types are not available as continuous cell lines.

Finite cell cultures

Finite cell cultures are formed after the first subculturing (passaging) of a primary cell culture. These cultures will proliferate for a limited number of cell divisions, after which they will senesce. The proliferative potential of some human finite cell cultures can be extended by introduction of viral transforming genes (e.g., the SV40 transforming-antigen genes). The phenotype of these cultures is intermediate between finite cultures and continuous cultures. The cells will proliferate for an extended time, but usually the culture will eventually cease dividing, similar to senescent primary cells. Use of such cells is sometimes easier than use of primary cell cultures, especially for generation of stably transfected clones.

Continuous cell lines

Finite cell cultures will eventually either die out or acquire a stable, heritable mutation that gives rise to a continuous cell line that is capable of unlimited proliferative potential. This alteration is

commonly known as in vitro transformation or immortalization and frequently correlates with tumorigenicity.

Rodent primary cell cultures form continuous cell lines relatively easily, either spontaneously or following exposure to a mutagenic agent. In contrast, human primary cell cultures rarely, if ever, become immortal in this way and require additional genetic manipulation to form a continuous cell line. However, cell cultures derived from human tumors are often immortal.

Continuous cell lines are generally easier to work with than primary or finite cell cultures. However, it should be remembered that these cells have undergone genetic alterations and their behavior in vitro may not represent the in vivo situation.

Back to top

Safety and handling considerations for animal cell culture

Legislation and regulatory guidelines

Before undertaking any work with human or animal tissue (e.g., to establish a primary cell culture), it is necessary to ensure that the nature of the work conforms to the appropriate medical-ethical and animal-experiment legislation and guidelines. It may be necessary to seek approval from the relevant regulatory authorities and/or individuals.

Safety considerations and biohazards

When working with potentially hazardous material, it is important to be aware of the possible risks associated with both the material and the experimental protocol. All cell cultures are considered a biohazard because of their potential to harbor an infectious agent (e.g., a virus).

The degree of hazard depends on the cells being used and the experimental protocol. Primary cell cultures in particular should be handled carefully as these cultures have a high risk of containing undetected viruses. Although commonly used cell lines are generally assumed to be free of infectious agents, care should still be exercised when working with these cell lines as it is possible that they contain infectious agents, such as latent viruses. Cell cultures used to study specific viruses should be assumed to have the same degree of hazard as the virus under study.

We recommend handling all material as potentially infectious to ensure the safest possible working environment. Work should be performed in an approved laminar flow hood using aseptic technique, and the creation of aerosols should be avoided (see Handling cell cultures). After the work is complete, all waste media and equipment (i.e., used flasks, pipets, etc.) should be disinfected by autoclaving or immersion in a suitable disinfectant according to institutional and regional guidelines.

Handling cell cultures

Adherence to good laboratory practice when working with cell cultures is essential for two reasons: first, to reduce the risk of exposure of the worker to any potentially infectious agent(s) in the cell culture, and second, to prevent contamination of the cell culture with microbial or other animal cells (see Aseptic technique and minimization of aerosols).

Aseptic technique and minimization of aerosols

Aseptic technique and the proper use of laboratory equipment are essential when working with cell cultures. Always use sterile equipment and reagents, and wash hands, reagent bottles, and work surfaces with a biocide or 70% ethanol before beginning work.

Creation of aerosols should be avoided — aerosols represent an inhalation hazard, and can potentially lead to cross-contamination between cultures. To avoid aerosols, use TD (to deliver) pipets, and not TC (to contain) pipets; use pipets plugged with cotton; do not mix liquids by rapidly pipetting up and down; do not use excessive force to expel material from pipets; and do not bubble air through liquids with a pipet. Avoid releasing the contents of a pipet from a height into the receiving vessel. Expel liquids as close as possible to the level of liquid of the receiving vessel, or allow the liquid to run down the sides of the vessel.

Proper use of equipment can also help minimize the risk of aerosols. For example, when using a centrifuge, ensure the vessel to be centrifuged is properly sealed, avoid drops of liquid near the top of the vessel, and use centrifuge buckets with caps and sealed centrifuge heads to prevent contamination by aerosols.

Laminar flow hoods

For the most efficient operation, laminar flow hoods should be located in an area of the laboratory where there is minimal disturbance to air currents. Avoid placing laminar flow hoods near doorways, air vents, or locations where there is high activity. Hoods are often placed in dedicated cell culture rooms.

Tips:

- Keep laminar flow hoods clean, and avoid storing equipment inside the hood.
- Before starting work, disinfect the work surface of the hood as well as the outside of any bottles (e.g., by wiping with 70% ethanol), and then place everything needed for the cell culture procedure in the hood.
- Arrange equipment, pipets, waste containers, and reagent bottles so that used items are not placed near clean items, and avoid passing used items over clean items.
- Place used items (e.g., pipets) in a container inside the hood, and disinfect or seal before removing from the hood.

Contamination

The presence of microorganisms can inhibit cell growth, kill cells, and lead to inconsistent results. Contamination of cell cultures can occur with both cell culture novices and experts.

Potential contamination routes are numerous. For example, cultures can be infected through poor handling, from contaminated media, reagents, and equipment (e.g., pipets), and from microorganisms present in incubators, refrigerators, and laminar flow hoods, as well as on the skin of the worker and in cultures coming from other laboratories.

Bacteria, yeasts, fungi, molds, mycoplasmas, and other cell cultures are common contaminants in animal cell culture. To safeguard against accidental cell culture loss by contamination, we recommend freezing aliquots of cultured cells to re-establish the culture if necessary (see Freezing and viability staining of cells).

Microbial contamination

The characteristic features of microbial contamination are presented in the table Characteristic features of microbial contamination. The presence of an infectious agent sometimes can be detected by turbidity and a sharp change in the pH of the medium (usually indicated by a change in the color of the medium), and/or cell culture death. However, for some infections, no turbidity is observed and adverse effects on the cells are not easily observed.

Cell cultures should be routinely evaluated for contamination. Mycoplasmal infections are one of the more common and difficult-to-detect infections; their detection and eradication are described in further detail below.

Characteristic features of microbial contamination Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drop with most infections	pH change with heavy infections	pH changes sometimes
Cloudy medium: Under microscope (100–400x)	Shimmering in spaces between cells; rods or cocci may be observed	Round or ovoid particles that bud off smaller particles	Thin filamentous mycelia; sometimes clumps of spores

Mycoplasmal infection — detection

Mycoplasmas are small, slow-growing prokaryotes that lack a cell wall and commonly infect cell cultures. They are generally unaffected by the antibiotics commonly used against bacteria and fungi. Furthermore, as mycoplasma do not overgrow cell cultures and typically do not cause turbidity, they can go undetected for long periods of time and can easily spread to other cell cultures. The negative effects of mycoplasmal contamination include inhibition of metabolism and growth, as well as interference with nucleic acid synthesis and cell antigenicity. Acute infection causes total deterioration of the cell culture, sometimes with a few apparently resistant colonies that may, in fact, also be chronically infected. There are two main approaches to detect mycoplasma — Hoechst 33258 staining (1, 3) and mycoplasma-specific DNA probes. Alternatively, a PCR-based, mycoplasma-testing service is offered by the ATCC or other organizations on a fee-for-service basis.

Mycoplasmal infection — eradication

The best action to take with a culture containing chronic mycoplasmal infection is to discard it by either autoclaving or incineration. Only if the cell culture is absolutely irreplaceable should eradication be attempted. This process should be performed by experienced personnel in an isolated hood that is not used for cell culture, preferably in a separate room. Elimination of mycoplasma is commonly achieved by treatment with various commercially available antibiotics such as a quinolone derivative (Mycoplasma Removal Agent), ciprofolxacin (Ciprobay), enrofloxacin (Baytril), and a combination of tiamulin and minocycline (BM-Cyclin). Treatment procedures and appropriate antibiotic concentrations can be found in the suppliers' instructions and in references 1 and 3.

Cross-contamination of cell lines

Cross-contamination of one cell culture with fast-growing cells from another culture (such as HeLa) presents a serious risk. To avoid cross-contamination, only use cell lines from a reputable cell bank; only work with one cell line at a time in the hood; use different pipets, bottles of reagents, and bottles of media for different cell lines; and check cells regularly for the correct morphological and growth characteristics.

Back to top

Cell culture conditions

Media and serum

The choice of cell culture medium is extremely important, and significantly affects the success of cell culture experiments. Different cell types have highly specific growth requirements, and the most suitable medium for each cell type must be determined experimentally. Common basal media include Eagle minimal essential medium (MEM), Dulbecco's modified Eagle medium

(DMEM), RPMI 1640, and Ham F10. These contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and are available either as a powder or as a liquid from various commercial suppliers.

Basal media are usually supplemented just before use with serum, L glutamine, and antibiotics and/or fungicides to give complete medium (also called growth medium). Serum is a partially undefined material that contains growth and attachment factors, and may show considerable variation in the ability to support growth of particular cells. Fetal calf serum (FCS) is the most frequently used serum, but for some applications, less expensive sera such as horse or calf serum can be used. Different serum batches should be tested to find the best one for each cell type. L-glutamine is an unstable amino acid that, with time, converts to a form that cannot be used by cells, and should be added to medium just before use. Antibiotics and fungicides can be used as a supplement to aseptic technique to prevent microbial contamination. The working concentration of commonly used antibiotics and fungicides is provided in the tables Commonly used antibiotics for animal cell culture and Commonly used fungicides for animal cell culture. Some cell types, particularly primary cells, require additional supplements (e.g., collagen and fibronectin, hormones such as estrogen, and growth factors such as epidermal growth factor and nerve growth factor) to attach to the cell culture vessel and proliferate.

Media, serum, and supplements should be tested for sterility before use by incubation of a small aliquot at 37°C for 48 hours. If microbial growth has occurred after this incubation, the medium or supplement should be discarded.

Adapted from reference 4.

Commonly used antibiotics for animal cell culture Antibiotic	Working concentration	Effective against	Stability at 37°C
Penicillin	50–100 U/ml	Gram-positive bacteria	3 days
Streptomycin	50–100 µg/ml	Gram-negative bacteria	5 days
Kanamycin	100 µg/ml	Gram-positive and gram-negative bacteria; mycoplasma	5 days
Gentamycin	5–50 µg/ml	Gram-positive and gram-negative bacteria; mycoplasma	5 days

Adapted from reference 4.

Commonly used fungicides for animal cell culture Antibiotic	Working concentration	Effective against	Stability at 37°C
Nystatin	100 U/ml	Yeasts and molds	3 days
Amphotericin B	0.25–2.5 µg/ml	Yeasts and molds	3 days

Incubation conditions

The incubation conditions used to culture cells are also important. Cell cultures should be incubated in an incubator with a tightly regulated temperature (e.g., a water-jacketed incubator) and CO_2 concentration. Most cell lines grow at 37°C and 5% CO_2 with saturating humidity, but some cell types require incubation at lower temperatures and/or lower CO_2 concentrations.

Cell culture vessel

The choice of growth vessel can influence the growth of adherent cells. Sterile, disposable dishes and flasks that have been treated to allow attachment of animal cells to the growing surface are available commercially.

Cell banking

For some cell cultures, especially those that are valuable, it is common practice to maintain a two-tiered frozen cell bank: a master cell bank and a working cell bank. The working cell bank comprises cells from one of the master bank samples, which have been grown for several passages before storage. If future cell samples are needed, they are taken from the working cell bank. The master cell bank is used only when absolutely necessary. This ensures that a stock of cells with a low passage number is maintained, and avoids genetic variation within the cell culture.

Culture instability

The growth rate of cells that have been repeatedly subcultured may sometimes unexpectedly decrease, and the cytotoxicity of, for example, a transfection process may unexpectedly increase. This instability can result from variations in cell culture conditions, genomic variation, and selective overgrowth of constituents of the cell population. We recommend using cells with a low passage number (<10 splitting cycles). To safeguard against instability in continuous cell lines, avoid senescence or transformation in finite cell lines, and maintain consistency in

transfection experiments, we recommend creating cell banks by freezing aliquots of cells to recall into culture if and when necessary.

Back to top

Essential protocols for animal cell culture

Maintaining cell cultures

Establishment and maintenance of animal cell cultures require standardized approaches for media preparation, feeding, and passaging (or subculturing) of the cells. Cultures should be examined regularly to check for signs of contamination and to determine if the culture needs feeding or passaging.

The cell culture protocols below have been adapted from the following sources: *Culture of Animal Cells; a Manual of Basic Technique* (1), *Current Protocols in Molecular Biology* (4), and *Cells: A Laboratory Manual* (2). These protocols are examples of methods for general cell culture, and have not been rigorously validated and optimized by QIAGEN. There are many alternative protocols in current use.

IMPORTANT: Potentially biohazardous materials (e.g., cells, culture medium, etc.) should be sterilized before disposal, and disposed of according to your institution's guidelines.

Cell thawing

1. Heat a water bath to 37°C, and warm the growth medium into which the cells will be plated.
2. Add prewarmed growth medium to an appropriately sized cell culture vessel.
3. Remove a vial of frozen cells from liquid nitrogen, and place in the water bath until thawed.
   IMPORTANT: Wear protective goggles and gloves when thawing vials that have been stored in liquid nitrogen. Vials may explode when removed from liquid nitrogen.
   IMPORTANT: Proceed to step 4 as soon as the cells have thawed. Do not allow the cells to warm up before transferring them into growth medium.
4. Wash the outside of the vial with 70% ethanol or another suitable disinfectant.
5. Slowly pipet the thawed cell suspension into the cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.
   Note: Immediate removal of DMSO may sometimes be necessary, especially for suspension cells, primary cells, and sensitive cell types. For such cell types, pipet the thawed cell suspension into a sterile centrifuge tube containing prewarmed medium, centrifuge at 200 x *g* for 2 min, aspirate the supernatant, resuspend the cells in fresh growth medium, and then transfer to an appropriate cell culture vessel.
   IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of the cells throughout the vessel.
6. Incubate cells overnight under their usual growth conditions.
7. The next day, replace the growth medium.

Trypsinizing cells

Trypsinization is a technique that uses the proteolytic enzyme trypsin to detach adherent cells from the surface of a cell culture vessel. This procedure is performed whenever the cells need to be harvested (e.g., for passaging, counting, or for nucleic acid isolation).

1. Aspirate the medium and discard.
2. Wash cells with PBS (phosphate-buffered saline) or HBSS (Hanks balanced salt solution) (see tables 1x PBS and 1x HBSS), aspirate, and discard. Repeat.
   The volume of PBS or HBSS should be approximately the same as the volume of medium used for culturing the cells.
3. Add enough warmed 1x trypsin–EDTA solution (see table 1x trypsin–EDTA solution) to cover the monolayer, and rock the flask/dish 4–5 times to coat the monolayer.
4. Place the flask/dish in a CO_2 incubator at 37°C for 1–2 min.
5. Remove flask/dish from incubator and firmly tap the side of the flask/dish with palm of hand to assist detachment.
   If cells have not dislodged, return the flask/dish to the incubator for a few more minutes.
   IMPORTANT: Do not leave cells in 1x trypsin–EDTA solution for extended periods of time. Do not force the cells to detach before they are ready to do so, or clumping may occur.
   Overly confluent cultures, senescent cells, and some cell lines may be difficult to trypsinize. While increasing the time of trypsin exposure may help to dislodge resistant cells, some cell types are very sensitive to trypsin and extended exposure may result in cell death. In addition, some cell lines will resist this treatment and will produce cell clumps.
6. Once dislodged, resuspend the cells in growth medium containing serum.
   Use medium containing the same percentage of serum as used for growing the cells. The serum inactivates trypsin activity.
7. Gently pipet the cells up and down in a syringe with a needle attached to disrupt cell clumps.
   If pipetted too vigorously, the cells will become damaged. Ensure that pipetting does not create foam.
8. Proceed as required (e.g., with passaging, freezing, nucleic acid isolation, etc.).

The pH should be 7.4 without adjustment. Store at room temperature.

1x PBS Composition
137 mM NaCl
2.7 mM KCl
4.3 mM Na_2HPO_4

1.47 mM KH_2PO_4

The pH should be 7.4 without adjustment. Store at room temperature.

1x HBSS Composition
5 mM KCl
0.3 mM KH_2PO_4
138 mM NaCl
4 mM $NaHCO_3$
0.3 mM Na_2HPO_4
5.6 mM D-glucose

* Store 1x trypsin-EDTA solution at –20°C. Small aliquots can be stored at 2–8°C for 1–2 weeks. Work quickly when using trypsin during cell culture, since trypsin degrades and enzymatic activity declines at 37°C..

1x trypsin–EDTA solution Composition
0.05% (w/v) trypsin
0.53 mM EDTA
Dissolve trypsin and EDTA in a calcium- and magnesium-free salt solution such as 1x PBS or 1x HBSS*

Passaging cells

Many adherent cell cultures will cease proliferating once they become confluent (i.e., when they completely cover the surface of cell culture vessel), and some will die if they are left in a confluent state for too long. Adherent cell cultures therefore need to be routinely passaged, that is, once the cells are confluent, a fraction of the cells need to be transferred to a new cell culture vessel. Suspension cells will exhaust their culture medium very quickly once the cell density becomes too high, so these cultures similarly require regular passaging.

IMPORTANT: Although regular passaging is necessary to maintain animal cell cultures, the procedure is relatively stressful for adherent cells as they must be trypsinized. We do not recommend passaging adherent cell cultures more than once every 48 h.

1. Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium containing serum.
   The volume of medium used to resuspend the cells depends on the split ratio required (see step 2) and the size of the cell culture vessel. If too small a volume is used, it may be difficult to accurately pipet the desired volume to the new culture vessel.
   Conversely, if too large a volume is used, the culture vessel may be too full following transfer of the cells.
   Removal of trypsin may sometimes be necessary following harvesting of adherent cells, especially for primary and sensitive cell types. Centrifuge the cells at 200 x *g* for 5 min, carefully aspirate the supernatant, and resuspend the cells in an appropriate volume of prewarmed medium containing serum.
2. Transfer an appropriate volume of the resuspended cells to a fresh cell culture vessel containing prewarmed growth medium. Swirl the vessel gently to mix the cells with the medium.
   IMPORTANT: Thoroughly mix the cells in the cell culture vessel to ensure even distribution of cells.
   IMPORTANT: Some cell types will not survive if too few cells are transferred. We do not recommend high split ratios for primary cells, sensitive cell types, or senescent cultures.
   For adherent cells, we recommend adding enough cells so that the culture takes approximately one week to reach confluence again. This minimizes the number of times the cells are trypsinized as well as the handling time required to maintain the culture. When determining how many cells to transfer to the new cell culture vessel, it can be helpful to think in terms of how many cell divisions will be required for the culture to reach confluence again. For example, if half the cells are transferred, then it will take the culture one cell division to reach confluency again; if a quarter of the cells are transferred then it will take 2 cell divisions, and so on. If a culture divides once every 30 h or so, then in one week it will undergo approximately 5 cell divisions. A split ratio of 1:32 (1:25) should therefore be appropriate for the cells to reach confluency in about one week. In step 1, resuspend the cells in 8 ml medium, and transfer 0.25 ml to the new cell culture vessel.
3. Incubate cells under their usual growth conditions.

Back to top

Counting cells

Cell counting using a hemocytometer

It is often necessary to count cells, for example, when plating cells for transfection experiments. One method for counting cells is to use a hemocytometer. A hemocytometer contains 2 chambers

(see figure Counting cells using a hemocytometer). Each chamber is ruled into 9 major squares (volume of 0.1 mm^3 or 1 x 10^{-4} ml each). Cell concentration is determined by counting the number of cells within a defined area of known depth (volume).

This protocol is adapted from references 1, 2, and 4. It should be noted that there are many other protocols also in use.

1. Clean the surface of the hemocytometer with 70% ethanol or another suitable disinfectant, taking care not to scratch the surface of the central area. Dry with lens paper.
2. Clean the coverslip, wet the edges very slightly, lay it over the grooves and central area of the hemocytometer and gently press down.
   It is important that the coverslip is properly attached to obtain the correct chamber depth. The appearance of Newton's rings (bright and dark rings caused by interference in the air between the coverslip and the glass surface of the hemocytometer) will confirm that the coverslip is attached properly.
3. Harvest the cells, either by trypsinization (adherent cell cultures; see Trypsinizing cells) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium. At least 10^6 cells/ml are required for accurate counting.
   Tip: It may be necessary to centrifuge cells and resuspend in a smaller volume to obtain the desired cell concentration for counting. For adherent cells, it is important to produce a single-cell suspension after trypsinizing. Cell clumping will make counting difficult and inaccurate.
4. Mix the cell suspension sample thoroughly. Using a pipet, immediately transfer 20 µl to the edge of one side of the coverslip to fill one chamber of the hemocytometer. Repeat for the second chamber.
   The cell distribution should be homogeneous in both chambers. The cell suspension is drawn under the coverslip and into the chamber by capillary action.
   The cell suspension should just fill the chamber. Blot off any surplus fluid without disturbing the sample underneath the coverslip.
5. Transfer the slide to the microscope, and view a large square ruled by 3 lines using a 10x objective and 10x ocular.
   Count the total number of cells in 5 of the 9 major squares. Count cells that overlap the top and left border of squares but not those overlapping bottom and right borders. This prevents counting overlapping cells twice. If the cell density is too high, the cell suspension should be diluted, noting the dilution factor.
6. Repeat the counting for the second chamber to give a total of 10 squares.
7. Add the number of cells counted in all 10 squares together to give the number of cells in 1 x 10^{-3} ml.Multiply by 1000 to give the number of cells/ml.
   IMPORTANT: If the original cell suspension was diluted for counting, multiply by the dilution factor to obtain the number of cells/ml.
8. Clean the hemocytometer and coverslip by rinsing with 70% ethanol and then with distilled water. Dry with lens paper.

Back to top

Freezing and viability staining of cells

For some cell cultures, especially valuable ones, it is common practice to maintain a two-tiered frozen cell bank: a master and a working cell bank. The working cell bank comprises cells from one of the master bank samples, which have been grown for several passages before storage. If and when future cell samples are needed, they are taken from the working cell bank. The master cell bank is used only when absolutely necessary. This ensures that a stock of cells with a low passage number is maintained, and avoids genetic variation within the culture.

1. Check that cells are healthy, not contaminated, and have the correct morphology.
2. Change the medium 24 h before freezing the cells.
   Adherent and suspension cell cultures should not be at a high density for freezing. We recommend freezing cells when they are in the logarithmic growth phase.
3. Adherent cultures: harvest the cells by trypsinization, resuspend in medium containing serum, centrifuge at 200 x *g* for 5 min, and then resuspend cells in freezing medium (see table Freezing medium) at a density of 3–5 x 10^6 cells/ml.
   Suspension cultures: centrifuge the cells at 200 x *g* for 5 min, and resuspend in freezing medium at a density of 5–10 x 10^6 cells/ml.
   IMPORTANT: Freezing medium containing DMSO is hazardous and should be handled with caution.
4. Transfer 1 ml of the cell suspension (approximately 3–5 x 10^6 adherent cells or 5–10 x 10^6 suspension cells) into each freezing vial. Label vials with the name of cell line, date, passage number, and growth medium.
   Tip: It may also be useful to note the cell density in the freezing vials before storing. This enables determination of the cell density that provides optimal recovery after thawing.
5. Place freezing vials in racks and transfer to a polystyrene box (with walls approximately 15 mm thick) lined with cotton wool. Store box in a –80°C freezer overnight.
   It is important that cells are frozen at a rate of 1°C/min. A controlled-rate freezing device can be used instead of the polystyrene box and cotton wool method.
6. The next day, quickly transfer the vials to a liquid nitrogen chamber, making sure that the vials do not begin to thaw.

Most suspension cells are frozen in freezing medium containing DMSO.
Store at –20°C.

Freezing medium
Composition
Growth medium (RPMI, DMEM, etc.) containing 10–20% FBS and 5–20% glycerol or DMSO

Viability staining

Trypan blue staining provides a method for distinguishing between viable (i.e., capable of growth) and nonviable cells in a culture. This staining method is based on "dye exclusion": cells with intact membranes exclude (i.e., do not take up) the dye and are considered viable.

1. Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of pre-warmed growth medium to give a cell density of at least 10^6 cells/ml.
2. Add 0.5 ml 0.4% (w/v) trypan blue (see table Trypan blue) and 0.3 ml PBS or Hank's balanced salt solution (HBSS; see tables 1x PBS and 1x HBSS) to 0.1 ml of the cell suspension. Mix thoroughly, and let stand for 1–2 min. Alternatively, add 0.4 ml trypan blue directly to 0.4 ml of cells in growth medium.
   At least 10^6 cells/ml are required for accurate counting.
3. Count the stained and unstained cells using a hemocytometer (see Cell counting using a hemocytometer). Blue-stained cells are nonviable and unstained cells are viable.
   No. of viable cells/ Total no. of cells = % viability

Trypan blue	
Component	Amount
Trypan blue	0.4 g
1x PBS or 1x HBSS	100 ml

Cell, Tissue, and Organ Culture

Charity Waymouth

The principal purpose of cell, tissue and organ culture is to isolate, at each level of organization, the parts from the whole organism for study in experimentally controlled environments. It is characteristic of intact organisms that a high degree of interrelationship exists and interaction occurs between the component parts. Cultivation *in vitro* places cells beyond the effects of the organism as a whole and of the products of all cells other than those introduced into the culture. Artificial environments may be designed to imitate the natural physiological one, or varied at will by the deliberate introduction of particular variables and stresses.

Virtually all types of cells or aggregates of cells may be studied in culture. Living cells can be examined by cinephotomicrography, and by direct, phase-contrast, interference, fluorescence, or ultraviolet microscopy. Fixed cells from culture are suitable for cytological, cytochemical, histological, histochemical, and electron microscopical study. Populations of cells from

monolayers or suspension cultures are used for nutritional, biochemical, and immunological work.

Organ culture, the cultivation of whole organs or parts thereof, is particularly suitable for studies of development, of inductive interactions, and of the effects of chemical and physical agents upon the physiological functions of specific organs.

Both cell and organ culture have applications in pathology, *e.g.*, for comparative, developmental, and diagnostic studies of tissues from normal and diseased donors, for investigations on carcinogenesis, somatic cell genetic variation, viral susceptibility, etc. Cell cultures are widely used in microbiological studies, for investigations of the effects of radiation, and for screening drugs, especially carcinogenic, mutagenic, and radiomimetic agents.

Cell nutrition has been generally excluded from material selected for this chapter, since this topic has been fully reviewed elsewhere (Waymouth, 1954, 1960, 1965). The researchers to be referred to here have been chosen because they (1) contribute significantly to our understanding of the biology of the mouse, as distinguished from observations of general interest in cell biology (*e.g.*, the use of mouse cells for testing or screening drugs or carcinogens), (2) refer to cells obtained from inbred strains of mice or from mutants, or (3) suggest application to such cells.

CELL AND TISSUE CULTURES

Techniques

Tissues were first cultured before the turn of the century and since that time a multitude of techniques, each designed for the solution of a particular problem, has been devised. Basic information on technical procedures and their numerous applications in mammalian biology may be found in the textbooks of Parker (1961), Paul (1961), White (1963), and Merchant *et. al.* (1964).

Principal cell types

Although the chick embryo was the most generally used source of material for cultivation in the period 1910 to 1940, tissues of the embryonic and adult mouse have gained favor spectacularly since 1940, particularly since the introduction of antibiotics, which have reduced the need for strictly aseptic techniques in some types of experiment. The extensive cultivation of mouse cells owes much to the pioneering work of Earle, Evans, Sanford, and their colleagues at the National Cancer Institute. Among the major contributions of this group have been the improvement and standardization of techniques, including mass-culture methods and nutrition by chemically defined media, the development of cell lines and clones, the comparative study of the cytological and biochemical properties of long-established cell lines, and the investigation of malignant transformation *in vitro*.

Each of the basic cell types (fibrocytes or mechanocytes, epitheliocytes, and amebocytes (Willmer, 1960*a*, 1960*b*)) has its special characteristics *in vitro*. Experience has shown that it is important, when describing cells grown *in vitro*, to give details of the species, age, and sex of the source of the cells, the tissue of origin, and to state whether they are normal or neoplastic. Cells and tissues freshly isolated from the animal are designated "primary cultures" (Fedoroff, 1966). A "primary cell line" refers to a population of cells derived by direct isolation from an animal and is not necessarily capable of serial propagation indefinitely. An established cell line refers to a population of cells which has been serially transplanted at least 60 time *in vitro*. Primary or established cell lines or cell strains should receive designations according to the principles recommended by the 1957 International Tissue Culture Meeting (Anon., 1958; Paul, 1958; Fedoroff, 1966).

Applications of cell culture

Only a few representative examples of the use of cell cultures in mouse biology can be cited.

The mitotic process and its modification by stimulants or suppressors have been studied in many cell types (Fell and Hughes, 1949). Exact chromosome counts, to establish the degree of divergence from diploidy, have been made on 10-day fetal mouse cells in culture by Hungerford (1955). The chromosome complements of newborn and adult mice can be counted, without killing the animals, in primary tissue cultures of tail tips or ear fragments (Edwards, 1961). The mitotic cycle has been analyzed by Defendi and Manson (1963), actinomycin D-resistant and -sensitive systems or RNA synthesis identified by Paul and Struthers (1963), and the duration of the DNA synthetic period of mouse somatic cells shown to be probably constant (Cameron, 1964).

Visible light has some inhibitory effects upon living cells. The lethal effects of X-irradiation can be quantified on mouse cells (Reid and Gifford, 1952), and the effects of radiation upon cell constituents (Whitmore *et al.*, 1958) and upon DNA and RNA synthesis (Whitfield and Rixon, 1959; Till, 1961*a*) can be studied. Also methods of chemical protection of irradiated cells (Whitfield *et al.*, 1962) can be applied. X-ray-induced chromosome aberrations can be analyzed (Chu and Monesi, 1960). Ultraviolet light, which inhibits cell division is strain L cells, does not significantly affect DNA synthesis (Whitfield *et al.*, 1961). The survival of irradiated cells can be compared *in vivo* and *in vitro*, as has been done by McCulloch and Till (1962) for mouse bone marrow exposed to Co^{60} γ-rays.

Differentiation at the cellular level has mostly been studied in organ, rather than cell, cultures. However, Ginsburg (1963) has seen the differentiation of mouse thymus lymphoid cells into mast cells, which can be produced in large numbers and grown in suspension (Ginsburg and Sachs, 1963). Muscle differentiation can also be followed in tissue culture, and the tissue culture technique has been applied by Pearce (1963) to the study of muscular dystrophy.

The uses of tissue culture in the study of cancer have been reviewed by Murray (1959). More recent work includes that of Mitsutani *et al.* (1960) on the development of a near-diploid cell strain from a smoke condensate-induced leiomyosarcoma in a male C3H mouse and that of Fernandes and Koprowska (1963) on cell lines from normal cervix uteri of C3H mice and on cells from uteri treated for varying lengths of time with benzpyrene.

Comparison of enzyme activities in cells in culture with those from the mouse have been made (*e.g.*, of β-galactosidase by Maio and Rickenberg, 1960). Estimations of β-glucuronidase in cell lines from C3H mice (which have a lower activity in respect of this enzyme than most inbred mouse strains, especially in their livers) have demonstrated that most cell lines have activities many times higher than those of the highest activity mouse livers (Kuff and Evans, 1961). On the other hand, mouse cell strains after long cultivation *in vitro* seem uniformly to have a very low catalase activity in comparison with freshly isolated mouse tissue (Peppers *et al.*, 1960). Long-term cultures of fibroblasts seem to undergo greater variations in enzyme content than, of example, liver cells. Westfall *et al.* (1958) found that the arginase and rhodanese activities in liver cell lines were high, as in the tissue of origin, but that fibroblast lines varied widely in the activities of one or both of these enzymes. This variation may be related to Klein's (1960, 1961) experience that induction of arginase depends upon other factors than the substrate. In most cases arginase induction requires the presence of RNA as well as arginase. The enzyme patterns of established cell strains are among the most useful traits for characterizing cells in culture (Westfall, 1962; Conklin *et al.*, 1962).

Stable and unstable characters of cell cultures

Cloning. In certain respects cells cultivated *in vitro* exhibit considerable stability; in others they undergo extensive alterations from the parent cells. Cloning — the process of deriving a population of cells from a single cell — has enabled cell lines of common origin to be followed and has given many new insights into the potentialities of cells and into the circumstances under which they retain or lose characteristics derived from their parents (Sanford *et al.*, 1948; Hobbs *et al.*, 1957; Sanford *et al.*, 1961*b*).

Variations within clones. The many cell lines and clones established from C3H mice by Sanford *et al.* (1961*e*) usually underwent morphological, biochemical, and chromosomal changes quite early in the culture history. But, once established, many of the characteristics were of remarkable stability and persisted over many years of serial cultivation. A clone of sarcoma-producing cells (originating from normal C3H connective tissue) gave rise to "high" and "low" sarcoma-producing lines (Sanford *et al.*, 1954; Sanford *et al.*, 1958), and sublines of these were characterized by widely differing patterns of chromosome number and character (Chu *et al.*, 1958), of several enzyme activities (Sanford, 1958; Westfall *et al.*, 1958; Sanford *et al.*, 1959; Scott *et al.*, 1960; Peppers *et al.*, 1960; Sanford *et al.*, 1961*e*), and of glycolytic activity (Woods *et al.*, 1959).

Stable characteristics of cell lines. Some functions persist over many transplant generations in culture. These include the production of melanin by a mouse melanoma (Sanford *et al.*, 1952) and of steroids by an adrenal tumor (Sato and Buonassisi, 1961) and the synthesis of 5-hydroxytyrptamine, histamine, and heparin by the mouse mast-cell tumor P-815 (Dunn and Potter, 1957; Schindler *et al.*, 1959; Green and Day, 1960; Day and Green, 1962). The polynucleotide sequences of DNA characteristic of the mouse are retained in L cells after more than 20 years *in vitro* in spite of the very abnormal karyotype exhibited (McCarthy and Hoyer, 1964).

Immunological characteristics. Immunological specificity persists over very long periods of cultivation in homologous, heterologous, or chemically defined media, particularly mouse-strain specificity in tumors cultivated *in vitro*. Immunological methods have been used for species identification of cells in culture (Coombs *et al.*, 1961; Coombs, 1962; Fedoroff, 1962; Brand and Syverton, 1962), for studying antigenic differences between cell lines (Coriell *et al.*, 1958; Kite and Merchant, 1961; McKenna and Blakemore, 1962), and for identification of particular antigens, such as the *H-2* transplantation antigen (Manson *et al.*, 1962*a*, 1962*b*; Cann and Herzenberg, 1963*a*, 1963*b*).

Neoplastic transformations. It has been repeatedly observed that cells of normal origin undergo malignant change after more or less prolonged cultivation *in vitro* (Earle and Nettleship, 1943; Sanford *et al.*, 1950; Evans *et al.*, 1958; Sanford *et al.*, 1961*d*, 1961*e*). Neoplastic transformation of cells originating from normal tissues and the maintenance or loss of the capacity to produce tumors on transplantation into suitable hosts have been intriguing problems illustrating the range of capabilities of the cell. Many of these transformations have been "spontaneous" (*i.e.*, unexplained) (Earle and Nettleship, 1943; Sanford, 1958, 1962; Evans *et al.*, 1958; Sanford *et al.*, 1959; Shelton *et al.*, 1963). Others have been deliberately induced by chemical carcinogens (Earle, 1943; Earle *et al.*, 1950; Sanford *et al.*, 1950; Shelton and Earl, 1951; Berwald and Sachs, 1963) or by viruses (Dawe and Law, 1959; Dulbecco and Vogt, 1960; Vogt and Dulbecco, 1960; Sanford *et al.*, 1961*c*; Sachs and Medina, 1961; Pearson, 1962).

Tumors retain their capacity to grow in histocompatible hosts and in general do not acquire the ability to transcend histocompatibility barriers, except after being stored at -70°C (Morgan *et al.*, 1956), though some degree of immunological incompatibility can develop in long-term cultures (Sanford *et al.*, 1954, 1956). Strains of cells, originating from normal cells and acquiring within one or two years the ability to produce tumors, may undergo a progressive reduction in tumor-producing capacity after several more years *in vitro* (Earle *et al.*, 1950). The cells with reduced tumor-producing ability could, however, grow in animals which had received X-irradiation (Sanford *et al.*, 1956). The strain L, for example, after 10 year *in vitro* could produce tumors in 15 per cent of unirradiated and in 64 per cent of irradiated C3H hosts. This raises the question whether to progressive immunological incompatibility is due to changes in the cell line or to changes in the inbred strain over the period of 10 or more years since the cell isolation. After 13 years the L cells retained their C3H specificity, *i.e.*, would not grow in any of several other

strains of mice. The differences in the "high" and "low" cancer-producing lines, both of which produce some immunity in C3H mice, and the fact that the "low" line will grow in irradiated C3H hosts, led to the conclusion (Sanford *et al.*, 1958) that the faster-growing "high" tumor line can establish a tumor before resistance develops in the host. Cell strains and derived single-cell clones, established in culture from C3H carcinomas carrying mammary tumor agent, were found to vary in the persistence of the agent. In some cell strains the agent was demonstrable after 6 to 12 months of rapid cell proliferation *in vitro*; in others the agent disappeared (Sanford *et al.*, 1961*a*).

Experimental control of malignant change. Attempts have been made to place the "spontaneous" transformation of normal to malignant cells under experimental control. Evans *et al.* (1964) have followed the progressive changes in cultures initiated from minced C3H embryos by testing their ability to produce tumors on intraocular implantation. No tumors resulted from a limited number of cultures grown for up to 211 days in a chemically defined medium. Cells grown in a medium supplemented with 10 per cent horse serum were able to produce tumors from about 120 days.

Barski and Cassigena (1963) aimed to produce parallel malignant and nonmalignant cell lines from adult female C57BL lung, analogous to the spontaneously derived parallel C3H lines of Sanford *et al.* (1950), for use in their studies of cell hybridization (see below). Such pairs of lines were derived, one from a culture frequently subcultured with the aid of trypsin, the other from a less frequently transferred culture, subcultured by mechanical dispersion and not exposed to trypsin. Both lines early became aneuploid (mean chromosome number in both cell lines at the 10th passage of the trypsinized line and the sixth passage of the nontrypsinized line was 68). The trypsinized line (PT) was highly malignant from the 16th passage (184 days), whereas the nontrypsinized line (PG) was not malignant up to the 37th passage (436 days). Further evidence is needed to determine whether the differences in morphology and malignancy are causatively related to the trypsin treatment. Todaro and Green (1963) suggested that the process of establishment of cell lines may require a reduction in the "leakiness" of cells to small molecules, and trypsin treatment increases the "leakiness" (Phillips and Terryberry, 1957; Magee *et al.*, 1958).

Sarcomatous change in carcinomas. Tissue cultures have contributed to the elucidation of the well-known "sarcomatous change" frequently observed in transplanted carcinomas. In an extensive study Sanford *et al.* (1961*d*) examined 18 different cell strains derived from C3H mammary gland tumors. In general, tumors maintained in culture for up to 25 weeks grew as differentiated mammary carcinomas on retransplantation into mice. Cells transplanted after this time grew as sarcomalike tumors. It appeared that tumors which had been carried in mouse serial passage were morphologically more stable than primary tumors put into culture. The "sarcomatous change" was apparently not a unitary process. In some instances, with hepatomas, melanomas, and thyroid tumors (Sanford *et al.*, 1952), as well as with mammary tumors, the stroma may undergo malignant change. In other cases, the carcinoma cells themselves change

morphologically and assume a fibroblastic appearance. The opposite change (from fibroblastic to epithelioid cell types) has been studied in malignant origin by Ludovici *et al.* (1962*a*, 1962*b*), who produced a significantly higher (64 per cent) proportion of cultures showing this alteration by treatment with a trypsin-antibiotic mixture, than was seen in controls not so treated (7 per cent).

Chromosomal variation and somatic cell genetics. Nutrient-dependent and nutrient-independent, drug-resistant and drug-sensitive, radiation-resistant and radiation-sensitive clones (Hauschka, 1957; Fedoroff and Cook, 1959; Fisher, 1959; Hsu and Kellogg, 1959; Biesele *et al.*, 1959; Roosa and Herzenberg, 1959; Hsu, 1961; Cann and Herzenberg, 1963*a*, 1963*b*) are among the tools making possible the study of the genetics of mammalian cell and neoplastic cell population (Merchant and Neel, 1962; Harris, 1964; Krooth, 1964).

Rothfels and Parker (1959) reported, what is now a rather common experience, that freshly explanted tissues (in their case from CF_1 mice) grow rapidly at first, then pass into a long (6 months or more) period of survival without growth, and finally, in some instances, enter a new phase of proliferation from which cell lines may be established. The chromosomes of such cell lines are usually heteroploid and heterotypic (*i.e.*, contain chromosomes differing markedly from the normal 40 telocentrics of the mouse). Bimodal chromosome distributions are not uncommon, as in the case of Rothfels and Parker's culture 23855-8 from CF_1 kidney, which contained approximately equal numbers of cells with around 38 and 70 chromosomes respectively, a pattern which persisted through at least 14 subcultures (12 months). Hsu (1961) and Chu (1962) commented upon the rapid departure from diploidy observed even in primary cultures with mouse cells and contrast this with the greater karyotypic stability of man, rat, and many other mammals. Todaro and Green (1963) developed established cell lines from trypsin-disaggregated 17- to 19-day mouse embryos using trypsin at each transfer. The usual decline in growth rate during early passages was encountered but, at from 15 to 30 generations *in vitro*, the growth rate rose. At the beginning of the third phase which, under their conditions, was less than 3 months, the cells responsible for the upturn in growth rate were diploid, but they shifted (often rapidly) to the tetraploid range. Marker chromosomes appeared later.

Long-established cell strains, like most cancers (which Hauschka(1958) describes as "multiclonal mosaics of altered karyotypes"), have characteristic and identifiable karyotypes, even though within a strain there may be considerable variation of numbers and types of chromosomes. It is rare to find in cultures of mouse cells that the chromosomes are all, or even sometimes predominant, telocentric. However, an analysis by Levan and Hsu (1960) of NCTC 2940 — a cell line which originated from a C3H mammary carcinoma — after being carried for about 2 years *in vitro*, showed only telocentrics in a stem line number of $s = 84$ chromosomes. Another mammary tumor cell line, NCTC 2777, of hypertetraploid number ($s = 73$), contained only telocentrics, with the marked exception of one large, bizarre, and multiform heterometacentric chromosome.

Five established strains of mouse cell (three of them sublines of NCTC clone 929, strain L) were found by Hsu and Klatt(1958) to exhibit karyotypic polymorphism and to contain "marker" chromosomes highly characteristic of individual cell strains and wholly distinct from normal mouse chromosomes. In another study Hsu (1959) observed modal chromosome numbers of 67 to 73 in 12 mouse cell strains. Highly polyploid cells are not uncommon. Levan and Biesele(1958) observed a gradual increase in the number of polyploid cells in cultures of mouse embryo cells. Polyploid cells can usually be found early in the life of cultures, and their proportion in the population can be increased by treatment with colchicine (Hsu and Kellogg, 1960). One subline (L-P59) of NCTC clone 929 strain L, studied by Hsu (1960), contained 63 to 65 chromosomes, including a very conspicuous long subtelocentric (chromosome D). Derived subline Amy from L-P59 had an average chromosome number of 128 (with two D chromosomes), and subline Barbara had 58 to 59 without the D marker but with a large metacentric chromosome known as Victoria. In mixed cultures the stem line L-P59 rapidly overgrew Amy or Barbara. Moreover, the proportion of D chromosomes in L-P59 cultures was found to be variable according to the frequency of subculture. Old cultures, or cultures subdivided only every 2 weeks, contained on an average more than 1.5 D chromosomes per cell. In cultures subdivided twice a week, the population changed to one with less than one D chromosome per cell. The D chromosome and a probable isochromosome T were lost from one subline (L-M) (Hsu and Merchant, 1961) and new distinctive markers were reported 2 years after the first study. Hsu (1961) has reviewed the topic of chromosomal evolution in cell populations.

Occasionally mouse cell lines of diploid mode have been observed. Billen and Debrunner(1960) had cells from normal mouse bone marrow which remained diploid for more than 1 year. A line (H_2), started from cells from the peritoneum of a C3H mouse, retained its diploid character for at least 5 months before becoming predominantly tetraploid with a minority of hypodiploid (38) cells, which gradually diminished (Hsu *et al.*, 1961). Another series of intriguing hypodiploid cell lines are the MB III lymphoblasts which originated in 1935 from a spontaneous lymphosarcoma T86157 in a 286-day-old female mouse (De Bruyn *et al.*, 1949). The primary line (MB I) of lymphosarcoma cells contained a mixed population of tumor-producing lymphoblasts (s = 40 or 41) and tumor-negative fibroblasts (s = 56). The MB III lines are sublines of lymphoblasts, free from fibroblasts, which have become tumor-negative and hypodiploid (s = 30 to 32). In contrast to many normal cells which undergo "spontaneous" transformation *in vitro* into tumor-producing cells, neither MB III (lymphoblasts) nor MB II (fibroblasts), in spite of great morphological variability and frequent mitotic disturbances, produces tumors *in vivo* after about 27 years of life *in vitro* (De Bruyn and Hansen-Melander, 1962).

The radiosensitivity of sublines of strain L mouse cells, as measured by their ability to form macroscopic colonies, was found to be independent of chromosome number in cell lines with mean chromosome numbers between 53 and 109 (Till, 1961*b*). Chromosomal anomalies

acquired during *in vivo*, by injecting a teratogen during pregnancy, persisted in the fetal tissues during cell culture, the treated cells showing 50 per cent polyploidy, and the controls only 2 per cent (Ingalls *et al.*, 1963).

Cell hybridization. By making cultures of populations of two cell types, each containing conspicuous marker chromosomes, cells can be produced containing both sets of chromosomes. Sorieul and Ephrussi(1961), Barski (1961), and Barski *et al.* (1961*a*) found such "hybrid" cells in mixed cultures of cells from NCTC 2472 (a high-cancer line) and NCTC 2555 (a low-cancer line). Both of these lines took their ultimate origin from a single clone of normal cells, which produced the original "high" and "low" lines (Sanford *et al.*, 1954) later designated NCTC 1742 and NCTC 2049 respectively. NCTC 2472 was derived from NCTC 1742 (Sanford *et al.*, 1961*e*) and NCTC 2555 from NCTC 2049 (Woods *et al.*, 1959). The high-cancer line NCTC 2472 (N1) has a modal chromosome number of 55 telocentrics, one being very long (Barski *et al.*, 1961*b*). The low-cancer line NCTC 2555 (N2) has a modal chromosome number of 62, with from 9 to 19 two-armed chromosomes. By 104 days in mixed culture M type cells began to appear, with 115 to 116 chromosomes, of which 9 to 15 were metacentric, and in which the extra-long telocentric chromosome could usually be identified. Cells of the M type were never found in cultures of N1 or N2 cells alone (Barski and Cornefert, 1962) but M cells could be produced *in vivo* as well as *in vitro,* in tumors produced by inoculating C3H mice with mixed-cell populations. The hybrid characteristics of cloned M lines remained stable for at least 1 year. Barski and Belehradek(1963) have demonstrated cinephotomicrographically that nuclear transfer can take place in mixed cultures of N1 cells with normal mouse embryo cells. This may occur repeatedly in mixed cultures, or, as Ephrussi and Sorieul (1962) point out, it is not impossible that hybrid populations "arose from a single mating event involving modal cells of the two parental lines, followed by rapid segregation."

ORGAN CULTURES

Techniques

Techniques for culturing organs are described in the textbooks of Parker (1961), Paul (1961), White (1963), and Merchant *et al.* (1964), and are referred to in the major papers and review articles of the principal practitioners of the method, *e.g.*, Wolff (1952), Fell (1953, 1954, 1955, 1958, 1964), Gaillard (1942, 1948, 1953), Borghese (1958), Kahn (1958), Lasnitzki (1958, 1965), Trowell (1959, 1961*b*), and Grobstein (1962).

Applications

Organ culture is used principally for (1) the maintenance of structural organization in tissues which are to be subjected to experimentally varied environments (*e.g.*, to hormones, drugs, or radiation); (2) the study of morphogenesis, differentiation, and function in excised organs or presumptive organs; and (3) for comparison of the growth and behavior of explanted organs with the growth and behavior of similar organs *in sit*.

Almost every organ of the mouse has been cultivated *in vitro*. Some of the principal references to the cultivation of mouse organs are listed in Table 25-1.

The environmental variables studied by means of organ cultures include: radiation (Trowell, 1961*a*; Lasnitzki, 1961*a*, 1961*c*, 1961*d*; Borghese, 1961), vitamins, mainly vitamin A (Fell and Mellanby, 1952, Lasnitzki 1958, 1961*b*, 1961*c*, 1962; New 1962) and carcinogens (Lasnitzki, 1958; Lasnitzki and Lucy, 1961). Organ culture is peculiarly suitable for the study of hormones (Fell, 1964) and provides an excellent way of distinguishing the effects of individual, or combinations of several, hormones on particular structures (Table 25-2).

The mammary gland has been one of the most commonly grown organs of the mouse and, besides its use for investigation of responses to hormones, has been studied for its secretory activity (Lasfargues, 1957*b*; Lasfargues and Feldman, 1963) and as a vehicle for the mammary tumor agent (Lasfargues *et al.*, 1958). The toxic effects of steroid hormones on mammary adenocarcinomas of C3H mice in organ culture have been examined (Rivera *et al.*, 1963).

The cultivation of mouse ova (Whitten, 1956, 1957; Tarkowski, 1959*a*, 1959*b*) has made possible the production *in vitro* of genotypically mosaic embryos from fused eggs (Tarkowski, 1961, 1963; Mintz, 1962*c*, 1963).

Organ culture has contributed significantly to our understanding of embryonic induction and of the control of morphogenesis by the juxtaposition of specific cell types. The effects of specific mesenchymal elements upon epithelial structures has been elucidated, *e.g.*, by Borghese (1950*a*, 1950*b*), Grobstein (1953*a*, 1953*b*, 1953*c*, 1955*a*, 1955*b*, 1956, 1957, 1959, 1962), Grobstein and Dalton (1957), Auerbach and Grobstein (1958), and Auerbach (1960, 1961*a*, 1961*b*). Cartilage induction has been studied in BALB/c x C3H embryos by Grobstein and Parker (1954) and Grobstein and Holtzer (1955) and in *T*/*T* embryos by Bennett (1958).

The pioneer work of Hardy (1949, 1951) in the growth of hair and hair follicles has been followed up by Cleffman (1963) with studies of pigment formation in the hair-follicle melanocytes of agouti mice.

Developmental anomalies and inherited diseases and defects have been less studied by means of organ culture than might be expected. Elegant examples of the possibilities are shown in the growth of normal retinas of CBA (Lucas and Trowell, 1958) and BALB/c (Sidman, 1961) mice, in the analysis of the changes occurring in inherited retinal dystrophy in C3H animals by Lucas (1958) and by Sidman (1961, 1963), and in the examinations by organ culture of the potentialities of the kidney rudiments of prospective kidneyless (*Sd*/*Sd*) mice (Gluecksohn-Waelsch and Rota, 1963).

What are the advantages of tissue culture?

Advantages of Tissue Culture: These techniques have certain **advantages** over traditional methods of propagation. They produce exact copies of plants required that have desirable traits. Multiple plants are produced in the absence of seeds or necessary pollinators to produce seeds.

Genetic engineering is the name of a group of techniques used for direct genetic modification of organisms or population of organisms using recombination of DNA. These procedures are of use to identify, replicate, modify and transfer the genetic material of cells, tissues or complete organisms (Izquierdo, 2001; Karp, 2002). Most techniques are related to the direct manipulation of DNA oriented to the expression of particular genes. In a broader sense, genetic engineering involves the incorporation of DNA markers for selection (marker-assisted selection, MAS), to increase the efficiency of the so called 'traditional' methods of breeding based on phenotypic information. The most accepted purpose of genetic engineering is focused on the direct manipulation of DNA sequences These techniques involve the capacity to isolate, cut and transfer specific DNA pieces, corresponding to specific genes (Lewin, 1999; Klug and Cummings, 2002).

The mammalian genome has a larger size and has a more complex organization than in viruses, bacteria and plants. Consequently, genetic modification of animals, using molecular genetics and recombinant DNA technology is more difficult and costly than in simpler organisms. In mammals, techniques for reproductive manipulation of gametes and embryos such as obtaining of a complete new organism from adult differentiated cells (cloning), and procedures for artificial reproduction such as in vitro fertilization, embryo transfer and artificial insemination, are frequently an important part of these processes (Murray et al. 1999; Izquierdo, 2001).

Current research in genetic engineering of animals is oriented toward a variety of possible medical, pharmaceutical and agricultural applications. Also, there is an interest to increase basic knowledge about mammalian genetics and physiology, including complex traits controlled by many genes such as many human and animal diseases (Houdebine, 1998; Lynch and Walsh, 1998; Montaldo and Meza-Herrera, 1998; Schimenti, 1998; Eggen, 2003). The interest in genetic engineering of mammalian cells is based in the idea of, for example, use gene therapy to cure genetic diseases such as cystic fibrosis by replacing the damaged copies of the gene by normal ones in foetuses or infants (gene therapy) (Izquierdo, 2001; NHGRI, 2001; Coutelle and Rodeck, 2002). Genetically engineered animals such as the 'knockout mouse', in which one specific gene is 'turned off', are used to model genetic diseases in humans and to discover the function of specific sites of the genome (Majzoub and Muglia, 1996). Genetically modified animals such as pigs will probably be used to produce organs for transplant to humans (xenotransplantation) (Murray et al. 1999; Prather et al. 2003). Other applications include production of specific therapeutic human proteins such as insulin in the mammary gland of genetically modified milking animals like goats (transgenic animals, bioreactors) (Murray et al. 1999; Wall, 1999). These techniques may be used to increase disease resistance and productivity in agriculturally

important animals by increasing the frequency of the desired alleles in the populations used in food production. This can be accomplished by transferring alleles or allele combinations, over expressing or eliminating the expression of particular genes (use of genetic engineering in animal breeding) (Woolliams and Wilmut, 1989; Cameron et al. 1994; Kinghorn, 1998; Fries and Ruvinsky, 1999; Smidt and Niemann, 1999; Hill, 2000; Karatzas, 2003; Felmer, 2004). In addition, these techniques open the possibility of using artificially modified genes to increase the biological efficiency of proteins (Kinghorn, 2003).

The objective of this paper is to review some advances on genetic engineering applications in animal breeding, including a description of the methods, some applications and ethical issues. Here I made emphasis in both the search and use of genomic information for selecting animals and to transfer and use their genes in commercial populations via marker-assisted selection (MAS) or transgenesis.

This review focuses mainly in the methodology to apply genetic engineering directly to animals for genetic improvement.

Several important biotechnological applications such as the production of recombinant proteins in bioreactors (Houdebine, 2002), disease diagnostic (McKeever and Rege, 1999), feedstuff processing (Bonneau and Laarveld, 1999) and production of vaccines (Eloit, 1998), proteins, stem cells, tissues and monoclonal antibodies for use in therapeutics are not included here. The impact of reproductive technologies on animal breeding, not directly related with gene transfer, are reviewed elsewhere (Van Vleck, 1981; Visscher et al. 2000). The possible role for cloning adult animals in breeding is also discussed

Use of genomic information in animal improvement

The use of genomic information (sequences or DNA marker polymorphisms) for the genetic improvement and selection of animals requires the knowledge of the effect of physically mapped genes with effects on economically important traits or quantitative trait loci (QTL). This information is also required in order to effectively use transgenesis and MAS for genetic improvement (Lynch and Walsh, 1998; Montaldo and Meza-Herrera, 1998; Van Marle-Koster and Nel, 2003). In MAS, the genomic information is combined with the classical performance records and genealogical information to increase selection accuracy, performing selection earlier in life and reducing costs (Boichard et al. 1998; Elsen, 2003). The traits on which the application of marker-assisted selection can be more effective, are those that are expressed late in the life of the animal, have low heritability, are sex-limited, are expensive to measure or are controlled by a few genes. Examples are longevity, carcass traits in meat producing animals, and diseases or defects of simple inheritance. Expected increments in selection response from MAS for a single complex trait, using known QTL genotypes plus linear model predictions (BLUP), compared to selection on BLUP alone, ranges from -0.7 to 64 percent. In practice, results will depend on

many parameters which are likely to be very different for each trait combination and population (Montaldo and Meza-Herrera, 1998; Dekkers and Hospital, 2002). The statistical properties of genetic evaluations (predictions) of animals for quantitative traits obtained through mixed model methodology using phenotypic records and genealogical information as inputs are known as BLUP. Best -means minimum variance of prediction, Linear -because predictions are linear functions of observations, Unbiased -means that the expected value of predictors obtained with linear model have an expected value equal to the expected value of the mean of the breeding values, conditional to data, and Prediction -because involves prediction of random breeding values).

Problems related to false positive detection of candidate genes are also common. Using crosses between two pig breeds, a polymorphism on the estrogen receptor locus (ESR) was associated to litter size in pigs with 1.5 piglet advantage for homozygous sows for the beneficial allele, and where followed by immediate recommendations for commercial use and patenting. Further research however did not confirm the effect. Different phases of linkage between the markers and the QTL could explain the fact that the effect of the ESR locus varied widely between populations. Thus, very probably, despite the ESR gene is probably a plausible 'candidate' from their inferred physiological functions, the gene involved seems to be another one, still unknown, or the effect initially observed was the product of several, interacting genes (epistasis).

Main problems related to the use of molecular genetics in the improvement of agricultural populations are:

1. Direct use of a discovered QTL effect for selection across families is not possible.
2. By the time the information about the inferred genotypes is known, frequently the animals involved in the study are not available as candidates for selection, because they will be too old.
3. Advantage from within-family selection for a QTL bracketed by markers over BLUP or phenotypic selection alone is frequently low and the methodology to exploit this information for selection is complex and relatively inefficient.
4. There are statistical estimation errors, causing both false positive and false negative effects, particularly when the effect of the QTL is small.
5. There is a lack of consistency of the effect of the same QTL between studies, caused by QTL by genetic background (epistasis) of QTL by environment interactions.
6. The net economic effect of the QTL may be lower than the effect on single traits, because unfavourable effects on other traits.
7. Selection using QTL is more complex than phenotypic selection alone. QTL information (whether the information on the QTL is direct or indirect), adds to the list of traits used as

selection criteria. Issues such as reduction of selection intensities and relative emphasis given to each trait, make optimal selection more difficult, with a need for adequate relative weights for the QTL, and the polygenic portions of the genetic variation for each trait at each generation (year).

8. Short-term gains due to MAS may be at the expense of medium to long-term polygenic responses for important traits.

Even with an unambiguous knowledge for the allele effects of a mayor gene on a complex trait, expected advantages from optimum use of genotyping alleles for a QTL for a multi-generation selection horizon is not always high. The polymorphism for the αs1-casein in goats has a strong effect on protein content and total protein output. The difference between homozygous for the highest and lowest effects for milk protein is approximately three phenotypic standard deviations for milk protein content. Favourable alleles have frequencies lower to 0.5 in populations undergoing selection, making a very favourable case for potential gains in protein content and production from MAS using this polymorphism. Indicated that when an efficient 'conventional' progeny testing selection program is underway for increased protein production, the advantages from MAS are low to moderate. Maximum possible increase on total genetic gain for protein yield was 26%. Emphasized the overlap that exists between marker and phenotypic information for the improvement of a multi-trait goal over several generations, using MAS. A very optimistic prospect from use of MAS as well as other biotechnologies is very common in popular commercial and non-refereed publications, based on approaches based on exploiting single gene effects, without consideration to polygenic effects, economic values or time for fixation. Research shows that the real situations are far more difficult for complex traits. These traits are controlled by several genes and environmental effects made a survey on the status of application of MAS in actual animal breeding programs for complex traits. He concluded that initial expectations for the use of MAS were high, but the current attitude is one of cautious optimism, with a need for careful examination of alternative selection strategies, business goals and integration of molecular and other technologies. made a detailed survey on the application of DNA technology for beef cattle improvement in USA. He concluded that current contribution of the new DNA technologies for beef cattle breeding is marginal, because they are encountering logistics and mechanical issues. For genomics technologies to impact fully on the beef industry, a higher level of sophistication of the genetic tests will be needed. Tests based on the genes themselves, rather than DNA markers associated with genes.

It is theoretically possible to predict accurately the breeding values of animals using many markers. From this knowledge, it is possible to develop a model for *in vitro* genetic improvement of animals. This is known as velogenetics. The model involves *in vitro* selection of cells containing the desired genes the use of totipotent embryonic stem cells (ES). The procedure uses

transfection of the desired genes, selection in vitro of the cells, and nuclear transfer of the desired genotypes into receptor oocytes. This approach is supposed to increase the rate of genetic improvement by obtaining many generations in a short time by avoiding rearing, reproduction and selection of 'real animals'. Selection on the basis of genomic information only, such in this *in vitro* system, even with major genes with known effects well localized, may be dangerous, because in these artificial populations, unlike in real populations, natural selection would not be allowed to act at each generation on fitness traits under real, perhaps changing, environmental conditions. Changes on economically important traits will not be evaluated directly. This may potentially reduce the responses on selected traits because of genotype x environment interactions. This is because selection is performed in artificial conditions that may deteriorate the fitness of the population and economic response.

Using MAS for improving health in animals by reducing disease prevalence (increasing disease resistance) or increasing resilience (the ability to withstand the disease without harmful effects), for infectious or parasitic diseases has been difficult. In most cases, excepting some rare examples such as Scrapie in sheep, complete resistance could not be obtained with the manipulation of a small number of genes. For most diseases, single-gene approaches are expected to have only a partial contribution.

For many diseases, heritabilities are often low. That indicates the existence of many environmental factors affecting both the probability of infection and the response of the host. In spite of responses attained using conventional selection for some traits that are used as indicators of disease, the result is not well known. The existence of contradictory results regarding associations between production and disease resistance, the complexities of immune and resistance mechanisms and the interaction with other methods of control such as vaccination, sanitation, management and chemotherapy, makes the whole issue of selecting for disease resistance more difficult, in principle, that selecting for production traits. Moreover, we know that heritable resistance or resilience to more virulent form of pathogens would be increased by natural selection. As heritabilities for survival are generally low, we know that the genetic control of disease may be very complex, making difficult to change the outcome by manipulating single genes.

There is one published result on a successful MAS selection program to reduce the prevalence of dermatophilosis, a tropical infectious disease in Zebu cattle. argue to have obtained a sharp reduction in clinical prevalence of the disease from 0.76 to 0.02 in a period of five years by selecting against only two type II BoLA alleles associated with a high susceptibility of the disease. The authors explained the observed change resulting from selection performed in an unknown number of animals of each sex in 1996. However, a complete description of the changes in allele frequencies and genotypes from the moment of selection and their association

with the evolution of prevalence by sex is not given. Considering the possibility of environmental changes and the presence of natural selection, in the absence of a control group, it is difficult to know if the observed change is the sole result of the mechanisms invoked by the authors through MAS.

We cannot at this moment forecast precisely the future of MAS in animal selection, but it is premature to conclude that methods based on phenotypic information will be replaced by methods based solely on genomic data. An integration of both types of data with the use of more sophisticated statistical models is needed. It is far from sure that total replacement of phenotypic information with gene-by-gene information, as selection criterion is possible or even desirable in the future.

Other very important applications of genetic markers in animal improvement include the optimization of mating strategies for non-additive genetic effects (estimation and managing of inbreeding and heterosis), parentage determination, genetic characterization of diverse animal breeds and populations using studies of between and within population (breeds) diversity (Oldenbroek, 1999) and marker-assisted introgression of particular alleles.

Cloning Adult Mammals

Cloning an animal is the production of a genetically identical individual, by transferring the nucleus of differentiated adult cells into an oocyte from which the nucleus has been removed. This is known as Nuclear Transfer and is how the Dolly sheep was produced. Since the publication of the original paper on cloning, there are several other reports on adult cloned animals involving mice, cattle, cats, goats, pigs, sheep and rabbits involving the same, and other cloning techniques.

In the case of Dolly, mammary gland cells in culture from a 6-year old donor ewe, where subjected to a reduction in the concentration of serum and thus obliged to enter in a quiescent state of the cell cycle (G0). Nuclear transfers to enucleated oocytes, was followed by electrical pulses for fusion of the donor cell nucleus and oocyte membranes and activate division.

Problems

Currently there is no doubts regarding the genetic similarity of the donor and the clone in the case of Dolly, however, besides low success rates, several health problems related to the technique have been described. Normal development of an embryo is dependent on the methylation state of the DNA contributed by the sperm and egg and on the appropriate reconfiguration of the chromatin structure after fertilization. Somatic cells have very different chromatin structure to sperm and 'reprogramming' of the transferred nuclei must occur within a few hours of activation of reconstructed embryos. Incomplete or inappropriate reprogramming will lead to de-regulation of gene expression and failure of the embryo or foetus to develop

normally or to non-fatal developmental abnormalities in those that survive. These facts indicate that there is a need for studies to determine further biological consequences of cloning. Cloning has important potential applications in gene transfer procedures.

Use of cloning in animal breeding

Use of cloning in animal genetic improvement may increase the rates of selection progress in certain cases, particularly in situations where artificial insemination is not possible, such as in pastoral systems with ruminants. Currently, high costs of cloning are one of the main factors limiting their use as a technique in practical animal breeding. Clonal groups, however more uniform than full sibs, will have all differences caused by the environmental fraction of variation for measured traits, which is usually more than 50% of total variation.

Selection among many cloned germlines allows the use of the non-additive genetic effects. These effects are not exploited when traditional selection methods involving sexual reproduction are used in animal improvement, but most of the observed genetic variation between animals is additive. Advantages in terms of additional genetic progress however, seems to be only marginal from clone evaluation in selection nucleus herds. Production based on clones of the best animals of the population, may allow for a one time large 'jump' in breeding value, so the commercial animals might be very close to those in the nucleus. However, further genetic improvement must be based in the continued use of the genetic variation by selection programs.

Transgenic Animals

Transgenesis is a procedure in which a gene or part of a gene from one individual is incorporated in the genome of another one. Transgenic animals have any of these genetic modifications with potential use in studying mechanisms of gene function, changing attributes of the animal in order to synthesize proteins of high value, create models for human disease or to improve productivity or disease resistance in animals. In the early 80´s, several research groups reported success in gene transfer and the development of transgenic mice. The definition of transgenic animal has been extended to include animals that result from the molecular manipulation of endogenous genomic DNA, including all techniques from DNA microinjection to embryonic stem (ES) cell transfer and 'knockout' mouse production. Since the early 1980s, the production of transgenic mice by microinjection of DNA into the pronucleus of zygotes has been the most productive and widely used technique. Using transgenic technology in the mouse, such as antisense RNA encoding transgenesis, it is now possible to add a new gene to the genome, increase the level of expression or change the tissue specificity of expression of a gene, and decrease the level of synthesis of a specific protein. Removal or alteration of an existing gene via homologous recombination required the use of ES cells and was limited to the mouse until the advent of nuclear transfer cloning procedures.

Transgenic methods

Microinjection of DNA and now nuclear transfer, are two methods used to produce transgenic livestock successfully. The steps in the development of transgenic models are relatively straightforward. Once a specific fusion gene containing a promoter and the gene to be expressed has been cloned and characterized, sufficient quantities are isolated, purified and tested in cell culture if possible and readied for preliminary mammalian gene transfer experiments. In contrast with nuclear transfer studies, DNA microinjection experiments were first performed in the mouse. While the transgenic mouse model will not always identify likely phenotypic expression patterns in domestic animals, there have not been a single construct that would function in a pig when there was no evidence of transgene expression in mice. Preliminary experimentation in mice has been a crucial component of any gene transfer experiment in domestic animals. While nuclear transfer might be considered inefficient in its current form, major advances in experimental protocols, can be anticipated. The added possibility of gene targeting through nuclear transplantation opens up a host of applications, particularly with regard to the use of transgenic animals to produce human pharmaceuticals. The only major technological advance since the initial production of transgenic farm animals has been the development of methods for the *in vitro* maturation of oocytes (IVM), *in vitro* fertilization (IVF) and subsequent culture of injected embryos prior to transfer to recipient females. Another highly efficient technique for transgenesis has been recently developed based on the use of lentiviral vectors to transform cow and pig oocytes . These vectors are more efficient than microinjection in terms of transformation and expression rates. One limitation is that the size of the transgene and the internal promoter has to be less than 8.5 kb in size.

Transgenesis in the improvement of production traits

The technology of transgenesis is potentially useful to modify characters of economic importance in a rapid and precise way. Contrary to the 'classical' selection programs, it is necessary a knowledge of the genes that control these characters and their regulation.

Following is a brief discussion of experiences with transgenesis to alter economically important traits in livestock.

Growth and meat traits

In most of the earlier work in domestic species (pig, sheep, rabbit) growth hormone was enhanced by the metallotionein promoter to control its expression. Subsequent efforts to genetically alter growth rates and patterns have included production of transgenic swine and cattle expressing a foreign c-ski oncogene, which targets skeletal muscle, and studies of growth in lines of mice and sheep that separately express transgenes encoding growth hormone-releasing factor (GRF) or insulin-like growth factor I (IGF-I) . Transgenic pigs and sheep with high levels of serum growth hormone were obtained, but an increment of its rate of growth was not observed, and only in some lines average daily gain increased with the supplement of the diet

with high levels of protein. The highest effects were observed in the reduction of body fat. A large number of different serious pathologies and a severe reduction in reproductive capacity were described in these animals. In a report about two studies with pigs, there is evidence for the use of transgenesis allowing to important reductions in body fat and increased diameter of muscle fiber by increased IGF-I levels and growth hormone without serious pathological side effects. Australian regulations avoided the commercial release of these animals.

Frequently the used promoters have not allowed an efficient control of the expression of the transgene. It was assessed that it is necessary to develop more complex constructions that activate or repress the expression of the transgene more precisely. Inconsistent results regarding the effect of a growth hormone construct in sheep on growth and meat quality.

Recently, a spectacular transformation was obtained by insertion of a plant gene in pigs. Saeki et al. (2004) generated transgenic pigs that carried the fatty acid desaturation 2 gene for a 12 fatty acid desaturase from spinach. Levels of linoleic acid (18:2n-6) in adipocytes that had differentiated in vitro from cells derived from the transgenic pigs were 10 times higher than those from wild-type pigs. In addition, the white adipose tissue of transgenic pigs contained 20% more linoleic acid (18:2n-6) than that of wild-type pigs. These results demonstrate the functional expression of a plant gene for a fatty acid desaturase in mammals, opening up the possibility of modifying the fatty acid composition of products from domestic animals by transgenic technology.

Wool production

The objectives are to improve production of sheep wool and to modify the properties of the fiber. Because cystein seems to be the limiting amino acid for wool synthesis, the first approach was to increase its production through transfer of cystein biosynthesis from bacterial genes to sheep genome. This approach did not achieve the efficient expression of these enzymes in the rumen of transgenic sheep.

Milk Composition

Milk proteins are coded by unique copy genes that can be altered to modify milk composition and properties. Among the different applications of milk modification in transgenic animals; the following can be highlighted:

1. To modify bovine milk to make it more appropriate to the consumption of infants. Human milk lacks β-lactoglobulin, has a higher relationship of serum proteins to caseins, and has a higher content in lactoferrin and lysozyme when compared to bovine milk. Lactoferrin is responsible for the iron transport and inhibits the bacterial growth. To introduce the human lactoferrin into the bovine milk, transgenic cows have been obtained

(Van Berkel et al. 2002). The elimination of the β-lactoglobulin in the cow milk would be another interesting objective because is one of the major allergens of cow's milk.

2. To reduce the content of lactose in the milk to allow their consumption to people with intolerance to lactose. It is considered that 70% of the world population is lacking theintestinal lactase, the enzyme required to digest the lactose. The reduction in lactose may be obtained by expressing β-galactosidase in the milk or diminishing the content of α-lactalbumin. Transgenic mouse with inactivated α-lactalbumin gene produce milk without lactose. However, a serious practical drawback of this method is that this milk is very viscous and it is not secreted to the exterior of the mammary gland, due to the importance of the lactose in the osmoregulation of the milk.
3. To alter the content of caseins of the milk to increase their nutritive value, cheese yield and processing properties. Research has intended to increase the number of copies of the gene of the κ-casein, to reduce the size of the micelles and modificating the κ-casein to make it more susceptible to the digestion with chymosin. This has only been done using the mouse as a model. Bovine foetal fibroblasts to express additional copies of transgenes encoding two types of casein: bovine β-casein and κ-casein. The modified cell lines of fibroblasts were used to create eleven cloned calves. Milk from the cloned animals was enriched for β- and κ-casein, resulting in a 30% increase in the total milk casein or a 13% increase in total milk protein, demonstrating the potential of this technology to make modified milk.
4. To express antibacterial substances in the milk, such as proteases to increase mastitis resistance. The objective is to alter the concentrations of antibacterial proteins such as lyzozyme or transferrin in the milk.

Future perspectives of transgenesis

The techniques for obtaining transgenic animals in species of agricultural interest are still inefficient. Some approaches that may overcome this problem are based on cloning strategies. Using these techniques it is feasible to reduce to less than 50% the number of embryo receptor females, which is one of the most important economic limiting factor in domestic species. It would also facilitate the further proliferation of transgenic animals. Recent results relate these techniques with still low success rates, high rates of perinatal mortality and variable transgenic expression that requires to be evaluated before generalizing their application.

Considerable effort and time is required to propagate the transgenic animal genetics into commercial dairy herds. Rapid dissemination of the genetics of the parental animals by nuclear transfer could result in the generation of mini-herds in two to three years. However, the existing inefficiencies in nuclear transfer make this a difficult undertaking. It is noteworthy that the

genetic merit of the 'cloned' animals will be fixed, while continuous genetic improvements will be introduced in commercial herds by using artificial insemination breeding programs.

In an alternative scenario of herd expansion, semen homozygous for the transgene may be available in four to five years. Extensive breeding programs will be critical in studying the interaction and co-adaptation of the transgene(s), with the background polygenes controlling milk production and composition. Controlling inbreeding and confirming the absence of deleterious traits so that the immediate genetic variability introduced by transgenesis is transformed into the greatest possible genetic progress is equally critical.

Another alternative strategy for transgenesis is based on the use of sperms as vectors in the integration of the transgenes. Initially described in mice. Results showed that this procedure might be efficient in sheep. In addition, a successful expression of a gene related to genetic modification of pigs for a gene related to xenotranplantation was obtained using this technique. Eighty percent of the pigs were transformed and 54% expressed the transgene consistentl. A very efficient modification of this technique that uses the co-injection of sperms and DNA, has been described in the mouse and given a high rate of transgenesis (20%), therefore, their application to domestic species seems promising. Intracytoplasmic sperm injection (ICSI) has been used recently for the stable incorporation and phenotypic expression of large yeast artificial chromosome (YAC) constructs of submegabase and megabase magnitude. This technique allowed for more than 35% of transgenesis. Another option for transgenesis is the use of insertional mutagenesis using natural transposons. A transposon system called "Sleeping Beauty", and active in a wide range of vertebrate cells, was used to transform mouse embryos with mRNA expressing the SB10 transposes enzyme targeted sequentially a system for primary fibroblasts cells that were used to knock out both alleles of a silent gene, the bovine gene encoding immunoglobulin-μ (IGHM), and the active gene encoding the bovine prion protein (*PRNP*) and produced both heterozygous and homozygous knockout calves. The procedure integrates homologous recombination to replace genes in cell culture, and rejuvenation of cell lines by production of cloned fetuses. A method for selective elimination of selection marker genes was also developed. This method allow for the production of double homozygous transgenic embryos in 21.5 months. In contrast, for cattle, the production of double homozygous from heterozygous founders would require approximately 5 years and generation for double homozygous from heterozygous founders is impractical. This method can be used to breed many types of cattle with improved disease resistance and values for increased productivity. A recent alternative consists on the transformation of somatic tissues of developed animals, using techniques similar to those used in gene therapy.

Discussion

Detecting genes related to disease and their expression in humans from studies on the genome, could lead to the development of therapies and the development of drugs for specific individuals,

and enhanced early diagnosis of individuals with high-risk genotypes, allowing for preventive or remedial actions, even gene therapy. In animals, this knowledge could lead, in addition, to select against defective genes.

In livestock, knowledge of effects of specific genes and gene combinations on important traits could lead to their enhanced control to create new, more useful populations. The use of specific gene information is not a panacea, but could help to increase rates of genetic improvement, and open opportunities for using additive and non-additive genetic effects of domestic species, provided wise improvement goals are used and this new technology is optimally used together with the so called 'traditional' or 'conventional' methods based on phenotypic and genealogical information.

These methods will help to increase our knowledge about the genetic architecture of complex quantitative traits in domestic animal populations and to estimate the distribution of the genetic variation across and within breeds and population. It will also aid in ascertaining the genetic merit of local, less known populations. Studies for using genetic diversity in structured populations using DNA markers are very useful in order to set priorities for conservation of distant or unique populations as reservoirs of potentially unique genes, because their contribution to biodiversity would be greater. Currently, however, the main practical application of DNA markers is for parenting determination and to trace products such as meat.

Despite its relatively low success rates and associated high costs, transgenic technology have a number of important potential applications in animal improvement such as increasing productivity, product quality and creating novel products.A major limitation to use transgenesis in the improvement of productive characters is the limited knowledge available on the identity and regulation of the genes that control these characters. The advance in the elaboration of genetic maps and fine positional cloning studies in the main species of interest will allow having a larger number of candidate genes susceptible of being manipulated. However, the road from genotype to phenotype is proving to be much more complex than previously thought for disease and production traits affected by many genes.

One promising applications of transgenesis is the synthesis of biomedical products of high commercial interest. Transgenic bioreactors and the use of exogenous or artificial genes interfering with particular cell mechanisms or with pathogens but not, or only marginally, with the physiology of the animals are potential applications. A greater knowledge on the mechanisms that determine the integration of the transgenes and genic regulation will allow a more precise control of the expression of the transgenes and it will probably facilitate a larger number of applications in the domestic species, including modifications beyond normal limits, such as to increase the number of copies of the gene and their expression. These transformations could be regarded as a form of mutation. The expressions of complex traits are the result of several

mechanisms involving both regulatory and structural portions of the genome. Advances in molecular genetics, genomics, proteomics and transcriptomics; might perhaps help to shorten the gap between the more 'holistic' approaches of quantitative genetics with the more 'reductionistic' approach of molecular genetics. The release of genome sequence information in cattle and pig, may allow for a more efficient use of MAS and also to address some consumers concerns regarding product quality and safety.

Use of genetic engineering for animal and plant improvement is in its infancy, therefore many questions regarding efficiency, safety and societal benefits in particular situations remain. Problems arising transgenic plants, including their lower-than expected productivity, are reviewed thoroughly by. Simplistic and overoptimistic views of biotechnology should be replaced by serious and scientifically based assessments of these new technologies by potential users on a case-by-case basis. We need to emphasize that in most cases, the use of MAS is not a revolution but just an evolution with regard to the traditional methods, because we are looking to improve more efficiently traits that already are actually or potentially improved in an efficient way using, for instance, mixed model (BLUP) based technologies for selection. Efficiency issues are very important. In order to increasing the efficiency of MAS, we need previously to:

1. Define with greater precision the selection goal and selection criteria.
2. Optimize the use of BLUP and other 'classical' breeding methodology.

The use of transgenic animals models for the study of gene regulation and expression has become commonplace in the biological sciences. Contrary to the early prospects related to commercial exploitation in agriculture, there are some challenges regarding their use that still lay ahead. The risks at hand can be defined not only by scientific evidence but also in relation to public concern (whether perceived or real) that exists in some people. Therefore, the central questions will revolve around the proper safeguards to employ and the development of a coherent and unified regulation of the technology.

Cloning is another technique that raises concerns both from the ethical and practical point of view. Whether it is acceptable to clone humans is a very difficult issue. In animals, besides the very low success rates, some abnormalities should suggest that more information is required on the consequences of such practices in humans but also in animals, before its routine use. Advantages for animal breeding programs derived from cloning with no use of transgenesis are like to be small.

These two examples illustrate that in spite most of the problems are technical in nature, implications of the use of this knowledge will be important for the society as a whole .

A reasonable degree of regulation, open information on the issues of genetic engineering technologies from the academic world and an involvement of the whole society in the developments of the laws concerning these issues, seems to be the best way to circumvent an exaggerated or negative reactions to some of these knowledge, and to avoid or reduce unethical or abusive use of these techniques. A specific set of conclusions regarding safety of food from genetically modified animals is available from a FAO/WHO expert consultation panel.

Concluding Remarks

Most of the important potential technical advances offered by genetic engineering technology in animal breeding are still ahead. Their use has both advantages and problems. Advantages are related to a more complete control over the animal genome. Problems are related to technical complexity, high costs, in some cases, public acceptance and ethical dilemmas.

It is not likely that this technology, will replace 'conventional' methods for genetic improvement. Instead, they probably will begin to be gradually incorporated into current genetic improvement programs that use efficiently classical improvement methods to achieve particular objectives.

Contained use - Animal cell cultures: Risk assessment and biosafety recommendations

Regulatory framework in Belgium

In Belgium, the contained use of genetically modified organisms and/or pathogens, including animal cell cultures that are genetically modified or that are deliberately infected with pathogens, is governed by regional regulations enforcing the containment provisions of European Directives. It implies that animal cell cultures which are not intentionally infected with pathogens or which are not genetically modified, are beyond the scope of the regulatory provisions.
Nevertheless, as animal cultures may carry adventitious pathogens, it is highly recommended to conduct a biosafety risk assessment and to implement adequate safety measures whenever animal cell cultures are manipulated.
(Note: cette page n'existe qu'en anglais - Nota: deze pagina bestaat enkel in het Engels)

Introduction

The use of animal and human cell cultures has become very beneficial for diverse applications in biotechnology and biomedical research. Originally used as substrates for the production of viral vaccines, animal and human cell cultures became an indispensable tool to produce a variety of

products, including biopharmaceuticals, monoclonal antibodies and products for gene therapy. The use of animal and human cell cultures constitute also adequate test systems for studying biochemical pathways, virus production, pathological mechanisms or intra- and intercellular responses.
Along with the increasing importance of the contained use of animal and human cell cultures, biosafety concerns have pointed to the risks with respect to human health and environmental considerations. A maximal reduction of these risks necessitates a thorough **biosafety risk assessment**, taking account of the **type of manipulation** and the biological hazards inherent to the use of cell cultures. The risk assessment should result in the implementation of appropriate **containment measures and work practices** in order to provide maximal protection of human health and environment.

Risk assessment

Risk assessment of human/animal cell cultures is based on both the **intrinsic properties** of the cell culture - including subsequent properties acquired as a result of **genetic modification(s)** - and the possibility that the cell culture may **inadvertently or deliberately be contaminated** with pathogens. In addition, risk assessment should be performed according to the **type of manipulation**.

Intrinsic properties of cell cultures

The following intrinsic properties of cell cultures should be considered while performing the risk assessment: source (species of origin), cell types or tissues, culture type.

Source (species of origin)	Cell types or tissues	Culture type
The closer the genetic relationship of the cell culture is to humans, the higher the risk is to humans since contaminating pathogens usually have specific species barriers. !! Be aware that some contaminating organisms might cross the usual species barrier (e.g. H5N1 influenza, BSE, SARS, etc.)	Consider the tumour inducing potential of cell types.	
increasing order of risk ⇩	**increasing order of risk** ⇩	**increasing order of risk** ⇩
avian and invertebrate cells	epithelial and fibroblastic cells	well-characterized cell lines

mammalian cells (other than human or primate)	gut mucosa	continuous cell lines
non-human primate cells	endothelium	primary cell lines
human cells	neural tissues	
	hematopoietic cells	

Properties acquired as a result of genetic modification

Recombinant cells may have increased (or decreased) abilities to cause harm to human health and environment compared to their non-recombinant counterparts. The properties that recombinant cells acquire following genetic modification must be determined as well. This includes an evaluation of all events intervening in the process of genetic modification:

- the recipient organism properties (host cell)
- the donor organism properties
- the characteristics and location of the inserted genetic material
- the vector

The Annex of Commission Decision 2000/608/EC provides guidance notes for risk assessment of genetically modified microorganisms.

- see example 1 : transfection of mouse T-lymphoma cells with human interleukin 2 using mammalian expression vector
- see example 2: retroviral packaging cell lines

Properties acquired as a result of infection with pathogenic agents

Deliberate infection with pathogens

Cells may be deliberately infected with pathogens. In this case, the determination of potential hazards related to infected cell cultures requires an examination of the inherent properties of the infecting pathogen. This implies an assessment of a list of criteria, which are specific to the pathogen, along with aspects such as the existence of effective therapies or prophylaxis. An evalutation of these criteria have been used to classify pathogens into classes of biological risk, also called risk groups. **The biological risk of infected cell cultures will depend on the biological risk of the infecting pathogen(s)**.

- see example : culture of bovine leukocytes infected with Theileria parva

Adventitious contaminating agents

When manipulating cell cultures, the presence of adventitious contaminating agents constitutes the main hazard to humans. Contamination may occur by the source (e.g. infected animals or tissues), during cell handling (repeated passages and use in the laboratory) or by using contaminated biological reagents (e.g. media and additives derived from bovine sources are often contaminated with bovine viral diarrhoea virus, BVDV).

Bacteria and Fungi

In general bacterial or fungal contamination can be readily detected in cell cultures because of their capacity to overgrow cell cultures. Bacterial and fungal infections are relatively easy to prevent and to treat.

Mycoplasma

Compared to bacterial or fungal infections, contaminations with mycoplasma give more problems in terms of incidence, detectability, prevention and eradication. Mycoplasma infection may go undetected for many passages, causing a variety of unpredictable effects causing harm to the host cell. Mycoplasma infection may also influence the sensitivity of host cells for growth of viruses. Beside the fact that this intracellular bacteria is one of the most common cell culture contaminants, it should be considered that some of contaminating Mycoplasma spp. belong to class of risk 2. Together with M. arginini, M.orale, M.pirum and M. fermentans, pathogenic organisms like M. gallisepticum (class of risk 3 for animals), M. hyorhinis (class of risk 2 for animals), and M. pneumoniae (class of risk 2 for humans) account for more than 96% of mycoplasma contaminants in cell cultures.

Viruses

Viral contamination needs particular attention because infection may be without cytopathic effect for the cell culture or may be latent (e.g. herpesvirus) and hard to detect. Human and non-human primate cultures are more likely to harbour viruses that are highly pathogenic to humans. Of particular concern are the blood-borne viruses such as Hepatitis B Virus (HBV), Human Immunodeficiency Virus (HIV) and others such as Hepatitis C virus (HCV) and Human T-cell lymphotropic viruses (HTLV). However, non-human cell cultures are not without risks as they may contain viruses with a broader host range able to infect humans such as rodent cell culture carrying hantavirus or primate cells harbouring Marburg virus.

- see example : Lymphocytic choriomeningitis virus (LCMV) contamination of murine cell cultures

Parasites

Adventitious contamination with parasites may be an issue when handling freshly prepared primary cell cultures or tissue cultures originating from a donor organism that is known or suspected to be infected with a specific parasite. Well-known intracellular protozoan parasites

for which laboratory-acquired infections have been reported are Toxoplasma gondii, Trypanosoma cruzi, Leishmania sp, Cryptosporidium parvum , Plasmodium sp. etc.

Prions

Though a limited number of cultured cell lines (e.g. mouse neuroblastoma cell lines Sc N2a) have been shown to promote, upon subpassaging, stable and persistent replication of PrP(Sc) as well as infectivity, most cell lines are resistant to prion infection. However, in contrast to most of the infectious agents, prions are particularly difficult to inactivate. In fact no method can guarantee total inactivation of these agents. So, one should bear these considerations in mind when using growth media of bovine origin.

Type of manipulation

In addition to the determination of biological risk of animal cell cultures, the consideration of the type of manipulation constitutes a key aspect in the completion of a thorough risk assessment. Issues regarding the type of manipulation include:

- the characteristics of the environment likely to be exposed
    - e.g. mucous membranes of the manipulator may be exposed when handling cell cultures out of a biosafety cabinet type II
- the characteristics of the activity (scale, type of procedure, culture conditions)
    - e.g. Altered culture conditions can have significant effects to safe handling of human or animal cell cultures as it may result in altered neoplasia, release of endogenous viruses or altered expression of cell surface receptors. Changing culture conditions may thus lead to altered susceptibility of the cultured cells to viruses.
    - e.g. human cell cultures that harbour infectious agents can represent lower risks for human health once they are fixed by glutaraldehyde or formaldehyde/aceton for immunostaining or flow cytometry.
- any non-standard operations
    - e.g. depletion of primary cells from tissues; manipulations likely to generate aerosols.

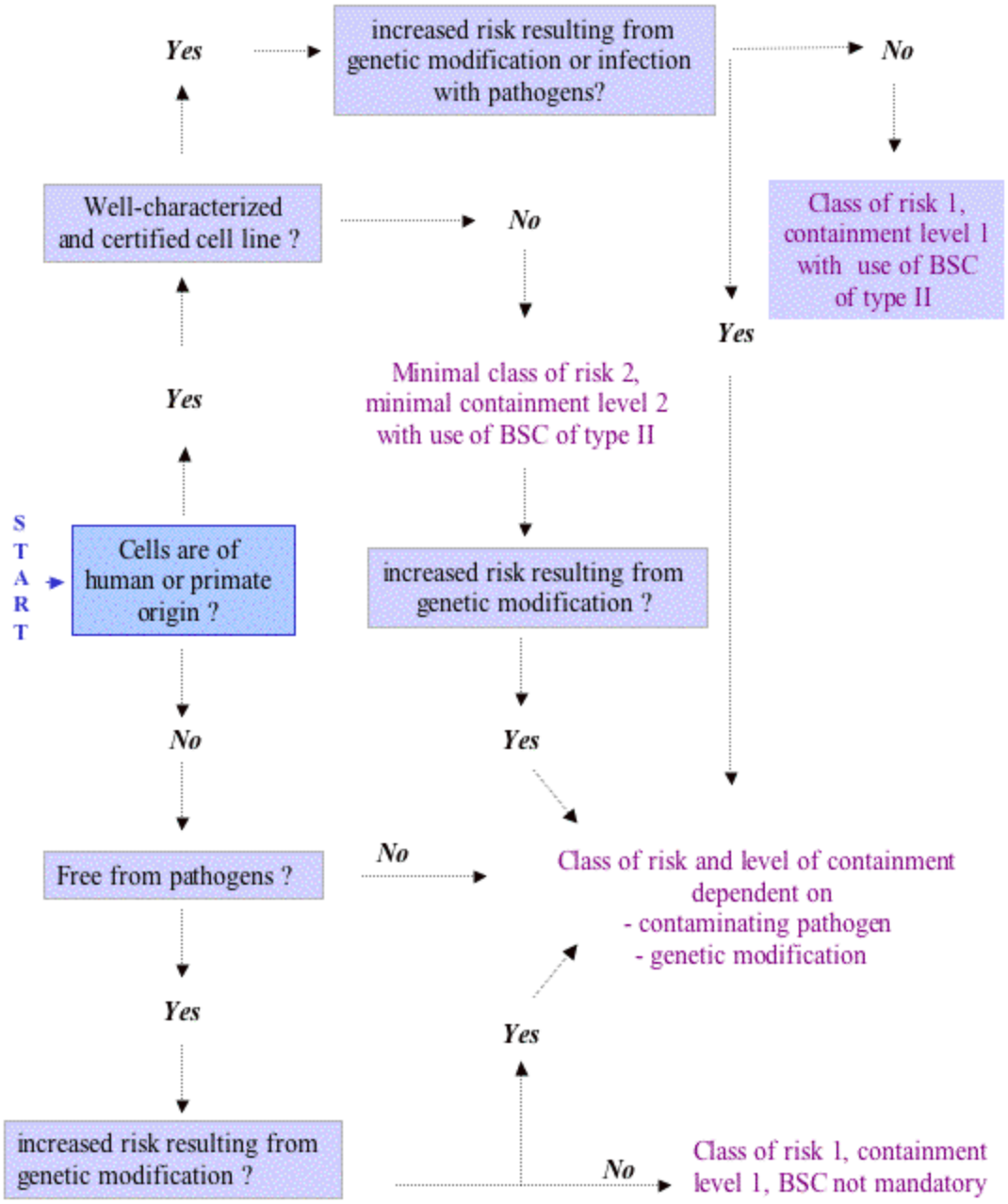
As some clinical approaches such as stem cell therapy, gene therapy, xeno- or allotransplantation involve ex vivo cell culturing, many aspects of the risk assessment – as mentioned above- should be applied. However, culturing cells for therapeutic purposes justifies more careful consideration regarding quality, efficacy, safety, ethical, social and regulatory issues that will not be addressed on this webpage. More information can be obtained on clinical trials with GMOs for human or veterinary use.

Biosafety recommendations and containment measures

The examination of biological risks related to animal cell cultures and the consideration of the type of manipulation allows the determination of adequate containment level in order to protect human and environmental health. The set up and implementation of an adequate containment level include a list of general and more specific work practices and containment measures.

- Precautionary measures should be applied whenever handling animal cell cultures. Much of these measures basically aim at reducing the risk of contamination with adventitious agents by ensuring protection of both operator and cell culture.
- Cells are reasonably considered **free of adventitious contaminating pathogens** if a number of conditions are fulfilled.
- If cell cultures are known to harbour an infectious etiologic agent or virus, containment measures should be the same as that recommended for the etiologic agent.

The flowchart hereunder offers a schematic guidance for the assignment of appropriate containment levels and is based on, but not limited to, key features of risk assessment as outlined above. This flowchart is only indicative and should be applied and /or reconsidered according to case specific conditions and risk assessments.



BSC type II = Biosafety cabinet type 2. Contrary to a biosafety cabinet of class I, which only protects the operator and the environment, the use of a biosafety cabinet of class II aims at protecting the operator, the environment and the cell culture, the latter being of importance in order to prevent contamination of cell cultures. In view of this, animal cell cultures should never be manipulated in a biosafety cabinet with horizontal laminar air flow.

Conclusions

The assignment of appropriate containment requirements cannot be generalised, it varies on a **case-by-case** basis and should rely on a thorough **risk assessment**, including considerations of intrinsic cell properties (including recombinant properties), potential contamination with pathogens and type of manipulation. Adventitious contaminating agents probably constitute the main hazard associated to the manipulation of cell cultures since they are often difficult to detect and therefore less verifiable.

1. The growth of animal cells in vitro in a suitable culture medium is called	a) Gene expression	b) Transgenesis	c) Plant tissue culture	d) Animal cell culture	d) Animal cell culture
2. Cells from kidney tissues cannot survive independently, it requires other surface for attachment and survival.	a) True	b) False			a) True
3. Name the type of culture which is prepared by inoculating directly from the tissue of an organism to culture media?	a) Primary cell culture	b) Secondary cell culture	c) Cell lines	d) Transformed cell culture	a) Primary cell culture
4. What is a cell line?	a) Multilayer culture	b) Sheep	c) Mouse	d) Drosophila	c) Mouse
5. Name the organism on which first cell line was observed?	a) E.coli	b) Continuous cell lines	c) Dependent on external growth factor	d) No contact inhibition	c) Dependent on external growth factor
6. Which of the following is the characteristics of a normal cell?	a) Anchorage independent	b) WISH	c) L	d) MRC-5	d) MRC-5
7. Name the cell line of the human embryonic lung?	a) HeLa	b) Serum	c) Carbon source	d) Inorganic salts	a) Starch
8. Which of the following is NOT the part of growth medium for animal culture?	a) Starch	b) Stimulate cell growth	c) Enhance cell attachment	d) Provide transport proteins	a) Promotion of tuber and bulb formation
9. Which of the following is NOT the major function of the serum?	a) Promotion of tuber and bulb formation	b) WISH	c) L	d) MRC-5	d) MRC-5
10. Name the cell line of the human embryonic lung?	a) HeLa	b) Continuous cell lines	c) Dependent on external growth factor	d) No contact inhibition	c) Dependent on external growth factor
11. Which of the following is the characteristics of a normal cell?	a) Anchorage independent	b) Sheep	c) Mouse	d) Drosophila	c) Mouse
12. Name the organism on which first cell line was observed?	a) E.coli	b) Transformed cells	c) Multiple growth of cells	d) Sub culturing of primary culture	d) Sub culturing of primary culture
13. What is a cell line?	a) Multilayer culture				
14. Name the type of culture which is prepared by inoculating directly from the tissue of an organism to culture media?	a) Primary cell culture	b) Secondary cell culture	c) Cell lines	d) Transformed cell culture	a) Primary cell culture
15.Cells from kidney tissues cannot survive independently, it requires other surface for attachment and survival.	a) True	b) False			a) True
16.The growth of animal cells in vitro in a suitable culture medium is called	a) Gene expression	b) Transgenesis	c) Plant tissue culture	d) Animal cell culture	d) Animal cell culture
17. Embryo initiation is facilitated by_____		b) BPA			
18. Which of the following culture is used for the production of primary and secondary metabolites?	a) GA3		c) ABA	d) 2,4 D	d) 2,4 D
	a) Cell suspension culture				
19. Which of the following does not act as a fusogen in protoplast fusion?	b) Callus culture	c) Protoplast culture	d) Somatic hybrid	a) Cell suspension culture	
20. Increase amount of auxin in callus culture will promote which part of the plant tissue?	a) 2,4 D	b) Polyethylene glycol	c) Calcium	d) PVA	a) 2,4 D
21. Name the asexual mode of embryo formation?	a) Multilayer tissues	b) Meristem	c) Shoot	d) Root	d) Root
22.Mark the INCORRECT statement about applications of the somatic embryogenesis?	a) Protoplast fusion	b) Callus culture	c) Somatic embryogenesis	d) Protoplast culture	c) Somatic embryogenesis
23. Which method is used to overcome cytoplasmic male sterility?	a) Mass multiplication of germplasm	b) Continuous cell lines	c) Production of artificial seeds	d) Production of primary and secondary metabolites	b) Continuous cell lines
24. Which of the following statement is NOT true for artificial seeds?	a) Callus culture	b) Artificial embryogenesis	c) Somatic embryogenesis	d) Cybrid	d) Cybrid
25. Prophylactic is also an antibiotic.	a) Higher cost method for multiplication	b) No transplantation is required	c) Bactericide and fungicide can be given	d) Artificial endosperm can be given	a) Higher cost method for multiplication
26. Hybridoma cells have an application to produce:	a) True	b) False			a) True
27. Monoclonal antibodies are also used for chromatographic separations.	a) Antigens	b) Antibodies	c) Cancer cells	d) Cell lines	b) Antibodies
28. Monoclonal antibodies are referred as	a) True	b) False			a) True
29. Antibody fragments lack fc domain.	a) Magic bullets	b) Magic gun	c) Magic shots	d) Magic bomb	a) Magic bullets
30. Antibody fragments are advantageous than Monoclonal antibodies.	a) True	b) False			a) True
31. Interferon is a virus.	a) True	b) False			a) True
32. Lymphokines are produced both by T-cell and B-cell.	a) True	b) False			b) False
33. Thymosins are:	a) True	b) False			b) False
34. Subunit vaccines from Virus contain viral DNA.	a) Small proteins	b) Medium proteins	c) Large proteins	d) Globular proteins	a) Small proteins
35. The poor antigen in a conjugate vaccine is:	a) True	b) False			b) False
36. Eicosanoids is a type of	a) Strong protein	b) Weak protein	c) A Polysaccharide	d) Non-polysaccharide	c) A Polysaccharide
37. Amines is a type of hormone.	a) Hormone	b) Antibiotic	c) Vaccine	d) Antigen	a) Hormone
38. Baculovirus infects insect cell lines and are also pathogenic to humans.	a) True	b) False			a) True
39. What do you mean by glycosylation?	a) True	b) False			b) False
40. The desire to maintain a safe laboratory environment for all begins with _____?	a) Addition of sugar	b) Non-addition of sugar	c) Lysis of sugar moieties	d) Blockage of sugar molecules	a) Addition of sugar
41. When a chemical splashes in the eye rinse for _____?	a. prevention	b. ubiquity	c. microbiology	d. accidents	a. prevention
42. 3. Which of the following type(s) of Personal Protective Equipment (PPE) is frequently used?	a. 10 seconds	b. 30 seconds	C. 5 minutes	d.15 minutes	D.15 minutes
43. Chemical, reagents or broth cultures should be pipetted by _____?	a. Safety glasses	b. Lab Coats	C. . Face Shields	d. All of the above	d. All of the above
	a. mouth	b. ear	c. pipetter	d. nose	c. pipetter
44. 5. Good work practices include	a. smelling and tasting chemicals	b. not washing hands before and after lab	c. confining long hair and loose clothing	d.using damaged equipment and glassware.	c. confining long hair and loose clothing
45. What is the name of the procedure performed under sterile conditions to eliminate contamination in hopes to obtain a pure culture of one type of microorganism?	a. sterilization technique	b. aseptic technique	c. disinfectant technique	pathogen technique	b. aseptic technique
46. After a biohazard spill is covered with paper towels and disinfectant solution, it must sit for _____ minutes?	a. 5	b. 30	c. 60	d. 20	b. 30
47. _____ is needed as a source of nutrient for the growth and reproduction of microbes.	a. Pathogens	b. bacteria	c. reagents	d. media	d.media
48. To prevent the contamination of microscopes and surrounding areas disenfect/clean used slides, prepared by student, with	a. 70% ethanol and lens paper	b. 5% methylene blue and lens paper	c. acetone and lens paper	d. water and lens paper	a. 70% ethanol and lens paper
49. Virulent strains of microorganisms are used in your laboratory?	a. False	b. true			a. False
50. 5. Good Laboratory Practice (GLP) is a method employed in a laboratory setting to prevent contamination, accidents and injuries.	a.true	b. False			a.true

Introduction

Cell culture is one of the major techniques in the life sciences. It is the general term used for the removal of cells, tissues or organs from an animal or plant and their subsequent placement into an artificial environment conducive to their survival and/or proliferation. Basic environmental requirements for cells to grow optimally are: controlled temperature, a substrate for cell attachment, and appropriate growth medium and incubator that maintains correct pH and osmolality. The most important and crucial step in cell culture is selecting appropriate growth medium for the *in vitro* cultivation. A growth medium or culture medium is a liquid or gel designed to support the growth of microorganisms, cells, or small plants. Cell culture media generally comprise an appropriate source of energy and compounds which regulate the cell cycle. A typical culture medium is composed of a complement of amino acids, vitamins, inorganic salts, glucose, and serum as a source of growth factors, hormones, and attachment factors. In addition to nutrients, the medium also helps maintain pH and osmolality.

Types of Cell Culture Media

Animal cells can be cultured either using a completely natural medium or an artificial/synthetic medium along with some natural products.

	Media Type	Examples	Uses
Natural media	Biological Fluids	plasma, serum, lymph, human placental cord serum, amniotic fluid	
	Tissue Extracts	Extract of liver, spleen, tumors, leucocytes and bone marrow, extract of the bovine embryo and chick embryo	
	Clots	coagulants or plasma clots	
Artificial media	Balanced salt solutions	PBS, DPBS, HBSS, EBSS	Form the basis of complex media
	Basal media	MEM DMEM	Primary and diploid culture
	Complex media	RPMI-1640, IMDM	Supports a wide range of mammalian

cells

Table 1.Types of natural and artificial media.

Natural media

Natural media consist solely of naturally occurring biological fluids. Natural media are very useful and convenient for a wide range of animal cell culture. The major disadvantage of natural media is its poor reproducibility due to lack of knowledge of the exact composition of these natural media.

Artificial media

Artificial or synthetic media are prepared by adding nutrients (both organic and inorganic), vitamins, salts, O_2 and CO_2 gas phases, serum proteins, carbohydrates, cofactors [1]. Different artificial media have been devised to serve one or more of the following purposes: 1) immediate survival (a balanced salt solution, with specific pH and osmotic pressure); 2) prolonged survival (a balanced salt solution supplemented with various formulation of organic compounds and/or serum); 3) indefinite growth; 4) specialized functions.

Artificial media are grouped into four categories:

Serum containing media

Fetal bovine serum is the most common supplement in animal cell culture media. It is used as a low-cost supplement to provide an optimal culture medium. Serum provides carriers or chelators for labile or water-insoluble nutrients, hormones and growth factors, protease inhibitors, and binds and neutralizes toxic moieties.

Serum-free media

Presence of serum in the media has many drawbacks and can lead to serious misinterpretations in immunological studies. A number of serum-free media have been developed. These media are generally specifically formulated to support the culture of a single cell type, such as Knockout Serum Replacement and Knockout DMEM from Thermo Fisher Scientific, and mTESR1 medium from Stem Cell Technologies, for stem cells, and incorporate defined quantities of purified growth factors, lipoproteins, and other proteins, which are otherwise usually provided by the serum. These media are also referred to as ‘defined culture media’ since the components in these media are known.

Chemically defined media

These media contain contamination-free ultra pure inorganic and organic ingredients, and may also contain pure protein additives, like growth factors. Their constituents are produced in bacteria or yeast by genetic engineering with the addition of vitamins, cholesterol, specific amino acids, and fatty acids.

Protein-free media

Protein-free media do not contain any protein and only contain non-protein constituents. Compared to serum-supplemented media, use of protein-free media promotes superior cell growth and protein expression and facilitates downstream purification of any expressed product. Formulations like MEM, RPMI-1640 are protein-free and protein supplement is provided when required.

Basic Components of Culture Media

Culture media contain a mixture of amino acids, glucose, salts, vitamins, and other nutrients, and available either as a powder or as a liquid form from commercial supplier. The requirements for these components vary among cell lines, and these differences are partly responsible for the extensive number of medium formulations. Each component performs a specific function, as described below:

Buffering systems

Regulating pH is critical for optimum culture conditions and is generally achieved by one of the two buffering systems:

Natural buffering system

In a natural buffering system, gaseous CO_2 balances with the CO_3/HCO_3 content of the culture medium. Cultures with a natural buffering system need to be maintained in an air atmosphere with 5-10% CO_2, usually maintained by a CO_2 incubator. A natural buffering system is low-cost and non-toxic.

HEPES

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

Phenol red

Most of the commercially available culture media include phenol red as a pH indicator, which allows constant monitoring of pH. During the cell growth, the medium changes color as pH is changed due to the metabolites released by the cells. At low pH levels, phenol red turns the medium yellow, while at higher pH levels it turns the medium purple. Medium is bright red for pH 7.4, the optimum pH value for cell culture. However, there are certain disadvantages of using phenol red as described below: 1) Phenol red mimics the action of some steroid hormones, particularly estrogen. Thus it is advisable to use media without phenol red for studies using estrogen-sensitive cells like mammary tissue. 2) Presence of phenol red in some serum-free formulations interferes with the sodium-potassium homeostasis. This effect can be neutralized by the inclusion of serum or bovine pituitary hormone in the medium. 3) Phenol red interferes with detection in flow cytometric studies.

Inorganic salt

Inorganic salt in the media helps to retain the osmotic balance and help in regulating membrane potential by providing sodium, potassium, and calcium ions.

Amino acids

Amino acids are the building blocks of proteins, and thus are obligatory ingredients of all known cell culture media. Essential amino acids must be included in the culture media as cells can not synthesize these by themselves. They are required for the proliferation of cells and their concentration determines the maximum achievable cell density. L-glutamine, an essential amino acid, is particularly important. L-glutamine provides nitrogen for NAD, NADPH and nucleotides and serves as a secondary energy source for metabolism. L-glutamine is an unstable amino acid, that, with time, converts to a form that can not be used by cells, and should thus be added to media just before use. Caution should be used when adding more L-glutamine than is called for in the original medium formulation since its degradation results in the build-up of ammonia, and ammonia can have a deleterious effect on some cell lines. L-glutamine concentrations for mammalian cell culture media can vary from 0.68 mM in Medium 199 to 4mM in Dulbecco's Modified Eagles's Medium. Invertebrate cell culture media can contain as much as 12.3 mM L-glutamine. Supplements like glutamax are more stable and can replace glutamine for long term culturing of slow cells. Typical suppliers of L-glutamine for cell culture isMilliporeSigma (G7513).

Nonessential amino acids may also be added to the medium to replace those that have been depleted during growth. Supplementation of media with non-essential amino acids stimulates growth and prolongs the viability of the cells.

Carbohydrates

Carbohydrates in the form of sugars are the major source of energy. Most of the media contain glucose and galactose, however, some contain maltose and fructose.

Proteins and peptides

The most commonly used proteins and peptides are albumin, transferrin, and fibronectin. They are particularly important in serum-free media. Serum is a rich source of proteins and includes albumin, transferrin, aprotinin, fetuin, and fibronectin. Albumin is the main protein in blood acting to bind water, salts, free fatty acids, hormones, and vitamins, and transport them between tissues and cells. The binding capacity of albumin makes it a suitable remover of toxic substances from the cell culture media.

Aprotinin is a protective agent in cell culture systems, stable at neutral and acidic pH and resistant to high temperatures and degradation by proteolytic enzymes. It has the ability to inhibit several serine proteases such as trypsin. Fetuin is a glycoprotein found in fetal and newborn serum at larger concentrations than in adult serum. It is also an inhibitor of serine proteases. Fibronectin is a key player in cell attachment. Transferrin is an iron transport protein that acts to supply iron to the cell membrane.

Fatty acids and lipids

They are particularly important in serum-free media as they are generally present in serum.

Vitamins

Many vitamins are essential for growth and proliferation of cells. Vitamins cannot be synthesized in sufficient quantities by cells and are therefore important supplements required in tissue culture. Again serum is the major source of vitamins in cell culture, however, media are also enriched with different vitamins making them suitable for a particular cell line. The B group vitamins are most commonly added for growth stimulation.

Trace elements

Trace elements are often supplemented to serum-free media to replace those normally found in serum. Trace elements like copper, zinc, selenium and tricarboxylic acid intermediates are chemical elements that are needed in minute amounts for proper cell growth. These micronutrients are essential for many biological processes, e.g., the maintenance of the functionality of enzymes.

Media supplements

The complete growth media recommended for certain cell lines requires additional components which are not present in the basal media and serum. These components, supplements, help sustain proliferation and maintain normal cell metabolism. Although supplements like hormones, growth factors and signaling substances are required for normal growth of some cell lines, it is always best to take the following precautions: since the addition of supplement can change the osmolality of the complete growth media which can negatively affect the growth of cells, it is always best to recheck the osmolality after supplements are added. For most of the cell lines, optimal osmolality should be between 260 mOSM/kg and 320 mOSM/kg.

The shelf life of the growth media changes after the addition of supplements. Complete media containing protein supplement tend to degrade faster than basal media alone.

Antibiotics

Although not required for cell growth, antibiotics are often used to control the growth of bacterial and fungal contaminants. Routine use of antibiotics for cell culture is not recommended since antibiotics can mask contamination by mycoplasma and resistant bacteria. Moreover, antibiotics can also interfere with the metabolism of sensitive cells. Penicillin-streptomycin preparations from Life Technologies (15140163) and MilliporeSigma (P0781) are typical choices. Plasmocin can eliminate mycoplasma contamination, and has been used in culturing glioma cell lines TS603, TS516, and BT260.

In addition, primary cell culture can also be contaminated by fungi, protozoa, and plant cells (for animal cell culture), due to the host or other circumstances. Roundup/glyphosate isopropylamine/(2-Oxo-2-hydroxyethyl)aminomethylphosphonicacid · isopropylamine can be applied for plant cells; amphotericin B (fungizone) and/or fluconazole for fungi; and pentamidine and/or atovaquone for protozoa.

Serum in media

Serum is a complex mix of albumins, growth factors and growth inhibitors. Serum is one of the most important components of cell culture media and serves as a source for amino acids, proteins, vitamins (particularly fat-soluble vitamins such as A, D, E, and K), carbohydrates, lipids, hormones, growth factors, minerals, and trace elements. Serum from fetal and calf bovine sources are commonly used to support the growth of cells in culture. Fetal serum is a rich source of growth factors and is appropriate for cell cloning and for the growth of fastidious cells. Calf serum is used in contact-inhibition studies because of its lower growth-promoting properties. Normal growth media often contain 2-10% of serum. Supplementation of media with serum serves the following functions :

- Serum provides the basic nutrients (both in the solution as well as bound to the proteins) for cells.
- Serum provides several growth factors and hormones involved in growth promotion and specialized cell function.
- It provides several binding proteins like albumin, transferrin, which can carry other molecules into the cell. For example: albumin carries lipids, vitamins, hormones, etc. into cells.
- It also supplies proteins, like fibronectin, which promote the attachment of cells to the substrate. It also provides spreading factors that help the cells to spread out before they begin to divide.
- It provides protease inhibitors which protect cells from proteolysis.
- It also provides minerals, like Na+, K+, Zn2+, Fe2+, etc.
- It increases the viscosity of the medium and thus, protects cells from mechanical damages during agitation of suspension cultures.
- It also acts a buffer.

Due to the presence of both growth factors and inhibitors, the role of serum in cell culture is very complex. Unfortunately, in addition to serving various functions, the use of serum in tissue culture applications has several drawbacks. Table 2 shows the advantages and disadvantages of using serum in the media.

Advantages of serum in media	Disadvantages of serum in media
Serum contains various growth factors and hormones which stimulates cell growth and functions.	Lack of uniformity in the composition of serum
Helps in the attachment of cells	Testing needs to be done to maintain the quality of each batch before using
Acts as a spreading factor	May contain some of the growth inhibiting factors

Acts as a buffering agent which helps in maintaining the pH of the culture media	Increase the risk of contamination
Functions as a binding protein	Presence of serum in media may interfere with the purification and isolation of cell culture products
Minimizes mechanical damages or damages caused by viscosity	

Table 2.advantages and disadvantages of using serum in the media

Others

For suspension culture, Pluronic F-68 at 0.1% can be added to reduce the water shear force and reduce foaming.

Preparation of Media

Culture medium is available in three forms from commercial suppliers:

1. Powdered form: it needs to be prepared and sterilized by the investigator.
2. Concentrated form: to be diluted by the investigator.
3. Working solution: to be used directly without further manipulation.

Powdered medium is the least expensive but needs to be sterilized. It is advisable to filter-sterilize it prior to the addition of serum as the foaming that occurs in the presence of serum denatures the protein. Fetal bovine or horse sera can be added after filtration. Media should always be tested for sterility by placing it in a $37^{o}C$ CO_2 incubator for 72 hours prior to utilization to ensure that the lot is contamination-free. Medium should be stored at $4^{o}C$. Since several components of the medium are light-sensitive, it should be stored in the dark.

Criteria for Selecting Media

Cell lines

The choice of cell culture media is extremely important, and significantly affects the success of cell culture experiments. The selection of the media depends on the type of cells to be cultured and also the purpose of the culture and resources available in the laboratory. Different cell types have highly specific growth requirements, therefore, the most suitable media for each cell type must be determined experimentally. In general, it's always good to start with MEM for adherent cells and RPMI-1640 for suspension cells. Table 3 describes commonly studied cell lines and recommended growth media.

Cell Line	Morphology	Species	Medium	Applications

HeLa B	Epithelial	Human	MEM+ 2mM Glutamine+ 10% FBS + 1% Non Essential Amino Acids (NEAA)	Tumourigenicity and virus studies
HL60	Lymphoblast	Human	RPMI 1640 + 2mM Glutamine + 10-20% FBS	Differentiation studies
3T3 clone A31	Fibroblast	Mouse	DMEM + 2mM Glutamine +5% New Born Calf Serum (NBCS) + 5% FBS	Tumourigenicity and virus studies
COS-7	Fibroblast	Monkey	DMEM+ 2mM Glutamine + 10% FBS	Gene expression and virus replication studies
CHO	Epithelial	Hamster	Ham′s F12 + 2mM Glutamine + 10% FBS	Nutritional and gene expression studies
HEK 293	Epithelial	Human	EMEM (EBSS) + 2mM Glutamine + 1% Non Essential Amino Acids (NEAA) + 10% FBS	Transformation studies
HUVEC	Endothelial	Human	F-12 K + 10% FBS + 100 µg/ml Heparin	Angiogenesis studies
Jurkat	Lymphoblast	Human	RPMI-1640 + 10% FBS	Signaling studies

Table 3. Common cell lines and recommended growth media

Primary cell culture

Primary cell culture provides unique and valuable research data, but most of the time cell number is the limitation. For such critical samples, especially from diseased human biopsies, a quality medium is required. Most of the life sciences companies are providing complete and ready to use, fully supplemented conditioned medium. This reduces the risk of contamination as well as save time, labor and money by eliminating the preparation steps and supplementation required. Moreover, all of these media are subjected to comprehensive quality control tests and each lot is routinely tested for growth promotion, the absence of cytotoxicity, and physical parameters such as osmolality and pH level. Table 4 describes the recommended media provided by different companies for commonly used primary cells.

Cells	Media
Endothelial cells	Human Endothelial-SFM (Life Technologies), Endothelial Basal Media, EndoGRO-LS Complete Media Kit (MilliporeSigma), HUVEC Basal Medium CB HUVEC (AllCells), Endothelial Cell Medium (ScienCell)
Bone marrow cells	MarrowMAX Bone Marrow Medium (Life Technologies), Bone Marrow Medium Plus (MilliporeSigma)
Glial cells	GIBCO® Astrocyte Medium
Epithelial cells	Epithelial cell medium (ScienCelly), EpiGRO primary epithelial cells (MilliporeSigma)
T cells	Human StemXVivo Serum-Free T cell Base Media (R&D Systems), Stemline T cell Expansion Medium (MilliporeSigma)
Hematopoietic stem cells	StemPro-34 SFM (Life Technologies), MethoCult (STEMCELL Technologies, Inc)

Table 4. Recommended media for common primary cells

Common cell culture media

Most commonly used culture media include the following.
Dulbecco's Modified Eagle's Medium (DMEM)

DMEM has almost twice the concentration of amino acids and four times the amount of vitamins as EMEM, as well as ferric nitrate, sodium pyruvate, and some supplementary amino acids. The original formulation contained 1,000 mg/L of glucose and was first reported for culturing embryonic mouse cells. A further variation with 4500 mg/L of glucose has been proved to be optimal for the culture of various types of cells. DMEM is a basal medium and contains no proteins or growth promoting agents. Therefore, it requires supplementation to be a "complete" medium. It is most commonly supplemented with 5-10% Fetal Bovine Serum (FBS). DMEM utilizes a sodium bicarbonate buffer system (3.7 g/L) and therefore requires artificial levels of CO_2 to maintain the required pH. Powdered media is formulated without sodium bicarbonate because it tends to gas off in the powdered state. Powdered media requires the addition of 3.7 g/L of sodium bicarbonate upon dissolving it in water. DMEM was used initially for the culture of mouse embryonic stem cells. It has been found to be widely applicable in primary mouse and

chicken cells, viral plaque formation and contact inhibition studies. It can also be used to culture hybridomas [43].

RPMI-1640

RPMI-1640 is a general purpose media with a broad range of applications for mammalian cells, especially hematopoietic cells. RPMI-1640 was developed at Roswell Park Memorial Institute (RPMI) in Buffalo, New York. RPMI-1640 is a modification of McCoy's 5A and was developed for the long-term culture of peripheral blood lymphocytes. RPMI-1640 uses a bicarbonate buffering system and differs from the most mammalian cell culture media in its typical pH 8 formulation. RPMI-1640 supports the growth of a wide variety of cells in suspension and grown as monolayers. If properly supplemented with serum or an adequate serum replacement, RPMI-1640 has a wide range of applications for mammalian cells, including the culture of fresh human lymphocytes, fusion protocols and growth of hybrid cells like mouse hybridoma cells for antibody preparations.

Eagle's Minimum Essential Medium (EMEM)

EMEM was among the first widely used media and was formulated by Harry Eagle from a simpler basal medium (BME). EMEM contains balanced salt solution, nonessential amino acids, and sodium pyruvate. It is formulated with a reduced sodium bicarbonate concentration (1500 mg/l) for use with 5% CO_2. Since EMEM is a non-complex medium, it is generally fortified with additional supplements or higher levels of serum making it suitable for a wide range of mammalian cells. Huang H et al, for example, maintained HepG2 (HB-8065)in EMEM medium from ATCC. EMEM was used to maintain HeLa cells.

Ham's nutrient mixtures

These were originally developed to support the clonal outgrowth of Chinese hamster ovary (CHO) cells. There have been numerous modifications to the original formulation including Hams's F-12 medium, a more complex formulation than the original F-10 suitable for serum-free propagation. Mixtures were formulated for use with or without serum supplementation, depending on the type of cells being cultured.

Ham's F-10: It has been shown to support the growth of human diploid cells, for example, human fibroblast cells, and white blood cells for chromosomal analysis.

Ham's F-12: It has been shown to support the growth of primary rat hepatocytes and rat prostate epithelial cells. Ham's F-12 supplemented with 25 mM HEPES provides more optimum buffering.

Coon's modification of Ham's F-12: It consists of almost two times the amount of amino acids and pyruvate as compared to F-12 and also includes ascorbic acid. It was developed for culturing hybrid cells produced by viral fusion.

DMEM/F12: It is a mixture of DMEM and Ham's F-12 and is an extremely rich and complex medium. It supports the growth of a broad range of cell types in both serum and serum-free formulations. HEPES buffer is included in the formulation at a final concentration of 15 mM to compensate for the loss of buffering capacity incurred by eliminating serum.

Iscove's Modified Dulbecco's Medium (IMDM)

IMDM is a highly enriched synthetic media well suited for rapidly proliferating, high-density cell cultures. IMDM is a modification of DMEM containing selenium, and has additional amino acids, vitamins and inorganic salts as compared to DMEM. It has potassium nitrate instead of ferric nitrate and also contains HEPES and sodium pyruvate. It was formulated for the growth of lymphocytes and hybridomas. Studies have demonstrated that IMDM can support murine B lymphocytes, hemopoietic tissue from bone marrow, B cells stimulated with lipopolysaccharide, T lymphocytes, and a variety of hybrid cells. Meertens L et al, for example, maintained HAP1 cells from Horizon Discovery, a cell line derived from near-haploid chronic myeloid leukaemia KBM7 cells, in IMDM medica.
Table 5 describes different cells/cell lines which can be cultured using above mentioned media:

Media	Tissue or cell line
MEM	Chick embryo fibroblast, CHO cells, embryonic nerve cells, alveolar type cells, endothelium, epidermis, fibroblast, glia, glioma, human tumors, melanoma
DMEM	Endothelium, fetal alveolar epithelial type II cells, cervix epithelium, gastrointestinal cells, mouse neuroblastoma, porcine cells from thyroid glands, ovarian carcinoma cell lines, skeleton muscle cells, Sertoli cells, Syrian hamster fibroblast
RPMI-1640	T cells and thymocytes, hematopoietic stem cells, human tumors, human myeloid leukemia cell lines, human lymphoblastoid leukemia cell lines, mouse myeloma, mouse leukemia, mouse erythroleukemia, mouse hybridoma, rat liver cells
Nutrient mixture F-10 and F-12	Chick embryo pigmented retina, bone, cartilage, adipose tissue, embryonic lung cells, skeletal muscle cells
IMDM	Bone marrow, hematopoietic progenitor cells, human lymphoblastoid leukemia cell lines

Table 5. Common media and their applications

Optimization of Cell Culture Media

The complexity of the composition of cell culture media provides many challenges to optimize individual components of media. Most of the classical culture media were devised for small-

scale low-density cultures and often require serum as a key nutrient. However, in the biotechnology industry where there is a need to sustain high cell densities and increase cellular productivity, development and optimization of culture media is very critical. Typically, media for the biotechnology industry are serum-free and have a much higher concentration of nutrients than classical media. Optimization of media requires the following parameters to be considered:

Product to be made

The type of product needed will determine the medium optimization strategy.

For the rapid generation of cell numbers, cell growth rate and viability are critical. So, cell culture media should support maximal cell growth and sustain cell viability at increasing cell densities.

For the production of virus, not just high cell densities are required but there must be abundant nutrients in the media to sustain virus replication after infection, for example, DMEM High Glucose H-21 for Lenti-X 293T cells.

For the production of recombinant protein, high cell density is required. However, nutrients required for the cell growth can compete with those required for the production of proteins. It is, therefore, very important to carefully determine the maximum cell densities a given medium can sustain for a required level of productivity. In addition, it is very important to consider that changes to the medium during optimization must not affect product quality.

Cell lines to be used

Different cell lines have different nutritional requirements because of the difference in metabolism which dictates media optimization methods. The most common cell lines used in the biotechnology industries are CHO cells, BHK-21, hybridoma cells, myeloma cells, and normal diploid fibroblasts. Certain cell lines have specific nutritional requirements, such as cholesterol for NS0 myeloma cells. Normal diploid fibroblasts require attachment factors to adhere and spread out on a surface for growth. They grow too much lower densities and therefore do not need nutrients in high quantities. Hybridoma cells lines are generally highly dependent on glutamine. They typically lack a stationary phase after reaching a peak cell density and then decline rapidly in viability. Optimization of medium, thus, would reduce the decline in viability and improve monoclonal antibody production.

Manufacturing process involved

Manufacturing process mode would not only affect the choice of cell culture medium but also approaches to optimization. Different manufacturing processes used are:

Batch Process: A single medium is used to sustain cell growth and productivity. Medium should therefore be rich in nutrients but remain in physiological limits of the cells.

Fed-batch: Several kinds of media are used over the course of the cell culture, depending on the stage of the process. A growth medium is designed in such a way that it has lower nutrient concentrations when cell densities are low during inoculation but maintain high rates of cell growth during culture scale-up and early production. A separate production medium which has increased nutrient concentrations can also be used when the culture reaches the production stage.

Growth Supplements:

Cell Culture Supplements

Cell cultures supplements are required for optimal cell expansion. Cultured cells do not grow properly without these definite components added to media. Amino acids, salts, and albumin are among the assorted components added to culture media. L-glutamine and B28 are used in neural cultures for most select survival. Specialty supplement mixtures are available for specific types of cells, such as hES Cell Cloning & Recovery Supplement for human embryonic stem cells. Astrocytes, microglia, dermal cells and keratinocytes have distinct supplement requirements. Over 400 cell culture supplements can be compared on our site for the most suitable components for your cell type.

Nutrient requirements

Sufficient amounts of nutrients in the medium are a prerequisite for cells to behave properly. Some cell types require higher levels of nutrients than others do, depending on their metabolic activity and proliferation rate. Such characteristics of cells should be taken into account in the selection of a medium. DMEM, for example, was designed originally to contain glucose at 5.6 mmol L^{-1}. Now, a modified version of DMEM with a high glucose concentration, 25 mmol L^{-1}, which can be used for cells requiring greater amounts of glucose, is available from various suppliers. One caveat when using this high-glucose medium for actively proliferating cells is the accumulation of metabolites, like lactate, and a plunge in the pH. Researchers are advised to replace the medium at proper intervals or to use HEPES to confer a stronger pH-buffering capacity onto the medium.

Glutamine, one of the essential amino acids, is an energy source for mammalian cells in culture, in addition to being a biosynthetic material for nucleic acids and proteins. The glutamine requirements for cells in culture are ~3-40-fold greater than those of other amino acids.[40] During the cultivation of cells with high nutrient requirements, the addition of glutamine could be helpful. In contrast, glutamine readily decomposes in culture media and generates cytotoxic ammonia. Consequently, researchers may consider adding glutamine to the medium immediately before use or to consider using glutamine derivatives, such as L-alanyl-L-glutamine or glycyl-L-glutamine, which are resistant to degradation.

Non-essential amino acids (NEAAs) can be biosynthesized in the cell. Thus, NEAAs are not included in some basal media, such as BME and MEM. Nevertheless, not every cell type can produce sufficient amounts of NEAAs in the cultured state. The addition of NEAAs to the

medium, therefore, could ensure more favorable culture conditions. Even cells that can biosynthesize NEAAs at a necessary rate might benefit from the addition of NEAAs to the medium because this action alleviates the biosynthetic load of the cells.

Branched-chain amino acids (BCAAs), including valine, leucine, and isoleucine, require special attention. They all belong to the group of essential amino acids and several cell types, including human fibroblasts and mouse myeloma cells, require larger amounts of BCAAs. For those cells, increased concentrations of BCAAs or replacing the medium at proper intervals can be considered in order to obtain the best results of an experiment.

SERUM FREE MEDIA:

To grow cells *in vitro* culture media must mimic *in vivo* conditions. This is commonly achieved by adding serum to a classical medium (DMEM, RPMI etc) at concentrations of 5 to 10%.
Growing concern over the poorly defined nature and variability of serum has led to the development of serum-free media and serum substitutes. Below you can read why we think it's a good idea to replace your current serum-containing media by serum-free alternatives.

Consistent, defined composition

Serum is a poorly defined culture medium component as its composition can vary considerably. Even though serum consistency has improved over the years, and new lots can be tested, the physiological variance can be quite high, leading to poor reproducibility or misinterpretation of results.

The composition of serum-free medium on the other hand is exactly known and the level of each component precisely defined. This ensures lot-to-lot consistency and eliminates the screening of new serum lots.

Lower risk of infectious agents

Contamination of serum with viruses, bacteria, fungi or BSE has been a concern for many years. For biopharmaceutical manufacturers this has led to the elimination of all animal derived components from the manufacturing process.

For research, serum-free media also offer the benefit of eliminating the risk of infectious agents while supporting the growth of a wide range of cell types.

Lower risk of interfering components

Serum components may also bind, degrade or otherwise interact with substances added to the medium. This may influence the observed effect and lead to false conclusions.

Serum-free medium has fewer possible interfering factors and if an interaction is observed, it can be more easily controlled.

Less contaminants

The concentration of serum proteins is generally much higher than the level of a recombinant protein produced by mammalian cells. Serum proteins are thus a major contaminant of the target protein. And if the target protein is related to a serum protein, it may be difficult to separate the two.

In serum-free media the protein concentration is low and, unlike serum, the composition is known. As a result, the target protein is easier to purify in fewer steps and with higher recovery.

Avoids ethical issues

Based on what is known about the harvesting process of fetal calf serum it can be said that animal welfare is better served by serum-free alternatives.

Drawbacks

Despite the major benefits of serum-free media described above, there are a few drawbacks that should be considered. First, it takes time to adopt a particular cell line to serum-free medium. The cells will have to be weaned from serum gradually and some cell lines may require the addition of growth factors.

Second, serum-free medium does not contain sufficient protein to protect cells against shear. The

addition of Pluronic F68 or PEG or alternative supplements may be required.

Finally, adherent cell lines require an extracellular matrix on the culture surface, part of which is provided by serum. Therefore, when using serum-free medium the culture plates should be pre-coated with a fibronectin, laminin or another suitable alternative.

Conclusion

Serum-free cell culture media are an excellent alternative to standard serum-containing media and offer several major advantages. The commercial availability of optimized serum-free medium and serum substitutes has made it easy to obtain and use. As a result serum-free media deserve serious consideration for the culture of a wide variety of cell types.

Balanced Salt Solution

Balanced salt solutions are used in life science for research, diagnostic and manufacturing applications. The various solutions include salt solutions, enzyme solutions, antibiotic solutions and concentrated nutrient supplements etc. Auxiliary balanced salt solutions are processed according to a Product Master Record (PMR). The master record defines the procedures for production, process control, quality control and final product release. Selected batches are produced and sterilized by filtration or steam sterilization under aseptic conditions. The sterile solution is aseptically dispensed into sterile containers and immediately stored as required.

Tissue Culture

Types, Techniques and Process

What is Tissue Culture?

In biological research, tissue culture refers to a method in which fragments of a tissue (plant or animal tissue) are introduced into a new, artificial environment, where they continue to function or grow. While fragments of a tissue are often used, it is important to note that entire organs are

also used for tissue culture purposes. Here, such growth media as broth and agar are used to facilitate the process.

* While the term tissue culture may be used for both plant and animal tissues, plant tissue culture is the more specific term used for the culture of plant tissues in tissue culture.

Types of Tissue Culture

Seed Culture

Seed culture is the type of tissue culture that is primarily used for plants such as orchids. For this method, explants (tissue from the plant) are obtained from an in-vitro derived plant and introduced in to an artificial environment, where they get to proliferate. In the event that a plant material is used directly for this process, then it has to be sterilized to prevent tissue damage and ensure optimum regeneration.

Embryo Culture

Embryo culture is the type of tissue culture that involves the isolation of an embryo from a given organism for in vitro growth.

*Note, the term embryo culture is used to refer to sexually produced zygotic embryo culture.

Embryo culture may involve the use of a mature of immature embryo. Whereas mature embryos for culture are essentially obtained from ripe seeds, immature embryo (embryo rescue) involves the use of immature embryos from unripe/hybrid seeds that failed to germinate. In doing so, the embryo is ultimately able to produce a viable plant.

For embryo culture, the ovule, seed or fruit from which the embryo is to be obtained is sterilized, and therefore the embryo does not have to be sterilized again. Salt sucrose may be used to provide the embryo with nutrients. The culture is enriched with organic or inorganic compounds, inorganic salts as well as growth regulators.

Tissue Culture By Linda Bartlett (Photographer) [Public domain or Public domain], via Wikimedia Commons

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Callus Culture

*Callus - This is the term used to refer to unspecialized, unorganized and a dividing mass of cells. A callus is produced when explants (cells) are cultured in an appropriate medium - A good example of this is the tumor tissue that grows out of the wounds of differentiated tissues/organs.

In practice, callus culture involves the growth of a callus (composed of differentiated and non-differentiated cells), which is the followed by a procedure that induces organ differentiation.

For this type of tissue culture, the culture is often sustained on a gel medium, which is composed of agar and a mixture of given macro and micronutrients depending on the type of cells. Different types of basal salt mixtures such as murashige and skoog medium are also used in addition to vitamins to enhance growth.

Callus Culture By Igge (Own work) [GFDL (http://www.gnu.org/copyleft/fdl.html)

Organ Culture

Organ culture is a type of tissue culture that involves isolating an organ for in vitro growth. Here, any organ plant can be used as an explant for the culture process (Shoot, root, leaf, and flower).

With organ culture, or as is with their various tissue components, the method is used for preserve their structure or functions, which allows the organ to still resemble and retain the characteristics they would have in vivo. Here, new growth (differentiated structures) continues given that the organ retains its physiological features. As such, an organ helps provide information on patterns of growth, differentiation as well as development.

There are number of methods that can be used for organ culture. These include;

- Plasma clot method - Here, the method involves the use of a clot that is composed of plasma and chick embryo extract (or any other extract) in a watch glass. This method is particularly used for the purposes of studying morphogenesis in embryonic organ rudiments and more recently for studying the actions of various hormones, vitamins and carcinogens of adult mammalian tissues.
- Raft method - For this method, the explant is placed on a raft of lens paper/rayon acetate and floated on a serum in a watch glass.
- Agar gel method - The medium used for this method is composed of a salt solution, serum as well as the embryo extract or a mixture of various amino acids and vitamin with 1 percent agar. The explant has to be subcultured every 5 to 7 days. The method is largely used for the study of developmental aspects of normal organs and tumors.

- Grid method - Grid method, as the name suggests involves the use of perforated stainless steel sheet, on which the tissue of interest is placed before being placed in a culture chamber containing fluid medium.

Protoplast Culture

*Protoplast -cells without cell walls. A protoplast is the term used to refer to cell (fungi, bacteria, plant cells etc) in which the cell wall has been removed, which is why they are also referred to as naked cells.

Protoplasts may be cultured in the following ways;

- Hanging-drop cultures
- Micro culture chambers
- Soft agars matrix

Once a protoplast has regenerated a cell wall, then it goes through the process of cell division to form a callus, which may then be subcultured for continued growth.

Protoplast culture is an important method that provides numerous cells (single cells) that can be used for various studies. These include;

- Protoplast culture regenerated into a whole plant
- Development of hybrids
- Cell cloning
- Genetic transformations
- Membrane studies

In protoplast culture, a number of phases can be observed. These include;

- Development of a cell wall
- Cell division
- Continuous growth or regeneration to a whole plant

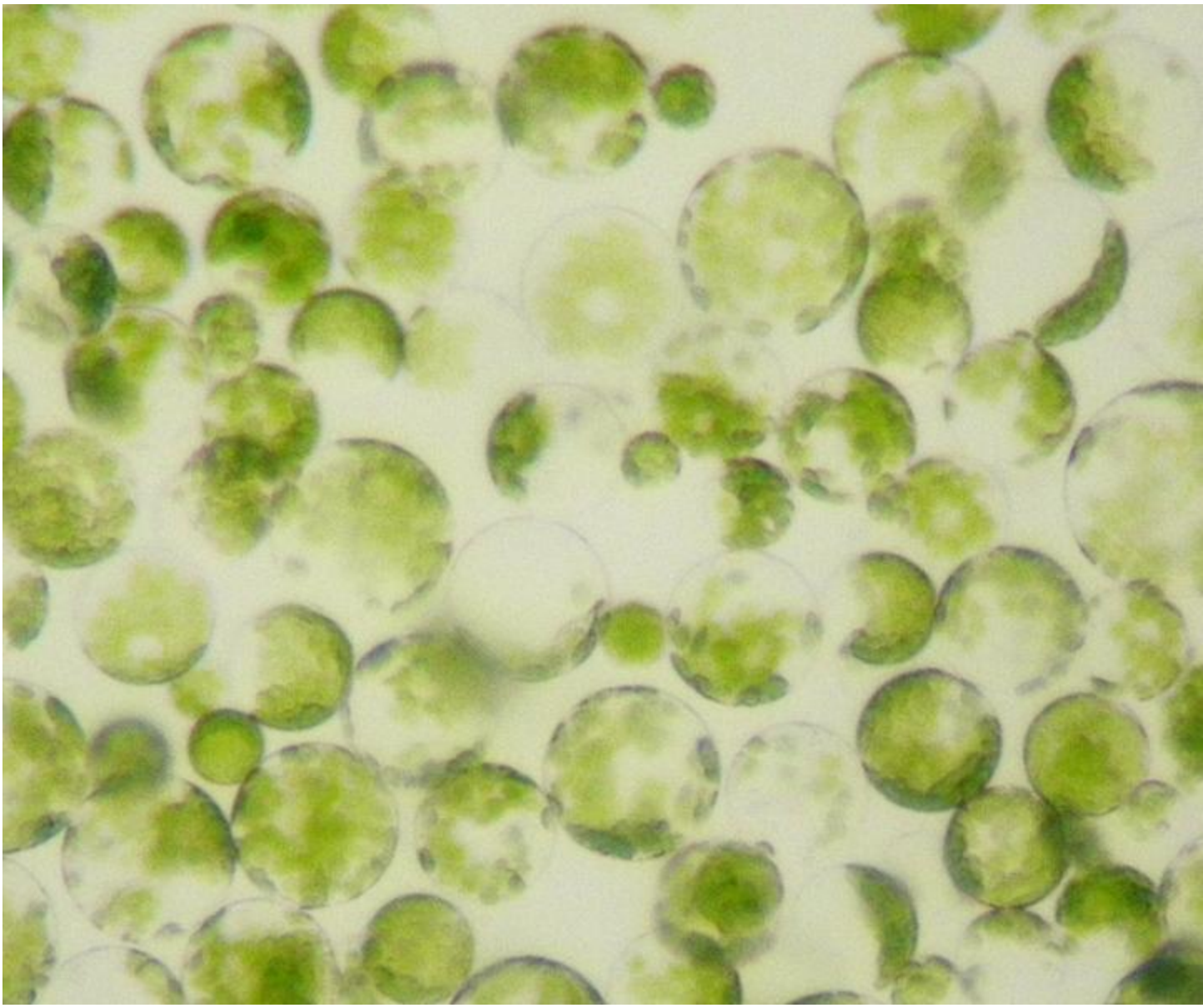
For plants, some of the special requirements include;

- Less amounts of iron and zinc and no ammonium
- Higher concentration of calcium
- High auxin/kinetic ratio for cell division and high kinetin/auxin ration for regeneration
- Glucose and vitamins

Some of the other types of tissue culture include;

- Single cell culture
- Suspension culture
- Anther culture
- Pollen culture

- Somatic Embryogenesis



Protoplast Solution, Cells of Petunia leaves By Mnolf (Own work) [CC BY-SA 3.0 (http://creativecommons.org/licenses/by-sa/3.0)

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Major Steps of Tissue Culture (Plants)

Initiation Phase (Stage 1)

The initiation phase is the first phase of tissue culture. Here, the tissue of interest is obtained and introduced and sterilized in order to prevent any microorganism from negatively affecting the process. It is during this stage that the tissue is initiated in to culture.

Multiplication Phase (Stage 2)

The multiplication phase is the second step of tissue culture where the in vitro plant material is re- divided and then introduced in to the medium. Here, the medium is composed of appropriate components for growth including regulators and nutrients. These are responsible for the proliferation of the tissue and the production of multiple shoots.

*This step is often repeated several times in order to obtain the desired number of plants

Root formation (Stage 3)

It is at this phase that roots are formed. Here, hormones are required in order to induce rooting, and consequently complete plantlets.

Plant Tissue Culture

Tissue culture is applied in plant research for such purposes as the growing of new plants, which in some cases undergo genetic alterations. Here, the plant of interest is taken through the tissue culture process and grown in a controlled environment.

The Process of Plant Tissue Culture

This process involves the use of small pieces of a given plant tissue (plant of interest). Once the tissue is obtained, it is then cultured in the appropriate medium under sterile conditions so as to prevent various types of microorganisms from affecting the process.

The following is a general procedure for plant tissue culture

Medium preparation

- The appropriate mixture (such as the MS mixture) is mixed with distilled water and stirred while adding the appropriate amount of sugar and sugar mixture. Here, sodium hydroxide or hydrochloric acid is used to adjust the pH - Contents used here will depend on the plant to be cultured and the number of tissues to be cultured.
- Agar is added to the mixture, heat and stirred to dissolve
- After cooling, the warm medium is poured into polycarbonate tubes (to a depth of about 4 cm)

- With lids sitting on the tubes, the tubes are placed in a pressure cooker and sterilized for 20 minutes

Plant preparation

- Cut the plant part in to small pieces (e.g. cauliflower can be cut to florets of about 1cm across). On the other hand, such parts as the African violet leaves can be used as a whole.
- Using detergent and water, wash the plant part for about 20 minutes
- Transfer the plant part in to sterilizing Clorox solution, shake for a minute and leave to sock for 20 minutes
- Using a lid, gently discard the Clorox and retain the plant part in the container and then cap the container

Transferring the plant material to a tissue culture medium

* 70 percent alcohol should be used for the sterilization of the equipment used and containers

- Open the container and pour sterile water to cover half the container
- Cover with a sterile lid again and shake the container for 2 to 3 minutes in order to wash the tissue and remove the bleach
- Pour the water and repeat this three times
- Using sterilized gloves, remove the plant part from the container and on to a sterile Petri dish
- Using a sterile blade cut the plant material to smaller pieces of about 2 to 3 mm across avoiding the parts that have been damaged by bleach
- Using sterile forceps, place a section of the plant in to the medium

Cauliflower - partly submerged in medium with flower bud facing up

Rose with shoots at level with medium surface

African violet leaf laid directly in surface of medium

*depending on the plant used, it is important to check and find out how it should be placed in the medium

Replace the lid/cap and close tightly

This procedure will result in the development of a callus, which then produces shoots after a few weeks. Once the shoots develop, then the plant section may be placed in the right environment (well lit, warmth etc) for further growth.

* Plant materials should be sterilized so as to remove any bacteria or spores that may be present.

For plants, the medium culture acts as a greenhouse that provides the explant with the idea environment for optimum growth. This includes being free of microorganisms, nutrients as well

as the right balance of chemicals and hormones. Such media as BAP, TDZ are used while such hormones as IBA and IAA are used to induce growth. Some of the major reasons tissue culture is used for plants include;

- To produce large quantities of a given plant
- To accelerate the production of new varieties of a plant
- To maintain a virus free stock of the plant of interest

Technique for Plant In Vitro Culture

Micropropagation - This technique is used for the purposes of developing high- quality clonal plants (a clone is a group of identical cells). This has the potential to provide rapid and large scale propagation of new genotypes.

Somatic cell genetics - Used for haploid production and somatic hybridization

Transgenic plants - Used for expression of mammalian genes or plant genes for various species it has proved beneficial for the engineering of species that are resistant against viruses and insects.

Conclusion

In reality, there are numerous methods used for tissue culture given that there are different types of tissues that require specific conditions for the culture process yield desired results. Both plant and animal tissue can be used for tissue culture purposes for a wide range of purposes. For instance, animal tissue culture may serve such purposes as preservation of an organ/tissue, studying the tutors or given tissues or for diagnosis purposes.

On the other hand, plant tissue culture may be used for cloning purposes, genetic modification of a given plant or simply to accelerate or increase yield of the plant of interest.

Tissue culture is therefore of great significance in biological studies due to its wide range of applications. The processes involved in tissue culture may be complex, requiring a lot of care to avoid such effects as contamination. Because of the complexities that may be involved in some of the steps, this may not be an experiment for everyone.

Here, learn more about Cell Culture, Cell Division, Cell Differentiation and Cell Staining as well as Gram Stain. And check out information on Cell Theory.

Animal Cell Culture: Fundamentals, Facilities, Advantages and Everything Else

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Read this article to learn about the fundamentals, facilities, aseptic conditions, advantages, disadvantages of animal cell culture and also about the risks & safety regulations in a tissue culture laboratory.

Introduction to Animal Cell Culture :

Animal cell culture basically involves the in vitro (in the laboratory) maintenance and propagation of animal cells in a suitable nutrient media. Thus, culturing is a process of growing cells artificially. Cell culture has become an indispensible technology in various branches of life sciences.

Historical Background:

It was in 1907, Ross Harrison first developed a frog tissue culture technique. He probably chose frog for two reasons—being a cold-blooded animal, no incubation is required and tissue regeneration is fast in frog. In 1940's chick embryo tissue became a favorite for culture techniques.

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Interest in culturing human tissues started in 1950's after it was demonstrated (by HeLa; Gey) that human tumor cells could give rise to continuous cell lines. Among the various animal cell cultures, mouse cell cultures are the most commonly used in the laboratory.

Terminology in Cell Culture:

The term tissue culture is commonly used to include both organ culture and cell culture.

Organ culture:

The culture of native tissue (i.e. un-disaggregated tissue) that retains most of the in vivo histological features is regarded as organ culture.

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Cell culture:
This refers to the culture of dispersed (or disaggregated) cells obtained from the original tissue, or from a cell line.

Histotypic culture:
The culturing of the cells for their re-aggregation to form a tissue—like structure represents histotypic culture.

Organotypic culture:
This culture technique involves the recombination of different cell types to form a more defined tissue or an organ.

Primary culture:
The culture produced by the freshly isolated cells or tissues taken from an organism is the primary culture. These cell are heterogenous and slow growing, and represent the tissue of their origin with regard to their properties.

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Cell line:
The sub-culturing of the primary culture gives rise to cell lines. The term continuous cell lines implies the indefinite growth of the cells in the subsequent sub-culturing. On the other hand, finite cell lines represent the death of cells after several subcultures.

Facilities for Animal Cell Culture:
While designing the laboratory for animal cell culture technology, utmost care should be taken with regard to the maintenance of aseptic conditions. The facilities required with regard to infrastructure and equipment are listed below :

Minimal Requirements for Cell Culture:

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i. Clean and quite sterile area

ii. Preparation facilities

iii. Animal house

iv. Microbiology laboratory

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v. Storage facilities (for glassware, chemicals, liquids, small equipment).

Equipment:

Laminar-flow, sterilizer, incubator, refrigerator and freezer (-20°C), balance, $C0_2$ cylinder, centrifuge, inverted microscope, water purifier, hemocytometer, liquid nitrogen freezer, slow-cooling device (for freezing cells), pipette washer, deep washing sink.

Besides the basic and minimal requirements listed above, there are many more facilities that may be beneficial or useful for tissue cultures. These include air-conditioned rooms, containment room for biohazard work, phase-contrast microscope, fluorescence microscope, confocal microscope, osmometer, and high capacity centrifuge and time lapse video equipment.

Culture Vessels:

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In the tissue culture technology, the cells attach to the surface of a vessel which serves as the substrate, and grow. Hence there is a lot of importance attached to the nature of the materials used and the quality of the culture vessels. The term anchorage dependent cells is used when the cells require an attachment for their growth. On the other hand, some cells undergo transformation, and become anchorage independent.

Materials used for culture vessels:

Glass:

Although glass was the original substrate used for culturing, its use is almost discontinued now. This is mainly because of the availability of more suitable and alternate substrates.

Disposable plastics:

Synthetic plastic materials with good consistency and optical properties are now in use to provide uniform and reproducible cultures. The most commonly used plastics are polystyrene, polyvinyl chloride (PVC), polycarbonate, metinex and thermonex (TPX).

Types of culture vessels:

The following are the common types of culture vessels.

i. Multiwall plates

ii. Petridishes

iii. Flasks

iv. Stirrer bottles.

The actual choice of selecting a culture vessel depends on several factors:

1. The way cells grow in culture—monolayer or suspension.

2. The quantity of the cells required.

3. The frequency of sampling for the desired work.

4. The purpose for which the cells are grown.

5. The cost factor.

In general, for monolayer cultures, the cell yield is almost proportional to the surface area of the culture vessel. The flasks are usually employed for this purpose. Any type of culture vessel can be used to grow suspension cultures. It is necessary to slowly and continuously agitate the suspended cells in the vessel.

Treatment of culture vessel surfaces:

For improving the attachment of cells to the surfaces, and for efficient growth, some devices have been developed. It is a common observation that the growth of the culture cells is better on the surfaces for second seeding. This is attributed to matrix coating of the surfaces due to the accumulation of certain compounds like collagen and fibronectin released by the cells of the previous culture. There are now commercially available matrices (e.g. matrigel, pronectin, and cell-tak).

Feeder layers:

Some of the tissue cultures require the support of metabolic products from living cells e.g. mouse embryo fibroblasts. In this case, the growing fibroblasts release certain products which when fed to new cells enhance their growth.

Alternate substrates as substitutes of culture vessels:

In recent years, certain alternatives for culture vessels have been developed. The important alternative artificial substrates are micro carriers and metallic substrates.

Micro-carriers:

They are in bead form and are made up of collagen, gelatin, polyacrylamide and polystyrene. Micro-carriers are mostly used for the propagation of anchorage-dependent cells in suspension.

Metallic substrates:

Certain types of cells could be successfully grown on some metallic surfaces or even on the stainless steel discs. For instance, fibroblasts were grown on palledium.

Use of Non-Adhesive Substrates in Tissue Culture:

The growth of anchorage independent cells can be carried out by plating cells on non-adhesive substrate like agar, agarose and methyl cellulose. In this situation, as the cell growth occurs, the parent and daughter cells get immobilized and form a colony, although they are non-adhesive.

Contamination, Aseptic Conditions, and Sterilization:

There are several routes of contamination in the tissue culture laboratory (Table 33.1). These include the various materials (glassware, pipettes), equipment (incubators, refrigerators, and laminar-flow hoods), reagents (media, solutions), contaminated cell lines and poor techniques.

Table 33.1 Major routes of contamination in a tissue culture laboratory

Equipment and facilities
Laminar-flow hoods
Dry incubators
CO_2 incubators
Humidified incubators
Wooden furniture, benches
Other instruments
Glassware and reagents
Pipettes
Screw caps
Culture glasses
Media bottles
Media and various solutions
Biological materials
Infected tissue samples
Cell lines
Operating techniques
Operator hands, hair, clothing, breathing
Work spaces
Pipetting, dispensing
Operating manipulations

The routes of contamination are mostly associated with the laboratory environment, and operating techniques.

Types of microbial contamination:

Several species of bacteria, yeasts, fungi, molds and mycoplasmas, besides viruses are responsible for contamination. Major problems of contamination are linked to the repeated recurrence of a single species. Despite utmost care taken, no laboratory can claim to be totally free from contamination. It is necessary to continuously monitor for contamination and eliminate the same at the earliest.

Aseptic Conditions:

Maintenance of proper aseptic conditions is necessary to eliminate various contaminants (due to different microorganisms and viruses). The following measures are suggested for minimizing contamination, and maintenance of aseptic conditions.

i. Strict adherence to standard sterile techniques and code of practices.

ii. Checking of reagents and media for sterility before use.

iii. Checking of cultures by eyes, and microscopes (phase contrast) every time they are used.

iv. Use of media and separate bottles for each cell line is advised.

v. Maintenance of clean and tidy conditions at work places.

vi. Personal hygiene of the staff is very important.

Sterilization:

The sterilization procedures are designed to kill the microorganisms, besides destroying the spores.

There are three major devices for sterilization:

1. Dry heat

2. Moist heat (autoclave)

3. Filters.

In the Table 33.2, the sterilization of major equipment, apparatus and liquids is given.

Table 33.2 Sterilization of major equipment, apparatus and liquids used in tissue culture

Sterilization device	*Items sterilized*
I *For equipment and apparatus*	
Dry heat	Glass slides
	Pipettes
	Ampoules (glass)
	Pasteur pipettes
	Instruments
	Test tubes
Autoclave	Ampoules (plastic)
	Apparatus with silicone tubing
	Filters (reusable)
	Glass bottles with screw caps
	Glass syringes
	Magnetic stirrer bases
	Screw caps
	Stoppers (rubber silicone)
II *For liquids and nutrients*	
Autoclave	Salt solutions
	Glucose–20%
	Agar
	Bacto–peptone
	Glycerol
	Lactalbumin hydrolysate
	Phenol red
	Tryptose
	HEPES
	EDTA
	Water
Filter	Serum
	Amino acids
	Vitamins
	Antibiotics
	Bovine serum albumin
	Collagenase
	Glutamine
	Drugs
	NaOH
	Trypsin
	Transferrin

Sterilization by dry heat:

This is carried out at a minimum temperature of 160°C for about one hour.

Sterilization by moist heat:

Certain fluids and perishable items can be sterilized in an autoclave at 121°C for 15-20 minutes. For effective moist heat sterilization, it is necessary that the steam penetrates to all the parts of the sterilizing materials.

Sterilization by filters:

The use of filters for sterilization of liquids often becomes necessary, since the constituents of these liquids may get destroyed at higher temperatures (dry heat or moist heat). Sterile filtration is a novel technique for heat- labile solutions. The size of micropores of the filters is 0.1-0.2 µm. Filters, made from several materials are in use. These materials include nylon, cellulose acetate, cellulose nitrate, polycarbonate, polyethersulfone (PES) and ceramics.

The filters are made in different designs-disc filters, cartridges and hollow fiber. In fact, many commercial companies (e.g. Millipore, Durapore) supply reusable and disposable filters, designed for different purposes of sterilization.

Advantages and Limitations of Tissue Culture:

Advantages of Tissue Culture:

Tissue culture technique has a wide range of applications.

The most important advantages of this technique are listed below:

1. Control of physicochemical environment- pH, temperature, dissolved gases (O_2 and CO_2), osmolarity.
2. Regulation of physiological conditions-nutrient concentration, cell to cell interactions, hormonal control.

3. The cultured cell lines become homogenous (i.e. cells are identical) after one or two subcultures. This is in contrast to the heterogenous cells of tissue samples. The homogenous cells are highly useful for a wide range of purposes.

4. It is easy to characterize cells for cytological and immunological studies.

5. Cultured cells can be stored in liquid nitrogen for several years.

6. Due to direct access and contact to the cells, biological studies can be carried out more conveniently. The main advantage is the low quantities of the reagents required in contrast to in

vivo studies where most of the reagents (more than 90% in some cases) are lost by distribution to various tissues, and excretion.

7. Utility of tissue cultures will drastically reduce the use of animals for various experiments.

Limitations of Tissue Culture:

There are several limitations of tissue culture; some of them are given below.

1. Need of expertise and technical skill for the development, and regular use of tissue culture.

2. Cost factor is a major limitation. Establishment of infrastructure, equipment and other facilities are expensive.

3. It is estimated that the cost of production of cells is about 10 times higher than direct use of animal tissues.

4. Control of the environmental factors (pH, temperature, dissolved gases, disposal of biohazards) is not easy.

5. The native in vivo cells exist in a three- dimensional geometry while in in vitro tissue culture, the propagation of cells occurs on a two dimensional substrate. Due to this, the cell to cell interactive characters are lost.

6. The cell lines may represent one or two types of cells from the native tissue while others may go unrepresented.

7. Tissue culture techniques are associated with the differentiation i.e. loss of the characters of the tissue cells from which they were originally isolated.

8. This happens due to adaptation and selection processes while culturing.

9. Continuous cell lines may result in genetic instability of the cells. This may ultimately lead to heterogeneity of cells.

10. The components of homeostatic in vivo regulation (nervous system, endocrine system, metabolic integration) are lacking in vitro cultures. Addition of hormones and growth factors has been started recently.

Risks in a Tissue Culture Laboratory and Safety:

There are several risks associated with tissue culture technology. Most of the accidents that occur in culture laboratories are due to negligence and casual approach while dealing with biological and radiological samples, besides improper maintenance of the laboratory. A broad categorization of risks and the contributory factors is given in Table 33.5.

TABLE 33.5 Risks in a tissue culture laboratory

Category	*Contributing factor(s)*
Maintenance risks	Age and condition of various equipment, leakage of disposals.
Personnel risks	Inadequate training, lack of concentration and interest.
Physical risks	Electric shocks, fire, intense cold.
Chemical risks	Toxicity due to poisons, carcinogens, mutagens, irritants, allergens.
Biohazards	Pathogenic organisms, viruses, genetic manipulations, culture cells and DNA (quality and quantity).
Radioisotope risks	Energy emission and its penetration, ionization.

Safety regulations:

Some of the developed countries have formulated general safety regulations to minimize the risks associated with tissue culture laboratories.

Selected examples:

1. "Biosafety in microbiological and biomedical laboratories", U. S. Department of Health and Human Sciences (1993).

2. "Safe working and the prevention of infection in clinical laboratories" U.K. Health Services Advisory Committee (1991).

Some of the general precautions for the safety of a tissue culture laboratory are listed here:

i. Strict adherence to recommendations of regulatory bodies.

ii. Periodical meetings and discussions of local safety committees.

iii. Regular monitoring of the laboratories.

iv. Periodical training of the personnel through seminars and workshops.

v. Print and make the standard operating procedures (SOPs) available to all staff.

vi. Good record keeping.

vii. Limited access to the laboratory (only for the trained personnel and selected visitors).

viii. Appropriate waste disposal system for biohazards, radioactive wastes, toxins and corrosives.

Biohazards:

The accidents or the risks associated with the biological materials are regarded as biohazards or biological hazards. There are two main systems that contribute to the occurrence of biohazards (Table 33.6).

TABLE 33.6 Sources that contribute to biohazards

Biological material(s)
Tissue samples and cultures with human pathogens.
Human cells infected with viruses (including retroviruses)
Cells subjected to various genetic manipulations.
Operating processes
Preparation of the media.
Development of primary cultures, cell lines and other laboratory works.

1. The direct sources of the biological materials.

2. The processes or operations involved in their handling.

Control of biohazards:

Biohazards can be controlled to a large extent by strict adherence to the regulatory guidelines and maintenance programmes. Some important aspects are listed.

i. Microbiological safety cabinet or biohazard wood with pathogen trap filters have been developed.

ii. Vertical laminar-flow hood (instead of horizontal laminar-flow hood) is recently in use. This minimizes the direct exposure of the operator to the samples/processes.

iii. Pathogen containing samples are treated in separate rooms with separate facilities (centrifuge, incubator, cell counting etc.).

iv. Sterilization of all wastes, solid glassware etc. and their proper disposal.

v. Facilities for change of clothing while entering and leaving the rooms.

vi. Strict adherence to the access of designated personnel to the culture rooms.

Related Articles:

1. Animal Tissue Culture in India: Laboratory and Facilities
2. Animal Cell Culture: General Considerations

Cultured Cells: Characteristics, Growth Parameters and Synchronization

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Read this article to learn about the characteristics, growth parameters and synchronization of cultured cells.

Characteristics of Cultured Cells:

Some of the important distinguishing properties of cultured cells are given below:

1. Cells which do not normally proliferate in vivo can be grown and proliferated in cultures.

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2. Cell to cell interactions in the cultured cells are very much low.

3. The three dimensional architecture of the in vivo cells is not found in cultured cells.

4. The hormonal and nutritional influence on the cultured cells differs from that on the in vivo cells.

5. Cultured cells cannot perform differentiated and specialized functions.

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6. The environment of the cultured cells favours proliferation and spreading of unspecialized cells.

Environmental influence on cultured cells:

The environmental factors strongly influence the cells in culture. The major routes through which environmental influence occurs are listed:

i. The nature of the substrate or phase in which cells grow. For monolayer cultures, the substrate is a solid (e.g. plastic) while for suspension cultures, it is a liquid.

ii. The composition of the medium used for culture nutrients and physicochemical properties.

iii. Addition of hormones and growth factors.

iv. The composition of the gas phase.

v. The temperature of culture incubation.

The biological and other aspects of cultured cells with special reference to the following parameters are briefly described:

1. Cell adhesion.

2. Cell proliferation.

3. Cell differentiation.

4. Metabolism of cultured cells.

5. Initiation of cell culture.

6. Evolution and development of cell lines.

Cell Adhesion:

Most of the cells obtained from solid tissues grow as adherent monolayers in cultures. The cells, derived from tissue aggregation or subculture, attach to the substrate and then start proliferating.

In the early days of culture techniques, slightly negatively charged glasses were used as substrates. In recent years, plastics such as polystyrene, after treatment with electric ion discharge, are in use.

The cell adhesion occurs through cell surface receptors for the molecules in the extracellular matrix. It appears that the cells secrete matrix proteins which spread on the substrate. Then the cells bind to matrix through receptors. It is a common observation that the substrates (glass or plastic) with previous cell culture are conditioned to provide better surface area for adhesion.

Cell adhesion molecules:

Three groups of proteins collectively referred to as cell adhesion molecules (CAMs) are involved in the cell-cell adhesion and cell-substrate adhesion.

Cell-cell adhesion molecules:

These proteins are primarily involved in cell-to-cell interaction between the homologous cells. CAMs are of two types — calcium-dependent ones (cadherin's) and calcium-independent CAMs.

Integrin's:

These molecules mediate the cell substrate interactions. Integrin's possess receptors for matrix molecules such as fibronectin and collagen.

Proteoglycans:

These are low affinity trans membrane receptors. Proteoglycans can bind to matrix collagen and growth factors. Cell adhesion molecules are attached to the cytoskeletons of the cultured cells.

Cell Proliferation:

Proliferation of cultured cells occurs through the cell cycle, which has four distinct phases (Fig. 35.1)

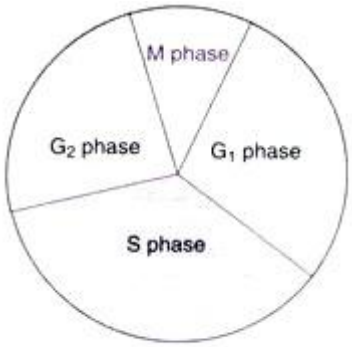


Fig. 35.1 : The cell cycle.

M phase:

In this phase (M = mitosis), the two chromatids, which constitute the chromosomes, segregate to daughter cells.

G_1 phase:

This gap 1 phase is highly susceptible to various control processes that determine whether cell should proceed towards DNA synthesis, re-enter the cycle or take the course towards differentiation.

S phase:

This phase is characterized by DNA synthesis wherein DNA replication occurs.

G_2 phase:

This is gap 2 phase that prepares the cell for reentry into mitosis. The integrity of the DNA, its repair or entry into apoptosis (programmed cell death) if repair is not possible is determined by two check points-at the beginning of DNA synthesis and in G_2 phase.

Control of cell proliferation:

For the cells in culture, the environmental signals regulate the cell cycle and thereby the cell proliferation. Low density of the cells in a medium coupled with the presence certain growth factors (e.g. epidermal growth factor, platelet-derived growth factor) allows the cells to enter the cell cycle.

On the other hand, high cell density and crowding of cells inhibits the cell cycle and thereby proliferation. Besides the influence of the environmental factors, certain intracellular factors also regulate the cell cycle. For instance, cyclins promote while p53 and Rb gene products inhibit cell cycle.

Cell Differentiation:

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

Among the factors that promote cell proliferation, the following are important:

i. Low cell density

ii. Low Ca^{2+} concentration

iii. Presence of growth factors

For the process of cell differentiation to occur, the proliferation of cells has to be severely limited or completely abolished.

Cell differentiation can be promoted (or induced) by the following factors:

i. High cell density.

ii. High Ca^{2+} concentration.

iii. Presence of differentiation inducers (e.g. hydrocortisone, nerve growth factor).

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

1. To optimize cell proliferation.

2. To optimize cell differentiation.

Maintenance of differentiation:

It is now recognized that the cells retain their native and original functions for long when their three dimensional structures are retained. This is possible with organ cultures. However, organ cultures cannot be propagated.

In recent years, some workers are trying to create three dimensional structures by per-fusing monolayer cultures. Further, in vitro culturing of cells on or in special matrices (e.g. cellulose, collagen gel, matrix of glycoproteins) also results in cells with three dimensional structures.

Dedifferentiation:

Dedifferentiation refers to the irreversible loss of specialized properties of cells when they are cultured in vitro. This happens when the differentiated in vitro cells lose their properties (Fig. 35.2). In the in vivo situation, a small group of stem cells give rise to progenitor cells that are capable of producing differentiated cell pool (Fig. 35.2A).

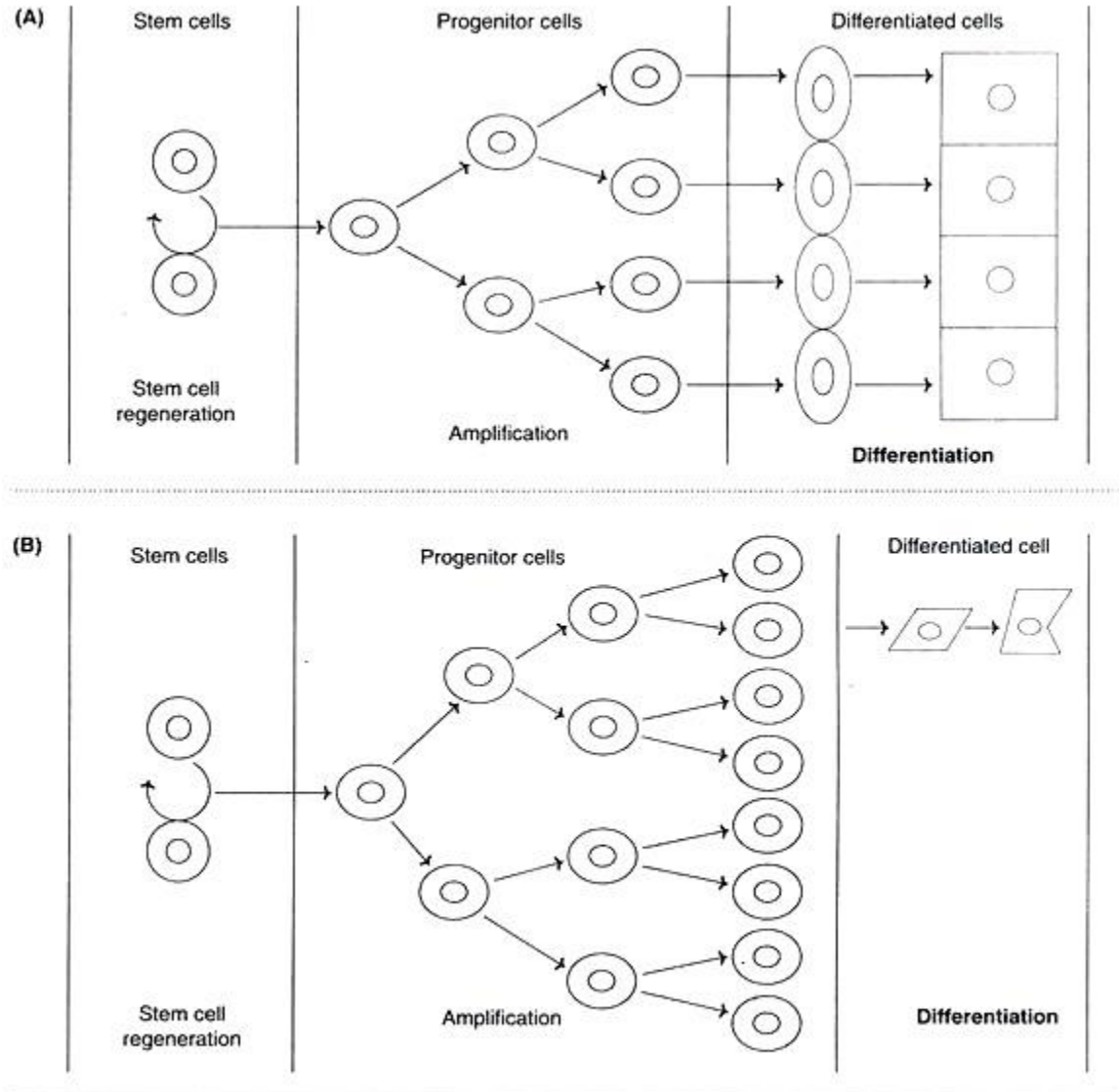


Fig. 35.2 : Differentiation of cells (A) In vivo differentiation of stem cells (B) Blocked differentiation in cultured cells.

On the other hand, in the in vitro culture system, progenitor cells are predominantly produced which go on proliferating. Very few of the newly formed cells can form differentiated cells (Fig. 35.2B). The net result is a blocked differentiation. Dedifferentiation implies an irreversible loss of specialized properties of the cells. On the other hand, de-adaptation refers to the re-induction of specialized properties of the cells by creating appropriate conditions.

Metabolism of Cultured Cells:

The metabolism of mammalian cultured cells with special reference to energy aspects is depicted in Fig. 35.3. The cultured cells can use glucose or glutamine as the source of energy. These two compounds also generate important anabolic precursors.

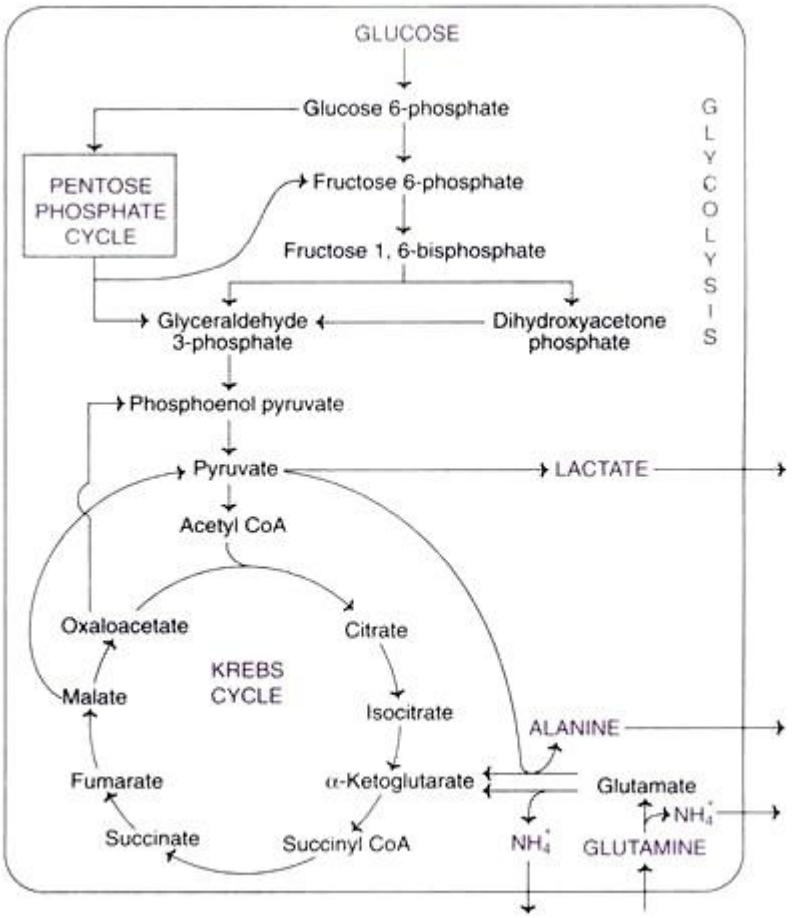


Fig. 35.3 : An outline of the glucose and glutamine metabolism in cultured mammalian cells.

As glucose gets degraded by glycolysis, lactate is mainly produced. This is because oxygen is in limited supply in the normal culture conditions (i.e. atmospheric oxygen and a submerged culture) creating an anaerobic situation. Lactate, secreted into the medium, accumulates.

Some amount of pyruvate produced in glycolysis gets oxidized through Krebs cycle. A small fraction of glucose (4-9%) enters pentose phosphate pathway to supply ribose 5-phosphate and reducing equivalents (NADPH) for biosynthetic pathways e.g. synthesis of nucleotides.

Glutamine is an important source of energy for the cultured cells. By the action of the enzyme glutaminase, glutamine undergoes deamination to produce glutamate and ammonium ions.

Glutamate, on transamination (or oxidative deamination) forms a-ketoglutarate which enters the Krebs cycle.

Pyruvate predominantly participates in transamination reaction to produce alanine, which is easily excreted into the medium. In the rapidly growing cultured cells, transamination reaction is a dominant route of glutamine metabolism.

Deamination of glutamine releases free ammonium ions, which are toxic to the cultured cells, limiting their growth. In recent years, dipeptides glutamyl-alanine or glutamyl-glycine are being used to minimize the production of ammonia. Further, these dipeptides are more stable in the medium.

As already stated, α-ketoglutarate obtained from glutamine (via glutamate) enters the Krebs cycle and gets oxidized to carbon dioxide and water. For proper operation of Kerbs cycle, balancing of the intermediates of the cycle is required.

Two metabolites of Kerbs cycle namely malate and oxaloacetate leave the cycle and get converted respectively to pyruvate and phosphoenol pyruvate. The latter two compounds can reenter the Krebs cycle in the form of acetyl CoA. Thus, the continuity of Kerbs cycle is maintained. Glucose as well as glutamine gets metabolised by the cultured cells to supply energy in the form of ATP.

Initiation of Cell Culture:

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

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

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

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

1. The closest morphological resemblance to the tissue of origin (i.e. parent tissue).

2. The expression of specialized functions of the cells comparable to that of the native cells.

Evolution and Development of Cell Lines:

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

Senescence:

The genetically determined event of cell divisions for a limited number of times (i.e. population doublings), followed by their death in a normal tissue is referred to as senescence. However, germ cells and transformed cells are capable of continuously proliferating. In the in vitro culture, transformed cells can give rise to continuous cell lines.

The evolution of a continuous cell line is depicted in Fig. 35.4. The cumulative cell number in a culture is represented on Y-axis on a log scale, while the X-axis represents the time in weeks. The time for development of a continuous cell line is variable. For instance, for human diploid fibroblasts, the continuous cell line arises at about 14 weeks while the senescence may occur between 10 to 20 weeks; usually after 30 and 60 cell doublings.

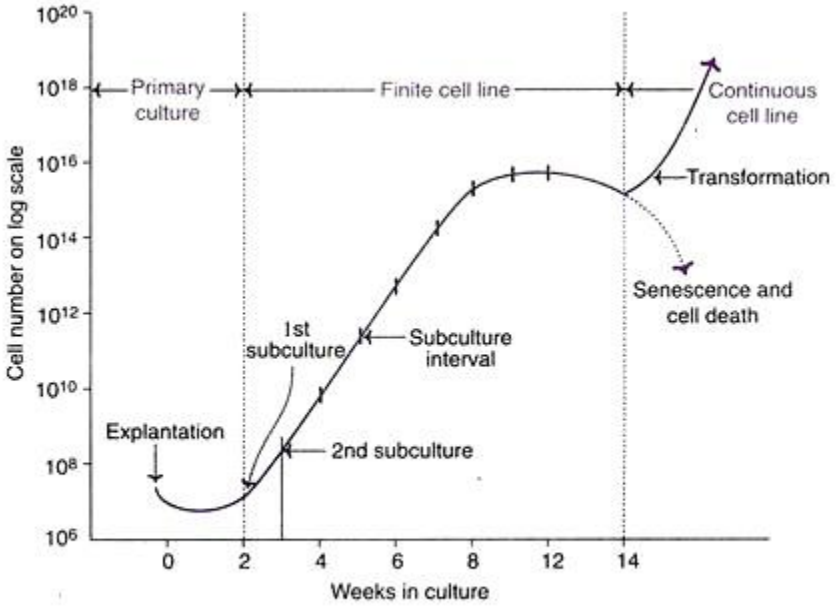


Fig. 35.4 : Diagrammatic representation of evolution of a cell line.

Development of continuous cell lines:

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

Genetic variations:

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

Normal cells and continuous cell lines:

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

Characterization of Cultured Cells:

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

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

1. Morphology of cells

2. Species of origin.

3. Tissue of origin.

4. Whether cell line is transformed or not.

5. Identification of specific cell lines.

Morphology of Cells:

A simple and direct identification of the cultured cells can be done by observing their morphological characteristics. However, the morphology has to be viewed with caution since it is largely dependent on the culture environment. For instance, the epithelial cells growing at the center (of the culture) are regular polygonal with clearly defined edges, while those growing at the periphery are irregular and distended (swollen).

The composition of the culture medium and the alterations in the substrate also influence the cellular morphology. In a tissue culture laboratory, the terms fibroblastic and epithelial are commonly used to describe the appearance of the cells rather than their origin.

Fibroblastic cells:

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

Epithelial cells:

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

Species of Origin of Cells:

The identification of the species of cell lines can be done by:

a. Chromosomal analysis.

b. Electrophoresis of isoenzymes.

c. A combination of both these methods.

In recent years, chromosomal identification is being done by employing molecular probes.

Identification of Tissue of Origin:

The identification of cell lines with regard to tissue of origin is carried out with reference to the following two characteristics:

1. The lineage to which the cells belong.

2. The status of the cells i.e. stems cells, precursor cells.

Tissue markers for cell line identification:

Some of the important tissue or lineage markers for cell line identification are briefly described.

Differentiated products as cell markers:

The cultured cells, on complete expression, are capable of producing differentiation markers, which serve as cell markers for identification.

Some examples are given below:

a. Albumin for hepatocytes.

b. Melanin for melanocytes

c. Hemoglobin for erythroid cells

d. Myosin (or tropomyosin) for muscle cells.

Enzymes as tissue markers:

The identification of enzymes in culture cells can be made with reference to the following characters:

a. Constitutive enzymes.

b. Inducible enzymes.

c. Isoenzymes.

The commonly used enzyme markers for cell line identification are given in Table 35.1.

TABLE 35.1 Some common enzyme markers for cell line identification

Enzyme	*Cell type*
Tyrosine aminotransferase	Hepatocytes
Tysosinase	Melanocytes
Glutamyl synthase	Brain (astroglia)
Creatine kinase (isoenzyme MM)	Muscle cells
Creatine kinase (isoenzyme BB)	Neurons, neuroendocrine cells
Non-specific esterase	Macrophages
DOPA-decarboxylase	Neurons
Alkaline phosphatase	Enterocytes, type II pneumocytes
Angiotensin–converting enzyme	Endothelium
Sucrase	Enterocytes
Neuron-specific esterase	Neurons

Tyrosine aminotransferase is specific for hepatocytes, while tyrosinase is for melanocytes. Creatine kinase (MM) in serum serves as a marker for muscle cells, while creatine kinase (BB) is used for the detection of neurons and neuroendocrine cells.

Filament proteins as tissue markers:

The intermediate filament proteins are very widely used as tissue or lineage markers.

For example:

a. Astrocytes can be detected by glial fibrillary acidic protein (GFAP).

b. Muscle cells can be identified by desmin.

c. Epithelial and mesothelial cells by cytokeratin.

Cell surface antigens as tissue markers:

The antigens of the cultured cells are useful for the detection of tissue or cells of origin. In fact, many antibodies have been developed (commercial kits are available) for the identification cell lines (Table. 35.2). These antibodies are raised against cell surface antigens or other proteins.

TABLE 35.2 A selected list of antibodies used for the detection of cell types

Antibody	*Cell type*
Cytokeratin	Epithelium
Epithelial membrane antigen	Epithelium
Albumin	Hepatocytes
α-Lactalbumin	Breast epithelium
Carcinoembryonic antigen (CEA)	Colorectal and lung adenocarcinoma
Prostate specific antigen (PSA)	Prostatic epithelium
Intracellular cell adhesion molecule (I-CAM)	T-cells and endothelium
α-Fetoprotein	Fetal hepatocytes
Human chorionic gonadotropin (hCG)	Placental epithelium
Human growth hormone (hGH)	Anterior pituitary
Vimentin	Mesodermal cells
Integrins	All cells
Actin	All cells

The antibodies raised against secreted antigen a-fetoprotein serves as a marker for the identification of fetal hepatocytes. Antibodies of cell surface antigens namely integrin's can be used for the general detection of cell lines.

Transformed Cells:

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

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

a. Growth rate

b. Mode of growth

c. Longevity

d. Tumorigenicity

e. Specialized product formation.

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

Identification of Specific Cell Lines:

There are many approaches in a culture laboratory to identify specific cell lines:

a. Chromosome analysis

b. DNA detection

c. RNA and protein analysis

d. Enzyme activities

e. Antigenic markers.

Chromosome analysis:

The species and sex from which the cell line is derived can be identified by chromosome analysis. Further, it is also possible to distinguish normal and malignant cells by the analysis of chromosomes. It may be noted that the normal cells contain more stable chromosomes. The important techniques employed with regard to chromosome analysis are briefly described.

Chromosome banding:

By this technique, it is possible to identify individual chromosome pairs when there is little morphological difference between them. Chromosome banding can be done by using Giemsa staining.

Chromosome count:

A direct count of chromosomes can be done per spread between 50-100 spreads. A camera Lucida attachment or a closed circuit television may be useful.

Chromosome karyotyping:

In this technique, the chromosomes are cut, sorted into sequence, and then pasted on to a sheet. The image can be recorded or scanned from the slide. Chromosome karyotyping is time consuming when compared to chromosome counting.

DNA detection:

The total quantity of DNA per normal cell is quite constant, and is characteristic to the species of origin, e.g. normal cell lines from human, chick and hamster fibroblasts. However, the DNA

content varies in the normal cell lines of mouse, and also the cell lines obtained from cancerous tissues. Most of the transformed cells are aneuploid and heteroploid. DNA analysis is particularly useful for characterization of such cells. Analysis of DNA can be carried out by DNA hybridization and DNA fingerprinting.

DNA hybridization:

The popular Southern blotting technique can be used to detect unique DNA sequences. Specific molecular probes with radioisotope, fluorescent or luminescent labels can be used for this purpose. The DNA from the desired cell lines is extracted, cut with restriction endonucleases, subjected to electrophoresis, blotted on to nitrocellulose, and then hybridized with a molecular (labeled) probe, or a set of probes. By this approach, specific sequences of DNA in the cell lines can be detected.

DNA fingerprinting:

There are certain regions in the DNA of a cell that are not transcribed. These regions, referred to as satellite DNA, have no known functions, and it is believed that they may provide reservoir for genetic evolution. Satellite DNA regions are considered as regions of hyper variability. These regions may be cut with specific restriction endonucleases, and detected by using cDNA probes.

By using electrophoresis and autoradiography, the patterns of satellite DNA variations can be detected. Such patterns referred to as DNA fingerprints are cell line specific. In recent years, the technique of DNA fingerprinting has become a very popular and a powerful tool to determine the origin of cell lines.

RNA and protein analysis:

The phenotype characteristics of a cell line can be detected by gene expression i.e. identification of RNAs and/or proteins. mRNAs can be identified by Northern blot technique while proteins can be detected by Western blot technique.

Enzyme activities:

Some of the in vivo enzyme activities are lost when the cells are cultured in vitro. For instance, arginase activity of the liver cells is lost within a few days of culturing. However, certain cell lines express specific enzymes that can be employed for their detection e.g. tyrosine aminotransferase for hepatocytes, glutamyl synthase activity for astroglia in brain. For more examples of enzymes useful in cell line detection, refer Table 35.1.

Isoenzymes:

The multiple forms of an enzyme catalysing the same reaction are referred to as isoenzymes or isozymes. Isoenzymes differ in many physical and chemical properties—structure, electrophoretic and immunological properties, K_m and V_{max} values.
The isoenzymes can be separated by analytical techniques such as electrophoresis and chromatography. Most frequently, electrophoresis by employing agarose, cellulose acetate, starch and polyacrylamide is used. The crude enzyme is applied at one point on the electrophoretic medium. As the isoenzymes migrate, they distribute in different bands, which can be detected by staining with suitable chromogenic substrates.

Isoenzymes are characteristic to the species or tissues. Isoenzymes of the following enzymes are commonly used for cell line detection:

a. Lactate dehydrogenase

b. Malate dehydrogenase

c. Glucose 6-phosphate dehydrogenase

d. Aspartate aminotransferase

e. Peptidase B.

Isoenzyme analysis is also useful for the detection of interspecies cross-contamination of cell lines. For instance, contamination of mouse cell line with hamster cell line can be identified by using peptidase B isoenzymes.

Antigenic markers:

Cell lines can be characterized by detection of antigenic markers through the use of antibodies. The antigenic markers may be located on the cell surface or secreted by the cells into the culture medium. Some of the antibodies in common use for the detection of different cell types are given in Table 35.2 (See p. 430).

Measurement of Growth Parameters of Cultured Cells:

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

a. Design culture experiments.

b. Routine maintenance of culture.

c. Measurement of cell proliferation.

d. Know the time for subculture.

e. Determine the culture response to a particular stimulus or toxin.

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

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

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

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

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

Cell density:
The number of cells per ml of the medium.

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

Growth Cycle of Cultured Cells:
The growth cycle of cultured cells is conventionally represented by three phases — the lag phase, the log (exponential) phase and the plateau phase (Fig. 35.5). The properties of the cultured cells vary in the phases.

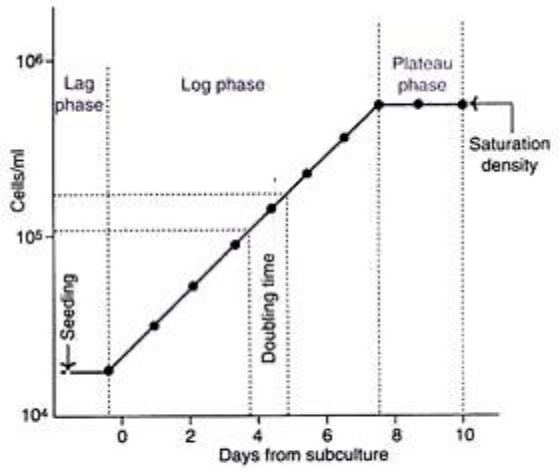


Fig. 35.5 : Growth curve of cultured cells (Note : The cell concentration is expressed in semilog plot).

The lag phase:

The lag phase represents a period of adaptation during which the cell forms the cell surface and extracellular matrix (lost during trypsinization), attaches to the substrate and spreads out. There is an increased synthesis of certain enzymes (e.g. DNA polymerase) and structural proteins, preparing the cells for proliferation.

The production of specialized products disappears which may not reappear until the cell proliferation ceases. The lag phase represents preparative stage of the cells for proliferation following subculture and reseeding.

The log phase:

The log phase is characterized by an exponential growth of cells, following the lag phase.

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

a. Seeding density.

b. Growth rate.

c. Density after proliferation.

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

The plateau phase:

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

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

a. Low motility of cells.

b. Reduced ruffling of plasma membrane.

c. Cells occupying minimum surface area.

d. Contact inhibition.

e. Saturation density.

f. Depletion of nutrients and growth factors.

g. Reduced synthesis of structural proteins.

h. Increased formation of specialized products.

The majority of normal cultured cells that form monolayers stop growing as they reach confluence. Some of the cells however, with replenishment of medium continue to grow (at a reduced rate) after confluence, forming multilayers of cells. The transformed cultured cells usually reach a higher cell density compared to the normal cells in the plateau phase (Fig. 35.6).

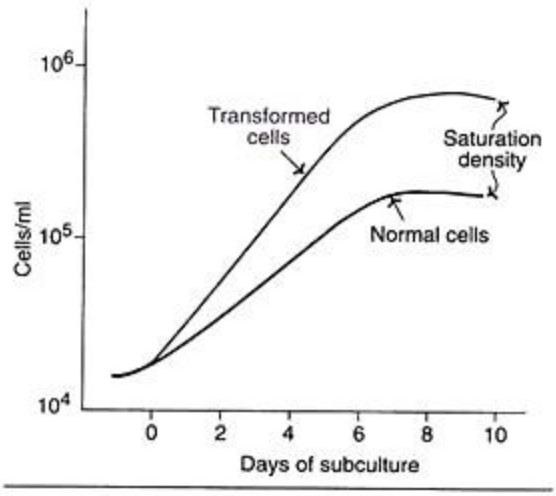


***Fig. 35.6** : Growth curves of transformed and normal cells (**Note** : The cell concentration is expressed in semilog plot).*

Plating Efficiency of Cultured Cells:

Plating efficiency, representing colony formation at low cell density, is a measure used for analyzing cell proliferation and survival.

When the cells, at low densities, are cultured in the form of single cell suspensions, they grow as discrete colonies. Plating efficiency is calculated as follows.

Plating efficiency = No. of colonies formed/No. of cells seeded × 100

The term cloning efficiency is used (instead of plating efficiency) when each colony grows from a single cell.

Seeding efficiency representing the survival of cells at higher densities is calculated as follows.

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

Cell Synchronization:

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

They are broadly categorized into two groups:

1. Physical fractionation for cell separation.

2. Chemical blockade for cell separation.

Cell Separation by Physical Means:

Physical fractionation or cell separation techniques, based on the following characteristics are in use:

a. Cell density.

b. Cell size.

c. Affinity of antibodies on cell surface epitopes.

d. Light scatter or fluorescent emission by labeled cells.

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

Centrifugal elutriation:

The physical characteristics—cell size and sedimentation velocity are operative in the technique of centrifugal elutriation. Centrifugal elutriator (from Beckman) is an advanced device for increasing the sedimentation rate so that the yield and resolution of cells is better. The cell separation is carried out in a specially designed centrifuge and rotor (fig. 35.7). The cells in the medium are pumped into the separating chamber while the rotor is turning.

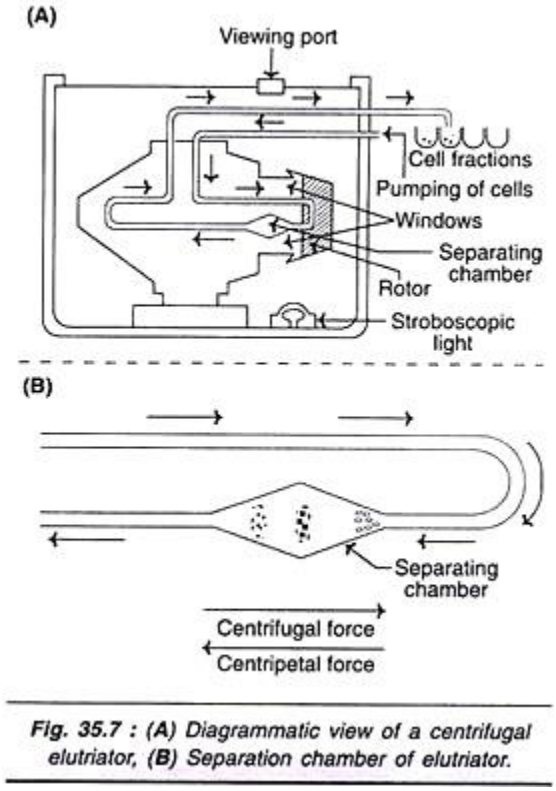


Fig. 35.7 : (A) Diagrammatic view of a centrifugal elutriator, (B) Separation chamber of elutriator.

Due to centrifugal force, the cell will be pushed to the edges. As the medium is then pumped through the chamber in such a way that the centripetal flow is equal to the sedimentation rate of cells. Due to differences in the cells (size, density, cell surface configuration), the cells tend to sediment at different rates, and reach equilibrium at different positions in the chamber.

The entire operation in the elutriator can be viewed through the port, as the chamber is illuminated by stroboscopic light. At the equilibrium the flow rate can be increased and the cells can be pumped out, and separated in collecting vessels in different fractions. It is possible to carry out separation of cells in a complete medium, so that the cells can be directly cultured after separation.

Fluorescence-activated cell sorting:

Fluorescence-activated cell sorting is a technique for sorting out the cells based on the differences that can be detected by light scatter (e.g. cell size) or fluorescence emission (by pretreated DNA, RNA, proteins, antigens). The procedure involves passing of a single stream of cells through a laser beam so that the scattered light from the cells can be detected and recorded. When the cells are pretreated with a fluorescent stain (e.g. chromomycin A for DNA), the fluorescent emission excited by the laser can be detected.

There are two instruments in use based on the principle of fluorescent-activated cell sorting:

1. Flow cytometer:

This instrument is capable of sorting out cells (from a population) in different phases of the cell cycle based on the measurements of a combination of cell size and DNA fluorescence.

2. Fluorescent-activated cell sorter (FACS):

In this instrument, the emission signals from the cells are measured, and the cells sorted out into collection tubes.

Comparison between physical methods:

For separation of a large number of cells, centrifugal elutriator is preferred. On the other hand, fluorescent-activated cell sorting is mostly used to obtain high grade pure fractions of cells from small quantities of cells.

Cell Separation by Chemical Blockade:

The cells can be separated by blocking metabolic reactions. Two types of metabolic blockades are in use — inhibition of DNA synthesis and nutritional deprivation.

Inhibition of DNA synthesis:

During the S phase of cell cycle, DNA synthesis can be inhibited by using inhibitors such as thymidine, aminopterine, hydroxyurea and cytosine arabinoside. The effects of these inhibitors are variable. The cell cycle is predominantly blocked in S phase that results in viable cells.

Nutritional deprivation:

Elimination of serum or isoleucine from the culture medium for about 24 hours results in the accumulation of cells at G_1 phase. This effect of nutritional deprivation can be restored by their addition by which time the cell synchrony occurs.

Some Highlights of Cell Synchronization:

a. Cell separation by physical methods is more effective than chemical procedures.

b. Chemical blockade is often toxic to the cells.

c. Transformed cells cannot be synchronized by nutritional deprivation.

d. A high degree of cell synchrony (>80%) can be obtained in the first cycle, and in the second cycle it would be <60%. The cell distribution may occur randomly in the third cycle.

Cellular Senescence and Apoptosis:

As the cells grow in culture, they become old due to aging, and they cannot proliferate any more. The end of the proliferative life span of cells is referred to as senescence.

Cellular Senescence:

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

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

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

The different parameters used for the measurement of cell growth in cultures are listed below:

a. Direct measure of cell number.

b. Determination of DNA/RNA content.

c. Estimation of protein/ATP concentration.

Measurement of Senescence:

The direct measurement of senescent cells is rather difficult.

Some of the indirect measures are:

a. Loss of metabolic activity

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

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

The assay consists of the following stages:
1. Wash the cells and fix them using a fixative (e.g. para formaldehyde), and wash again.

2. Add the staining solution (X-gal powder in dimethylformamide dissolved in buffer) to the fixed cells and incubate.

3. The senescent cells display a dense blue colour which can be counted.

Apoptosis:
The process of programmed cell death (PCD) is referred to as apoptosis. The cell death may be initiated by a specific stimulus or as a result of several signals received from the external environment. Apoptosis occurs as a result of inherent cellular mechanisms, which finally lead to self-destruction. The cell activates a series of molecular events that cause an orderly degradation of the cellular constituents with minimal impact on the neighbouring tissues.

Reasons for in situ apoptosis:
1. For proper development:
The formation of fingers and toes of the fetus requires the removal of the tissues between them. This is usually carried out by apoptosis.

2. Destruction of cells that pose threat to the integrity of the organism:
Programmed cell death is needed to destroy and remove the cells that may otherwise damage the organisms.

Some examples are listed:

a. Cells with damaged DNA during the course of embryonic development. If they are not destroyed, they may result in birth defects.

b. Cells of the immune system, after their appropriate immune function, undergo apoptosis. This is needed to prevent autoimmune diseases e.g. rheumatoid arthritis.

c. Cells infected with viruses are destroyed by apoptosis.

3. Cell destruction due to negative signals:

There are several negative signals within the cells that promote apoptosis. These include accumulation of free radicals, exposure to UV rays, X-rays and chemotherapeutic drugs.

Mechanism of apoptosis:

The programmed cell death may occur due to three different mechanisms:

1. Apoptosis due to internal signals.

2. Apoptosis triggered by external signals e.g. tumor necrosis factor-α (TNF-α), lymphotoxin.

3. Apoptosis triggered by reactive oxygen species.

Role of caspases in apoptosis:

A group of enzymes namely activated proteases play a crucial role in the programmed cell death. These proteases are actually cysteinyl aspartate specific proteinases or in short, commonly referred to as caspases. There are about ten different types of caspases acting on different substrates ultimately leading to cell death. For instance, capsase I cleaves interleukin 1β.

Inhibition of caspase activities:

Since the caspases are closely involved in apoptosis, it is possible to prevent cell death by inhibiting their activities. Certain specific peptides that can inhibit caspases, and thus apoptosis have been identified.

Measurement of Apoptosis:

A simple and easy way of detecting dead or dying cells is the direct microscopic observation. The dying cells are rounded with dense bodies which can be identified under phase contrast microscope. The cells that have undergone apoptosis contain fragmented chromatin which can be

detected by conventional staining techniques. In recent years, more sensitive and reliable techniques have been developed for measuring apoptosis.

Some of them are briefly described:

Determination ADP/ATP ratio:

Both the growth and apoptosis of cells require ATP. But when there is growth arrest, an elevation of ADP occurs. Thus measuring ADP/ATP ratio will throw light on the dead cells. In fact, some assay systems for measuring ADP/ATP ratios are commercially available.

TUNEL assay:

A significant biochemical event for the apoptosis is the activation of endogenous nuclease activity. This enzyme cleaves DNA into fragments with free 3-hydroxyl groups. The newly formed small DNA fragments can be extended by employing the enzyme DNA polymerase. If labeled nucleotides are used for DNA fragment extension, they can be detected.

TUNEL is an abbreviation for TdT-mediated dUTP nick end-labeling assay. TUNEL is very fast and effective for the determination of DNA fragments formed by endogenous nuclease activity. The apoptotic nuclei can be identified by a fluorescent technique using fluorescein isothiocyanate (FITC) and 4, 6-diaminophenylindole.

DNA laddering test:

During the course of apoptosis, the genomic DNA is cleaved to mono — and oligonucleosomal DNA fragments. These fragments can be separated by agarose electrophoresis, and detected. The nucleosomal fragments of apoptotic cells give a characteristic ladder pattern on electrophoresis.

Limitations of the test:

DNA laddering test is not very specific since several cells that have undergone apoptosis may not show DNA laddering. Further, some cells not subjected to apoptosis may also show DNA ladders, for these reasons, DNA laddering test is coupled with some other test for measurement of apoptosis.

Cell adhesion in animal cell culture: physiological and fluid-mechanical implications.

We have reviewed the general forces through which cells interact with substrata in their first nonspecific contact. The complex, fast-emerging biology of specific cell adhesion and the structure of the extracellular matrix were reviewed in substantial detail, and the most updated conceptual model of biological cell adhesion was assembled from past efforts and new literature

data. The chemistries of the various possible substrata for cell adhesion have been reviewed extensively in the past, and here only a brief summary was presented, with particular emphasis on the materials for traditional and porous microcarriers. The fascinating molecular and cellular implications of cell adhesion were reviewed in detail to establish that cell adhesion and the extracellular matrix provide more than structural support for the cells and their assemblies, and that in fact they constitute fundamental regulators of cell function, metabolism, and differentiation. We reviewed the fluid-mechanical mechanisms of cell damage in microcarrier systems and provided experimental evidence for the importance of the cell-adhesion quality in the ability of cells to withstand fluid forces in bioreactors. We provided evidence that the interplay of cell adhesion and fluid forces is likely to produce cell responses more complex than that of simple life and death, and we suggested that such responses are awaiting investigation and exploration for new applications and culturing possibilities. We also reviewed the experimental evidence on the importance of cell adhesion in cell and microcarrier aggregation and discussed the implications of such aggregation on the culturing environment and the operation of bioreactors. Finally, we discussed the possible implications of cell adhesion as it relates to the developing field of tissue engineering, using the example of bone marrow culture, which involves a large variety of cells and constitutes one of the most complex cell culture systems.

Cell Proliferation in Development and Differentiation

Early development is characterized by the rapid proliferation of embryonic cells, which then differentiate to produce the many specialized types of cells that make up the tissues and organs of multicellular animals. As cells differentiate, their rate of proliferation usually decreases, and most cells in adult animals are arrested in the G_0 stage of the cell cycle. A few types of differentiated cells never divide again, but most cells are able to resume proliferation as required to replace cells that have been lost as a result of injury or cell death. In addition, some cells divide continuously throughout life to replace cells that have a high rate of turnover in adult animals. Cell proliferation is thus carefully balanced with cell death to maintain a constant number of cells in adult tissues and organs.

Go to:

Proliferation of Differentiated Cells

The cells of adult animals can be grouped into three general categories with respect to cell proliferation. A few types of differentiated cells, such as cardiac muscle cells in humans, are no longer capable of cell division. These cells are produced during embryonic development, differentiate, and are then retained throughout the life of the organism. If they are lost because of injury (e.g., the death of cardiac muscle cells during a heart attack), they can never be replaced.

In contrast, most cells in adult animals enter the G_0 stage of the cell cycle but resume proliferation as needed to replace cells that have been injured or have died. Cells of this type include skin fibroblasts, smooth muscle cells, the endothelial cells that line blood vessels, and the epithelial cells of most internal organs, such as the liver, pancreas, kidney, lung, prostate, and breast. One example of the controlled proliferation of these cells, discussed earlier in this chapter, is the rapid proliferation of skin fibroblasts to repair damage resulting from a cut or wound. Another striking example is provided by liver cells, which normally divide only rarely. However, if large numbers of liver cells are lost (e.g., by surgical removal of part of the liver), the remaining cells are stimulated to proliferate to replace the missing tissue. For example, surgical removal of two-thirds of the liver of a rat is followed by rapid cell proliferation, leading to regeneration of the entire liver within a few days.

Cell Culture Fundamentals: Cell Types & Culture Characteristics

Immortalized Cell Lines

Continuous **immortalized cell lines** are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. Cell lines of a finite life are usually diploid and maintain some degree of differentiation. The fact that such cell lines senesce after approximately thirty cycles of division means it is essential to establish a system of Master and Working banks in order to maintain such lines for long periods.

Continuous immortalized cell lines that can be propagated indefinitely generally have this ability because they have been transformed into tumor cells. Tumor cell lines are often derived from actual clinical tumors, but transformation may also be induced using viral oncogenes or by chemical treatments. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original *in vivo* characteristics.

Primary Cell Lines

Primary cell lines are derived directly from excised tissue and cultures either as an explant culture or following dissociation into a single cell suspension by enzyme digestion. Such cultures are initially heterogeneous but later become dominated by fibroblasts. The preparation of primary cultures is labor intensive and they can be maintained *in vitro* only for a limited period of time. During their relatively limited lifespan primary cells usually retain many of the differentiated characteristics of the cell *in vivo*. Important Note: Primary cultures by definition have not been passaged, as soon as they are passaged they become a cell line and are no longer primary.

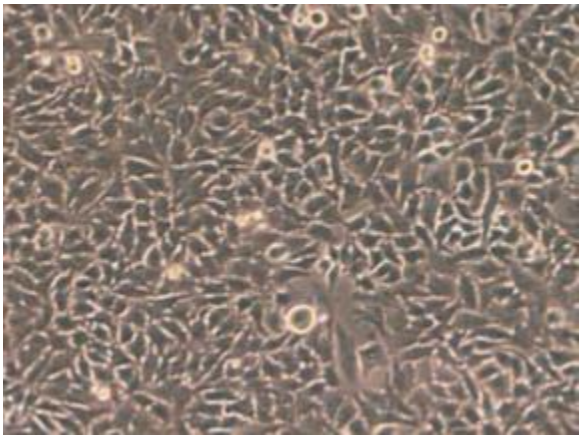
Stem Cell Lines

More recently, **stem cells lines** are been a popular cell model used in research due to their unlimited growth characteristics and plasticity. These cells have the unique ability to self-renew or to differentiate into various cell types in response to appropriate signals within the body. These properties provide stem cells with unique capabilities for tissue repair, replacement, and regeneration.

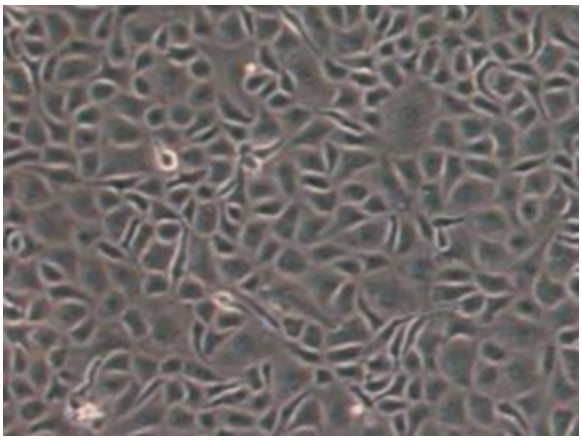
Cell Morphology Types

In terms of growth mode cell cultures take one of two forms, growing either in suspension (as single cells or small free-floating clumps) or as a monolayer that is attached to the tissue culture flask. The form taken by a cell line reflects the tissue from which it was derived. For example, cell lines derived from blood (leukemia, lymphoma) tend to grow in suspension whereas cells derived from solid tissue (lungs, kidney) tend to grow as monolayers. Attached cell lines can be classified as 1) endothelial such as BAE-1, 2) epithelial such as HeLa, 3) neuronal such as SH-SY5Y, or 4) fibroblast such as MRC-5.

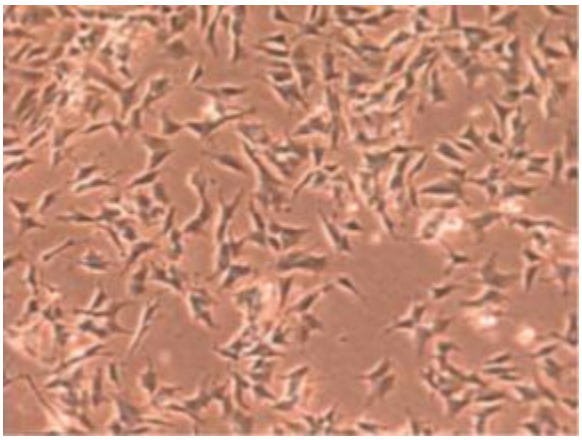
Epithelial Cell Type



Endothelial Cell Type



Neuronal Cell Type



Fibroblast Cell Type

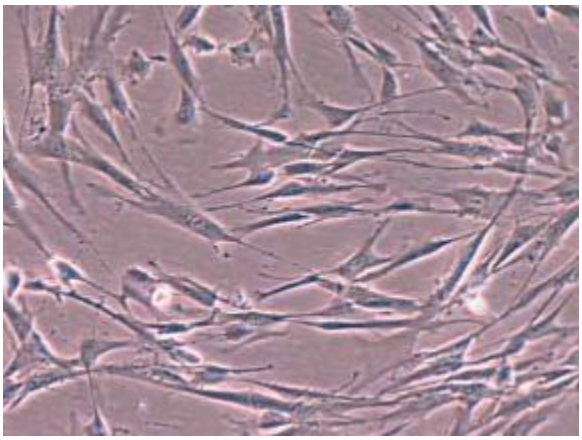


Figure 1.Examples of attached cell types. Cells are classified in 4 different cell type categories based on overall morphology 1) Epithelial 2) Endothelial 3) Neuronal or 4) Fibroblast.

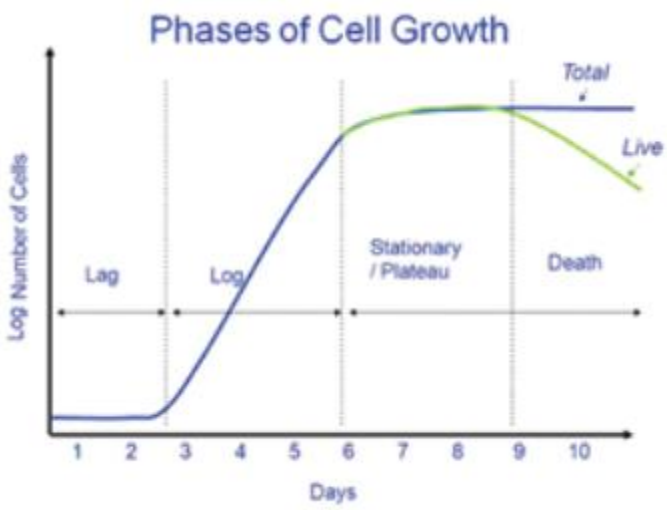
The European Collection of Authenticated Cell Cultures (ECACC) is one of the world's largest biological cell banks supplying a diverse range of authenticated cell lines. All 3000+ cell lines can be ordered through Sigma-Aldrich. **Browse the ECACC catalog**.

Attached Cell Lines		
Name	**Species and Tissue of Origin**	**Morphology**
MRC-5	Human Lung	Fibroblast
HeLa	Human Cervix	Epithelial
Vero	African Green Monkey Kidney	Epithelial
NIH3T3	Mouse Embryo	Fibroblast

L929	Mouse Connective Tissue	Fibroblast
CHO	Chinese Hamster Ovary	Fibroblast
BHK-21	Syrian Hamster Kidney	Fibroblast
HEK-293	Human Kidney	Epithelial
HepG2	Human Liver	Epithelial
BAE-1	Bovine Aorta	Endothelial
SH-SY5Y	Human Neuroblastoma	Neuronal
Suspension Cell Lines		
Name	**Species and Tissue of Origin**	**Morphology**
NS0	Mouse Myeloma	Lymphoblast
U937	Human Hystiocytic Lymphoma	Lymphoblast
HL60	Human Leukemia	Lymphoblast
WEHI231	Mouse B-cell Lymphoma	Lymphoblast
YAC1	Mouse Lymphoma	Lymphoblast
U266B1	Human Myeloma	Lymphoblast
Jurkat	Human T-cell Leukemia	Lymphoblast
THP-1	Human Monocyte Leukemia	Lymphoblast

Table 1. Commonly used cell lines of each cell morphology type

Phases of Cell Growth



It is important to know and record the growth characteristics of the cell line in use before starting any experiments including **cell viability and proliferation** rates. An alteration in cellular growth can indicate a significant problem within the cell line and if undetected, can have detrimental effects on experimental results.

A typical growth curve for cultured cells displays a sigmoid pattern of proliferation. The growth phases associated with normal cells are defined as:

1. **Lag Phase** – at this stage the cells do not divide. During this period the cells adapt to the culture conditions and the length of this phase will depend upon the growth phase of the cell line at the time of subculture and the seeding density.
2. **Logarithmic (Log) Growth Phase** – cells actively proliferate and an exponential increase in cell density arises. The cell population is considered to be the most viable at this phase; therefore, it is recommended to assess cellular function at this stage. Each cell line will show different cell proliferation kinetics during the log phase and it is therefore the optimal phase for determining the population doubling time. Cells are also generally passaged at late log phase. Passaging cells too late can lead to overcrowding, apoptosis and senescence.
3. **Plateau (or Stationary) Phase** – cellular proliferation slows down due to the cell population becoming confluent. It is at this stage the number of cells in the active cell cycle drops to 0-10% and the cells are most susceptible to injury.
4. **Decline Phase** – cell death predominates in this phase and there is a reduction in the number of viable cells. Cell death is not due to the reduction in nutrients, but to the natural progression of the cellular cycle.

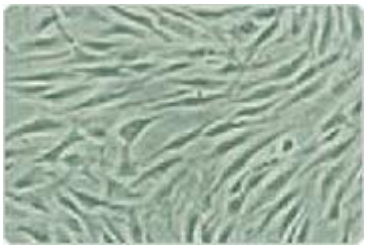
In Vitro Age of a Cell Culture

Two terms are predominantly used to define the age of a cell culture: (i) passage number - indicates the number of times the cell line has been subcultured and (ii) the population doubling (pd) number - indicates the number of cell generations the cell line has undergone i.e. the number of times the cell population has doubled. The in vitro age of a cell culture is particularly useful to know for cell lines with a finite lifespan or unstable characteristics that change over time in continuous culture.

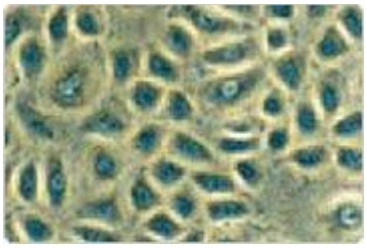
Mammalian Cell Morphology

1. Most mammalian cells in culture can be divided in to three basic categories based on their morphology.

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



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



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

3. In addition to the basic categories listed above, certain cells display morphological characteristics specific to their specialized role in host.

4. **Neuronal cells** exist in different shapes and sizes, but they can roughly be divided into two basic morphological categories, **type I** with long axons used to move signals over long distances and **type II** without axons. A typical neuron projects cellular extensions with many branches from the cell body, which is referred to as a dendritic tree. Neuronal cells can be unipolar or pseudounipolar with the dendrite and axon emerging from same process, bipolar with the axon and single dendrite on opposite ends of the soma (the central part of the cell containing the nucleus), or multipolar with more than two dendrites.

Cell Identification and Cell Lineage Analysis

Claudiu A. Giurumescu and Andrew D. Chisholm

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The publisher's final edited version of this article is available at Methods Cell Biol

Abstract

Go to:

I. Introduction

Cell lineage analysis refers to the tracing of cellular genealogies by following cell divisions and migrations over time, beginning with specific progenitor cells and ending with their postmitotic descendants. The development of almost all metazoan animals can in principle be described as a lineage tree whose origin is the single-cell zygote. However, the variability of normal development means that cell lineage relationships can in general only be described in probabilistic terms. In contrast, for some animal groups, including nematodes, molluscs, and tunicates, the pattern of cell divisions throughout development is highly invariant between individuals. In such animals, the invariant lineage constitutes a complete fate map of development with single-cell resolution.

The first descriptions of nematode cell lineages began in the late 19th century and were based on a series of fixed specimens. These studies established that the pattern of embryonic cell divisions was virtually invariant from animal to animal. In some cases, the cell lineage was thought to generate a fixed number of cells in the adult ("cell constancy" or eutely), or at least in certain tissues ("partial constancy") (van Cleave, 1932). However, it was not until the development of Nomarski DIC microscopy in the late 1960s (Allen *et al.*, 1969; Padawer, 1968) that it became feasible to observe cell divisions in live animals.

Using Nomarski DIC microscopy of live animals the complete cell lineage of *C. elegans* from zygote to adult was delineated in a series of classic studies, culminating in the complete description of the embryonic cell lineage in 1983 (Sulston *et al.*, 1983). All these descriptions were based on direct observation of live animals, without significant use of recording technology. Since then, cell lineages have been traced in over ten other nematode species (see Table I). The *C. elegans* "lineage papers" (Table II) remain an essential resource for learning cell identification and lineage analysis. For historical accounts of the early days of lineage analysis see Horvitz and Sulston (1990) and John Sulston's Nobel Lecture (Sulston, 2003).

Table I

Cell-lineage analysis in other nematode species

Species	Lineages studied	Reference
Panagrellusredivivus	Postembryonic lineages	(Sternberg and Horvitz, 1981, 1982)
Turbatrixaceti	Early embryo	(Sulston *et al.*, 1983)
Mesorhabditis etc.	Vulval lineages	(Sommer *et al.*, 1994)
Pristionchuspacificus	Vulval lineages	(Sommer, 1997)
Acrobeloides		(Wiegner and Schierenberg, 1998)
Oscheiustipulae	Vulval cell lineage	(Delattre and Felix, 2001)
Pellioditis marina	Complete embryonic lineage	(Houthoofd *et al.*, 2003)
Halicephalobusgingivalis		(Houthoofd and Borgonie, 2007)
Rhabditophanes	Embryonic lineage	(Houthoofd *et al.*, 2008)

Species	Lineages studied	Reference
C. briggsae	Embryonic lineage; automated lineage tracing	(Zhao *et al.*, 2008)
Diploscaptercoronatus	Embryonic lineage	(Lahl *et al.*, 2009)
Romanomermisculicivorax	Embryonic lineage	(Schulze and Schierenberg, 2008, 2009)

Table II

Key publications describing *C. elegans* lineages

Lineages	Reference
Ventral nerve cord	(Sulston, 1976)
Postembryonic nongonadal lineages (hermaphrodite)	(Sulston and Horvitz, 1977)
Gonadal lineages (both sexes)	Kimble and Hirsh, 1979)
Postembryonic nongonadal lineages (male)	(Sulston *et al.*, 1980)

Lineages	Reference
Embryonic lineage	(Deppe *et al.*, 1978; Sulston *et al.*, 1983)

With the advent of green fluorescent proteins (GFP) in the early 1990s (Chalfie *et al.*, 1994), cell identification entered a new phase. Now specific cells or cell types could be identified more rapidly, without the need for meticulous drawing out of cells and their positions. Expression of a fluorescent marker provided an unambiguous measure of cell fate. For example, cells could be identified even when misplaced in aberrant locations as a result of a cell migration defect. Most importantly, whereas under DIC observation only cell nuclei are typically resolved, GFP markers can be used to visualize the entire cell, or specific subcellular compartments. The ability to see axons, muscle arms, and other structures in live animals opened up whole new areas of analysis.

GFP transgenic markers have in many cases replaced DIC for cell identification. Nevertheless there are still reasons to learn the DIC anatomy. First, transgenic markers are not available for all cell types or subsets of cell types. Second, care is needed to ensure that the GFP marker (often a high-copy number transgene) does not itself interfere with cell differentiation. Issues of photobleaching or phototoxicity often limit the amount of observation possible, although this has been to some extent overcome in the automated analysis of embryonic cell divisions. Finally, analyzing a number of markers can involve considerable strain construction, work that can be avoided if the cells can be identified by DIC.

There has been rapid recent progress in automation of cell identification and lineage analysis. Cell lineage analysis in embryos can be partly automated as discussed in detail below. Automatic cell lineage analysis in larval stages has so far not been possible, largely due to the difficulty in immobilizing larvae in a way that allows normal development. However, digital atlases of cell positions at defined stages can be generated, allowing gene expression patterns to be mapped semiautomatically (Long *et al.*, 2009). At present such atlases represent ~65% of the nuclei in the L1 stage. However, many neuronal nuclei are too closely spaced to be reliably identified by automated analysis. Thus, to identify specific neuronal expression patterns a knowledge of the anatomy remains indispensable.

Go to:

II. Rationale

A. Analysis of Mutant Phenotypes

One of the most frequent goals in cell-lineage analysis is to address the developmental basis of a specific phenotype, whether caused by mutation, RNAi, or some other perturbation. For

example, using cell type specific GFP reporters it is straight-forward to screen for mutants affecting the number of cells that express a given reporter (Doitsidou *et al.*, 2008; Kanamori *et al.*, 2008). A change in the number of expressing cells could have a number of causes and lineage analysis can resolve these possibilities. For example, do excess GFP-expressing cells arise from ectopic expression of the reporter or from an overproliferation of specific precursors? Does failure to generate a given cell type reflect a cell fate transformation or an earlier defect in the lineage?

B. Cell Division Pattern as the Focus of Interest

In some cases, the pattern of cell divisions itself is the focus of interest, especially where no other molecular markers are available. For example, the role of the Wnt ligand LIN-44 in cell polarity was deduced from its effects on the polarity of certain cell divisions in the male tail (Herman and Horvitz, 1994). The stage specificity of cell-division patterns was critical in inferring the genetic control of developmental timing in larval development (Ambros and Horvitz, 1984). The regulative ability of certain tissues to undergo compensatory growth after damage was studied using cell-lineage analysis (Sulston and White, 1980).

Stem-cell-like division is inherently polarized. The stem-cell-like behavior of larval seam cells has been extensively analyzed by direct lineage analysis (e.g.Nimmo *et al.*, 2005). Analysis of cell-lineage mutants has also been important in understanding the genetic basis of cell cycle control (e.g. Kostić and Roy, 2002; Fukuyama *et al.*, 2003).

C. Cellular Patterns of Gene Expression

A common goal in cell identification is to define the cellular expression patterns of reporter genes. Some genes are expressed in relatively clear-cut patterns, for example, intestinal cells, all body wall muscles, all GABAergic neurons, and so on (cf. Chapter by Yan and JIn?). It is essential to learn to correlate such simple expression patterns first before attempting more complex patterns.

The nervous system poses the most daunting challenge for identification of gene-expression patterns. This is especially so in the large anterior ganglia, each of which contains 10–20 closely packed nuclei. However, the relative positions of most neuronal nuclei are fairly reproducible and can be learnt by reference to the maps in the L1 stage (Sulston *et al.*, 1983). Maps of these nuclei in the adult are based on serial section EM reconstruction (White *et al.*, 1986) and can be found in WormAtlas. Some neuronal nuclei display natural variability in location, and so cannot be conclusively identified based on position. Fortunately, identification of neurons is often made considerably easier by the distinctive disposition of the axons and dendrites of individual cell types.

D. Cell Killing by Laser Ablation

Cell ablation has been an important technique to define the developmental and physiological functions of cells (Bargmann and Avery, 1995). Individual cells can be killed with a laser microbeam focused on the cell nucleus. This depends on accurate cell identification, for which both DIC and GFP are now used. Ablation during development can be used to test the extent of

replacement regulation by other cells. Physiological functions of cells can be addressed by ablation unless they are subject to replacement or compensation.

E. Genetic Mosaic Analysis

In *C. elegans*, genetic mosaic analysis relies on spontaneous loss of unstable extrachromosomal arrays or chromosomal duplications during development. By identifying the pattern of cells in which the array or duplication has been lost, the "loss point" in the early lineage can be deduced. Such patterns are generally examined in late larval stages, that is, after the cell divisions are largely complete.

F. Comparative Developmental Biology

Cell lineages can be traced using Nomarski DIC in any optically transparent organism that can develop under continuous observation. As a result, direct cell-lineage analysis has now been undertaken in over a dozen different nematode species (Table I). Embryonic lineages have now been traced in several species. Although initial studies suggested a high degree of conservation in early embryonic lineages (Sulston *et al.*, 1983), subsequent studies of other species have revealed a remarkable diversity in the patterns of cell division within nematode embryos. Studies of vulval cell lineages in several species have been critical to our understanding of evolution of developmental mechanisms. As transgenic tools are now being developed in other nematode species, their use in automated analysis is likely to increase; the embryonic cell lineage of *C. briggsae* has already been followed using automated histone-GFP lineage tracing (Zhao *et al.*, 2008).

Go to:

III. Resources

The descriptions of cell lineages from the late 1970s remain the definitive descriptions of the cellular anatomy (Table II). In learning the anatomy an important initial goal is to compare one's own drawings with the diagrams in the following papers. In particular, the description by Sulston *et al.* (1983) remains the best resource for learning embryonic anatomy; an "embryo" section of WormAtlas is currently under construction.

WormAtlas (www.wormatlas.org) and the *C. elegans* Atlas book (Hall and Altun, 2008) are invaluable for understanding adult anatomy and for correlating cellular anatomy with electron micrographs. The web site contains a small section on cell identification. A good online guide to cell identification is in Wormbook (Yochem, 2006), with plentiful Nomarski DIC images of "landmark" cells. This is an important addition to the original lineage papers. However, in our experience the only way to successfully learn cell identification is to sit at the microscope and draw what one sees.

Go to:

IV. Nomenclature and Conventions

The nomenclature for cells was set out by Sulston and Horvitz (1977) and systematized by Sulston *et al.* (1983). Every cell in *C. elegans* can be named according to its ancestry, for example, ABpla. Terminally differentiated cells also have "functional" names that are either semiarbitrary (e.g., ASEL) or descriptive of terminal fate (hyp 7). For example, the cell ABalpppppppaa is the neuron ASEL.

Embryonic cells are named beginning with one of the five early embryonic "founder cells": AB, E, MS, C, D. The cells P_0 through P_4 denote the zygote and the precursors of the germ line, and should not be confused with the postembryonic blast cells P1–P12. Cells that go on to divide in postembryonic stages are renamed with a blast cell name (e.g., ABplapapaaa=QL), and their progeny named according to similar rules.

The suffixes in lineage names refer to the approximate orientation of the cell division relative to the overall axes of the embryo or larva: anterior/posterior, dorsal/ventral, left/right. Almost all cell divisions in *C. elegans* have a clear anterior–posterior orientation; indeed only ~8 embryonic cell divisions are predominantly in the transverse (left–right) axis. Cells are named according to the relative position of the daughters at the time of division, even if the daughters subsequently change relative position due to cell migration. In some places, such as at the anterior or posterior poles of the early embryo, steric constraints prevent the two daughters from remaining in strict anterior–posterior order, and their final positions are skewed relative to the initial orientation of the spindle.

A very small number of cells have variable ancestry. In several cases, a pair of cells constitutes an "equivalence group" in which each member of the pair can give rise to each fate. This is usually when pairs of cells formed on the left and right sides migrate to the ventral midline to form a single anterior–posterior series. For example, the cell ABplapaapp can become either of two ventral epidermal cells, P1 or P2, depending on whether it migrates to a midline position anterior or posterior to its contralateral homolog ABprapaapp. P1 is therefore denoted ABpl(lr)apaapp. Such fate choices involve an interaction between the members of the cell pair.

Go to:

V. Cell Identification and Lineage Analysis

A. Embryonic Cell Identification and Lineage Analysis

1. Manual Lineage Analysis

As all early embryonic blastomeres are very similar in morphology, early embryonic cell identification relies on the small number of cells involved and their invariant positions. Cell positions can be easily learnt up to the 28-cell stage (see Fig. 1). Between the 28-cell stage (100 min) and late gastrulation most cells can only be conclusively identified by following their lineage. After gastrulation (~240 min post first cleavage) cellular differentiation begins. Cells can be identified based on position relative to landmarks such as the first apoptotic cell deaths; maps of cell nuclei at 270 nuclei are in Figure 7 of Sulston *et al.* (1983).

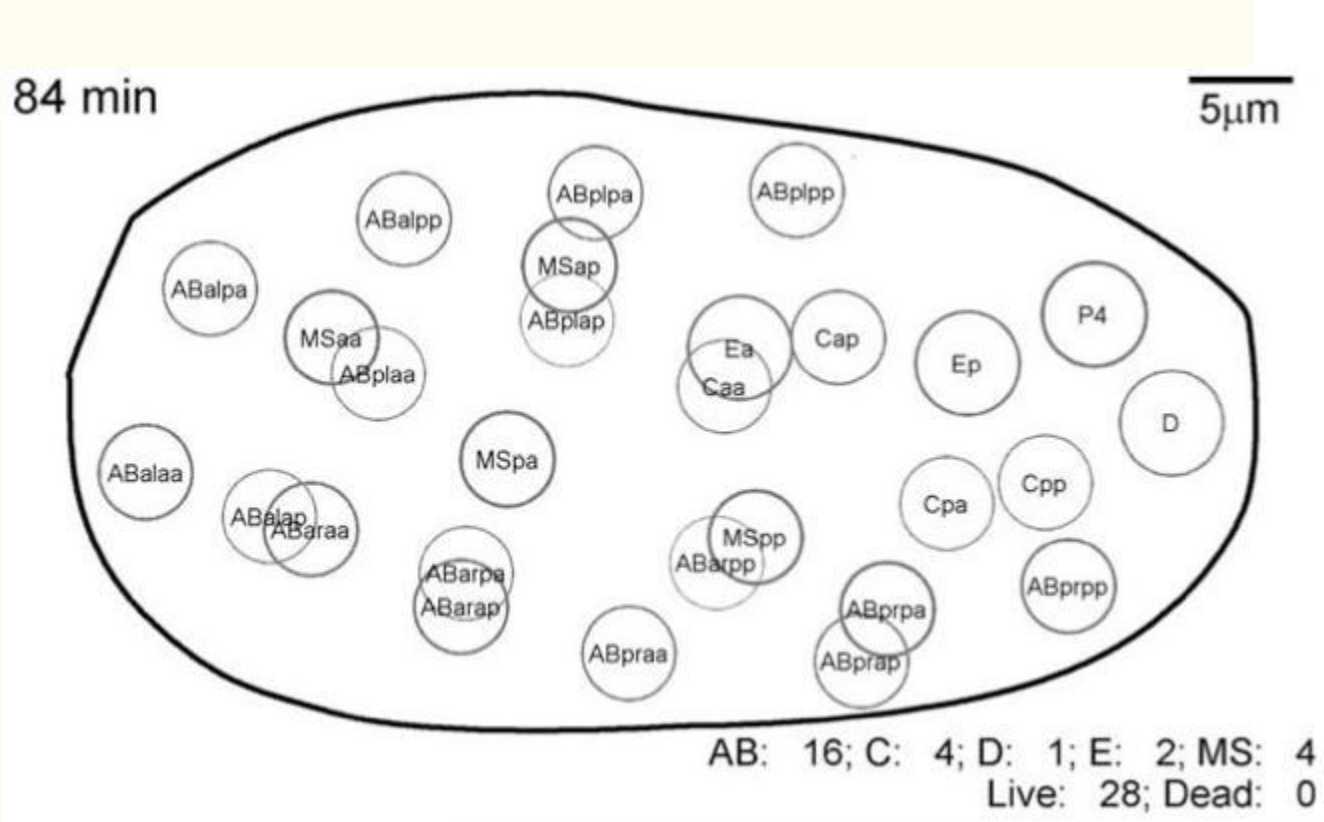

Fig. 1

Ventral view of wild-type embryo at 84 min/28-cell stage, redrawn from nuclear positions using NucleiTracker4D software (anterior is to left). Eggshell outline is an approximation based on nuclei filling in late embryogenesis. Depth is indicated by the thickness of the outline of each nucleus.

Cell and nuclear identification is more or less straightforward using the available maps up to the 1.75-fold stage when body wall muscle contractions begin. By the twofold stage the rolling activity of the embryo severely hampers attempts to identify cells in live specimens. To prevent this rapid movement the microscope objective can be transiently and reversibly cooled (Sulston *et al.*, 1983). Overall the last 3 h of embryonic development remain relatively little studied.

The cell lineage reported in Sulston *et al.* (1983) was a composite built up from partial lineages of hundreds of embryos. With the improvement of imaging and storage technology by the late 1980s it became possible to record the complete development of single embryos using timelapse imaging in multiple focal planes. Such “four dimensional” (4D) microscopy was developed by J. G. White and used for studies of early *C. elegans* embryos (Hird and White, 1993). Several groups developed software for 4D acquisition and playback (Fire, 1994; Thomas *et al.*, 1996). Commercial imaging software suites such as Improvision or Amira include options for automated 4D capture, as do the software suites for most commercial compound microscopes.

Finally the Open Source software suite Micro-Manager (http://valelab.ucsf.edu/~MM/MMwiki/index.php/Micro-Manager) can be used for automated 4D acquisition.

Cell-lineage tracing from DIC 4D movies requires manual identification of nuclei at each time point. Software such as SIMI Biocell (Schnabel *et al.*, 1997) (Table III) allows lineages to be generated in a straightforward manner through most of embryogenesis. The image degradation in deeper DIC focal planes limits complete lineaging of single embryos from DIC. Although computer image analysis can identify nuclei in DIC images (Hamahashi *et al.*, 2005) the low contrast of DIC images makes this computationally challenging.

Table III

Comparison of some methods for manual and semiautomatic embryonic lineage recording

Software name	**Data**	**Maximum nuclei tracked**	**Method**	**Time required**	**Error rate**	**Reference**
SIMI Biocell	DIC	385 cells full/114 cells up to 1.5 fold	Manual	N/A	N/A	(Schnabel *et al.*, 1997)
Angler	DIC	end of gastrulation/ 300 cells	Manual	N/A	N/A	(Martinelli *et al.*, 1997)
N/A	DIC	24 cells	Automated	N/A	N/A	(Hamahashi *et al.*, 2005)
StarryNite	GFP	350 cells	Automated	8 h up to	1–3% up to	(Bao *et al.*, 2006)

Software name	Data	Maximum nuclei tracked	Method	Time required	Error rate	Reference
				350 cells	194 cells, larger up to 350 cells	
Endrov	DIC/GFP	150 cells full/partial later stage	Manual	N/A	N/A	(Hench *et al.*, 2009)
NucleiTracker4D	GFP	566 live/ 669 total cells	Semiautomated	For GFP confocal = 4 h up to 350 cells 8–16 h to 525 cells	Up to 3% to 350 cells, up to 15% to 525 cells (at each time-point).	CAG & ADC, unpublished

2. Semiautomated Lineage Analysis

With the advent of histone-GFP reporters marking all embryonic nuclei, imaging and lineage analysis of embryo development could now be studied using the laser confocal microscope. The high fluorescence contrast of nuclear-localized GFP allows identification of nuclei by computer image segmentation in a more reliable fashion than from DIC data. Although photobleaching and phototoxicity limit the image quality, it is possible to image histone-GFP throughout embryonic

development. The histone-GFP itself is presumably synthesized anew during each cell cycle, partly compensating for photobleaching effects. The Waterston lab implemented the first histone::GFP imaging platform for following embryo development through gastrulation and morphogenesis. To track moving and dividing nuclei the Starrynite software was developed. Additional visualization software allows curation of the tracking data and extraction of lineage information.

The accuracy of the original Starrynite software declined in embryos with >350 nuclei. To allow nuclear tracking in later embryos several approaches are being tested. One approach is to optimize the segmentation algorithm for images of optically sectioned nuclei. Another approach, currently implemented in our laboratory (see below), is to curate nuclear identification at each time point, such that the automated tracking at the next time point t+1 starts with corrected information. A second modification to the search algorithm is to constrain the search for a particular nucleus (or its daughters) at t+1 only in the local neighborhood of its previous position at t. Rather than performing de novo segmentation, in this approach the maximum amount of information available at t is used for performing the segmentation and tracking for t+1.

3. Extent of Variation in the Wild-Type Lineage

The ability to completely track all nuclei in individual embryos has prompted further examination of the degree of variability in wild-type development. Automated lineage tracing has confirmed the high degree of invariance in the assignment of cell fates, with the known exceptions of the midline cell pairs mentioned above. Some cell-division axes in the C lineage display variability. Cell division times can vary by a factor of 10% between embryos, but within individual embryos the relative timing of cell divisions is highly consistent. The high degree of correlation of cell cycles within an individual embryo suggests the existence of a "developmental clock" controlling the rate of embryogenesis; the overall standard deviation in this developmental clock has been estimated at 4.5%.

Earlier lineage studies suggested that relative cell positions could show variability in midembryogenesis, although such variability decreases by the premorphogenetic stages. More recent studies have suggested that some of this variability may result from the slight compression introduced when an embryo is mounted on an agar pad or between slide and coverslip using beads. Compressed embryos display two stereotypical rotations. First, during gastrulation, the embryo turns from a left–right aspect to a dorsal–ventral aspect. Then, following epidermal enclosure, the embryo turns once again to display the left–right aspect and its comma shape. The increased variability in cell positions in such compressed embryos may reflect increased migration displacements in the flattened eggshell in conjunction with rotational movements. Unlike compressed embryos, freely mounted embryos attached to polylysine coated coverslips show less variability in cell positions and do not display the typical left–right/dorsal–ventral rotations. However, the increased depth of the uncompressed embryo leads to a slight loss of optical quality, and many laboratories continue to use slightly compressed embryos for optimal imaging (see chapter by Hardin).

B. Postembryonic Cell Identification and Lineage Analysis

Cell identification in larval and adult stages is facilitated by the increased separation of nuclei and the differentiation of cell types. However as development proceeds nuclei tend to have

slightly less stereotyped positions. Accurate identification of cells and nuclei is also complicated by the tendency of worms to move out of the field of view; at present there is no anesthetic or physical restraint that is compatible with long-term development.

Most cell types are readily identified by position and nuclear morphology. Complex cell groups such as the anterior ganglia require practice and tracing of cell positions from multiple animals. To begin identifying cells it is essential to start with simple easily recognized stages and tissues such as the 12-cell stage of vulval development. A novel approach to identifying new expression patterns is to analyze their intersection with previously characterized patterns using "split GFP".

Paralleling the automated lineaging efforts in the embryo, a 3D atlas of nuclear positions in L1 larvae. Generating a standard 3D representation of the L1 larvae nuclei is instrumental for mapping gene expression patterns or high-throughput computer-controlled functional screens. Atlas building depends on (1) reliable identification of larval nuclei, (2) registration of multiple larval samples into the same standard representation, and (3) mapping of novel samples onto this standard representation. To achieve these goals Long *et al.* used DAPI stained worms to mark all cell nuclei, followed by several image processing steps. First, the images of larvae are straightened to a rod shape. Next, nuclei are automatically identified by adaptive thresholding and rule- and training-based segmentation. Nuclei can be validated and curated using the volume-object image annotation system. In this way, 357 out of the 558 L1 larval nuclei could be faithfully identified.

Using fiduciary muscle nuclei GFP markers, Long *et al.* registered as many as 40 L1 larvae samples into a 3D standard representation. However, as few as 15 samples were sufficient to infer correct nuclear positions along the body. A comparison of cell positions in the atlas samples along the three axes of the body showed that nucleus-to-nucleus spatial relationships are invariant, especially among cells belonging to the same tissue. After building the standard representation, Long *et al.* developed an automated procedure for mapping and annotating novel samples, such as expression patterns, onto this reference. Overall, their automated segmentation can identify nuclei in certain tissues with >80% accuracy. Improving accuracy and the ability to segment all of the 558 nuclei of larvae will entail using higher resolution microscopy methods like selective plane illumination or stimulated emission depletion.

VI. Materials, Methods, and Protocols

A general protocol for mounting *C. elegans* on agar pads for live analysis is provided in the chapter by Shaham in Worm Methods (http://www.wormbook.org/toc_wormmethods.html); see also the methods appendix to the *C. elegans* I book.

A. Protocol 1: Analysis of Embryonic Cell Lineages

1. Mounting

Detailed protocols for mounting *C. elegans* embryos are provided in the chapter by Hardin. Traditionally *C. elegans* embryos have been mounted on agar pads with buffer and a coverslip. Although embryos are completely viable under such conditions, it is clear that this method

compresses the egg and eggshell. An uncompressed egg mounted in an aqueous medium is 50 μm long and 30 μm in diameter. Also Ref. Blanchoud *et al.*, 2010; *DevDyn* 239: 3285–96. Embryos mounted on agar pads are compressed to a thickness of ~20 μm. Mounting using spacer beads can also compress the embryo, depending on the bead size used. The lateral compression is helpful in reducing the number of optical sections needed for 4D lineage analysis and constraining the embryo to a fixed orientation for observation. However as mentioned in the text, compression may contribute to the variability in cell positions in early embryogenesis.

2. DIC Cell-Lineage Analysis and 4D Recording

Procedures for manual lineaging of embryos are described by . A number of software tools have been described over the years to allow time-lapse recording in multiple focal planes (4D recording) (see above). At present most commercial microscope vendors include 4D acquisition as an option. The minimal requirements are microscope equipped for Nomarski DIC optics, a motorized z-drive, a camera, and a computer workstation that controls the z-drive. A high-N.A. DIC objective (e.g., Zeiss Plan-Neofluar 100×) is essential for any lineage studies. Cell lineages can be traced manually from DIC 4D data sets. The software package SIMI Biocell is specifically designed to facilitate lineage construction from DIC 4D movies.

3. 4D Lineaging Using Histone-GFP

Procedures for 4D imaging of histone-GFP marked embryos. Briefly, single transgenic HIS-72::GFP(*zuIs*178) embryos are mounted between coverslips in 8 μL of a mixture of 20 μm polystyrene beads (Polysciences Inc., Warrington, PA) in 1% methylcellulose in M9 (15% v/v beads, 85% v/v 1% methylcellulose in M9). The coverslip sandwich can be flipped to display the desired late development dorsal or ventral aspect and then attached to a slide and sealed.

We use a Zeiss LSM510 confocal with a 30 mWArgon laser. We acquire confocal *z*-stacks of size $64 \times 35 \times 30$ μm^3 with resolution of $0.125 \times 0.125 \times 0.85$ μm^3/voxel every minute for the first 300 min of development then every 2 min for the next 180 min. Two-color (GFP/mCherry) movies can be acquired to correlate cell-specific expression patterns with the ubiquitously expressed histone-GFP. Laser power, detector, and acquisition configurations are loaded through the MultiTime macro in the Zeiss LSM software.

Precise temperature control is extremely important to maintain embryo viability over prolonged periods of confocal imaging. Although embryos will survive 4D DIC imaging throughout embryogenesis at 25 °C, we find the upper limit for confocal imaging is 24 °C; the viability of imaged embryos should be checked whenever 4D imaging is being set up for the first time. There are several options for control of specimen temperature; we have used a custom-designed aluminum casing for the objective. The casing is cooled or heated by a small Peltier element and a liquid cooling system designed for computer chips.

The analysis of 4D LSM data sets by Starrynite and Acetree is described. We provide here a brief overview of our nuclear tracking approach (Giurumescu *et al.*, in preparation). We analyze 4D LSM data sets with a user interface that combines the automated tracking and user-selected curation. At the first time point (usually 4–6 nuclei) the user manually identifies nuclei and names them according to the canonical wild-type lineage. For subsequent time *z*-stacks, the

software first performs an automatic segmentation and tracking step using a minimal movement algorithm and local neighborhood search. Nuclei that do not satisfy the strict minimal movement condition (i.e., those that move less than their radius from *t* to *t* + 1, usually less than 5% of nuclei), are flagged for manual curation. Nuclear divisions are also curated manually. At each time point the correct set of nuclei is annotated, preventing the accumulation of annotation errors. Our software does not search for nuclear radii as an additional free parameter. Our initial manual lineaging confirmed the initial observation that all nuclei in each of the major sublineages (AB, C, D, E, MS, and P4) show distinct radii values that linearly decrease with each round of division. Hence, our software prescribes nuclear radii values to all nuclei depending on their position in the lineage. Using this semiautomated approach it is possible to lineage essentially all nuclei up to the 1.5-fold stage (566 live nuclei, 103 cell deaths).

B. Protocol 2: Post-Embryonic Cell-Lineage Analysis

1. Worms to be lineaged must be in healthy, unstarved condition
2. Prepare a standard slide mount agar pad (cf. Sulston and Hodgkin methods appendix in *C. elegans* I). The agar should have been freshly prepared or melted.
3. Using a drawn-out capillary and mouth pipette pick up the worm(s) to be lineaged in a few microliters of M9 or S basal. Deposit the larva onto the agar pad together with a small volume of buffer. [If you are very dextrous, it is possible to do this with a worm pick, but small larvae are very easily injured. We recommend the mouth pipette.] Remove excess buffer by wicking with lens paper.
4. Using lens paper, wipe clean a small coverslip (18 × 18 mm OK, 12 × 12 mm best but can be hard to find). Using a worm pick, smear a small amount of OP50 *E. coli* onto the center of the coverslip. Place the coverslip gently over the buffer + worm so that the bacterial blob is within a couple of mm of the worm.
5. After 1–5 min the worm should become active and head toward the bacteria and start browsing contentedly. Under optimal circumstances, the worm will continue eating for hours, with occasional bouts of movement. If the worm does not move or begin eating within 10 min of mounting, it may be damaged.
6. If the worm appears healthy, trim the agar around the coverslip with a razor blade. Seal the edges of the coverslip with immersion oil or vaseline. Some brands of immersion oil are toxic and can interfere with long-term observation. Vaseline works fine unless your worm swims into it or you get some on the objective.
7. Find the area of interest in the animal and draw out everything you see as often as you can, identifying nuclei by reference to the standard maps in the papers in. With practice, multiple animals can be lineaged at a time, depending on the complexity of the lineage being traced. It is usually best to keep one animal per slide to avoid confusion.

1. Troubleshooting

The joy of observing a well-behaving worm is balanced by the frustration of a badly behaved worm that persistently heads for the edge of the agar pad, only to end up in the immersion oil or

vaseline. To avoid such frustration it is important to check your worm frequently (every 10 min) and to learn how to recover the worm intact from the slide mount. Practice sliding off the coverslip and getting the worm into a buffer-drop from which you can suck it back into your capillary.

If the worm stops moving and cells lose contrast, the animal may be dying, or it may be entering lethargus, the 1–2 h period of inactivity that precedes each molt. If the developmental stage makes the latter explanation unlikely, there may be too much bacteria under the slide, leading to hypoxia. The worm can be revived by removing it from the slide (slide off the coverslip and use mouth pipette + drawn-out capillary to retrieve the worm). Place the worm on an NGM agar plate to recover for a few minutes, then remount.

The microscope DIC optics should be optimized (Köhler illumination). A heat filter must be used to prevent specimen heating under the prolonged observation. Immersion oil should be used between the objective and coverslip and between slide and condenser top lens. Ensure there are no bubbles or debris in the agar pad; once a worm crawls into a bubble, it will not come out again.

VII. Discussion

Cell-lineage analysis allows rigorous definition of cell ancestries and positions, mutant phenotypes, and gene expression patterns to single-cell resolution. The labor-intensive nature of lineage tracing from live samples has tended to limit its popularity. The recent development of automated lineage analysis promises to reduce the effort needed for early embryonic lineage studies, but lineage tracing in later embryos and in larvae remains a labor of love. Further computational advances may help to return lineage studies to the center of *C. elegans* developmental biology.

1. The total number of cells in a culture is counted using the trypan blue exclusion assay and is found to be 2.7 x 106 cells/ml. The culture is diluted 1:27 and then 100µl seeded per well into a 96 well plate. What is the final cell density per well? 6 well plate requires 2 x 105 cells. How should the solution be diluted so that 1ml can be added to each well?	a) 1 x105	b) 2.7 x 10_4	c) 2.7 x 105	d) 1 x 104	d) 1 x 104
	a) 1:6.8 then 1:10	b) 1:10 then 1:2	c. c) 1:3.4 then 1:10	d) 1.3.4 then 1:2	c. c) 1:3.4 then 1:10
3. Subculturing a cell line always increase the passage number. Is this true or false?	a. True	b. False			a. True
4. Transferrin is present in serum media.	a) True	b) False			b) False
5. What is the full form of MEM in Eagle's cell culture medium?	a) Maximum evaporating medium	b) Maximum essential medium	c) Minimum essential medium	d) Minimum evaporating medium	c) Minimum essential medium
6. Serum does not require sterilization.	a) True	b) False			b) False
7. Vaccine is not a serum.	a) True	b) False			a) True
8. Hybridoma cells have an application to produce:	a) Antigens	b) Antibodies	c) Cancer cells	d) Cell lines	b) Antibodies
9. Baculovirus infects insect cell lines and are also pathogenic to humans.	a) True	b) False			b) False
10.Nontransformed cell lines form monolayer whereas transformed cell lines form multilayer.	a) True	b) False			a) True
11. Transformed cell lines are immortal.	a) True	b) False			a) True
12. EDTA is carcinogenic.	a) True	b) False			b) False
13. Which of the following is not a type of basic T-Flask?	a) T-25	b) T-55	c) T-75	d) T-175	b) T-55
14. What does "T" refers to in Tissue culture flask (T-Flask)?	a) Total volume of the flask	b) Total weight of the flask	c) Total surface area of the flask	d) Total mass of the flask	c) Total surface area of the flask
15. Primary plant cells are better formed aggregates than animal cells.	a) True	b) False			a) True
16. Embryo initiation is facilitated by_____	a) GA3	b) BPA	c) ABA	d) 2,4 D	d) 2,4 D
17. Which of the following culture is used for the production of primary and secondary metabolites?	a) Cell suspension culture	b) Callus culture	c) Protoplast culture	d) Somatic hybrid	a) Cell suspension culture
18. Which of the following does not act as a fusogen in protoplast fusion?	a) 2,4 D	b) Polyethylene glycol	c) Calcium	d) PVA	a) 2,4 D
19. What is the name of the tissues which helps in protection and support of the body?	a) Muscular tissue	b) Nervous tissue	c) Connective tissue	d) Epithelial tissue	c) Connective tissue
20. Which of the following does not belong to the class of covering and lining epithelium?	a) Simple squamous epithelium	b) Glandular epithelium	c) Simple cuboidal	d) Simple columnar	b) Glandular epithelium
21. Which of the following is NOT true regarding glandular epithelium?	a) Secretion is done only by a group of cells	b) Present in cluster form	c) Takes part in the secretion	d) Lie deep to the covering and lining epithelium	a) Secretion is done only by a group of cells
22. Name the tissues that are involved in the formation of membranes.	a) Epithelial tissue	b) Nervous tissue	c) Muscular tissue	d) Connective tissue	a) Epithelial tissue
23. Name the tissues which detect changes inside and outside the body and respond by action potential?	a) Epithelial tissue	b) Connective tissue	c) Muscular tissue	d) Nervous tissue	d) Nervous tissue
24. Group of cells, which has a common embryonic origin and function have been characterized as tissues.	a) True	b) False			a) True
25. Group of cells, which is similar in structure and function are structured into_____	a) Organ system	b) Muscles	c) Bone	d) Tissues	d) Tissues
26. Name the organism on which first cell line was observed?	a) E.coli	b) Sheep	c) Mouse	d) Drosophila	c) Mouse
27. Which of the following is the characteristics of a normal cell?	a) Anchorage independent	b) Continuous cell lines	c) Dependent on external growth factor	d) No contact inhibition	c) Dependent on external growth factor
28. Name the type of culture which is prepared by inoculating directly from the tissue of an organism to culture media?	a) Primary cell culture	b) Secondary cell culture	c) Cell lines	d) Transformed cell culture	a) Primary cell culture

Primary Cell Culture: 3 Techniques

This article throws light upon the three types of technique used for primary cell culture. The three types of technique are: (1) Mechanical Disaggregation (2) Enzymatic Disaggregation and (3) Primary Explant Technique.

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

With the experiences of the personnel working in tissue culture laboratories, the following criteria/ characteristics are considered for efficient development of primary cultures:

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

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

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

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

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

Techniques for Primary Culture:

Among the various techniques devised for the primary culture of isolated tissues, three techniques are most commonly used:

1. Mechanical disaggregation.

2. Enzymatic disaggregation.

3. Primary explant technique.

An outline of these techniques is depicted in Fig. 36.1, and the procedures are briefly described:

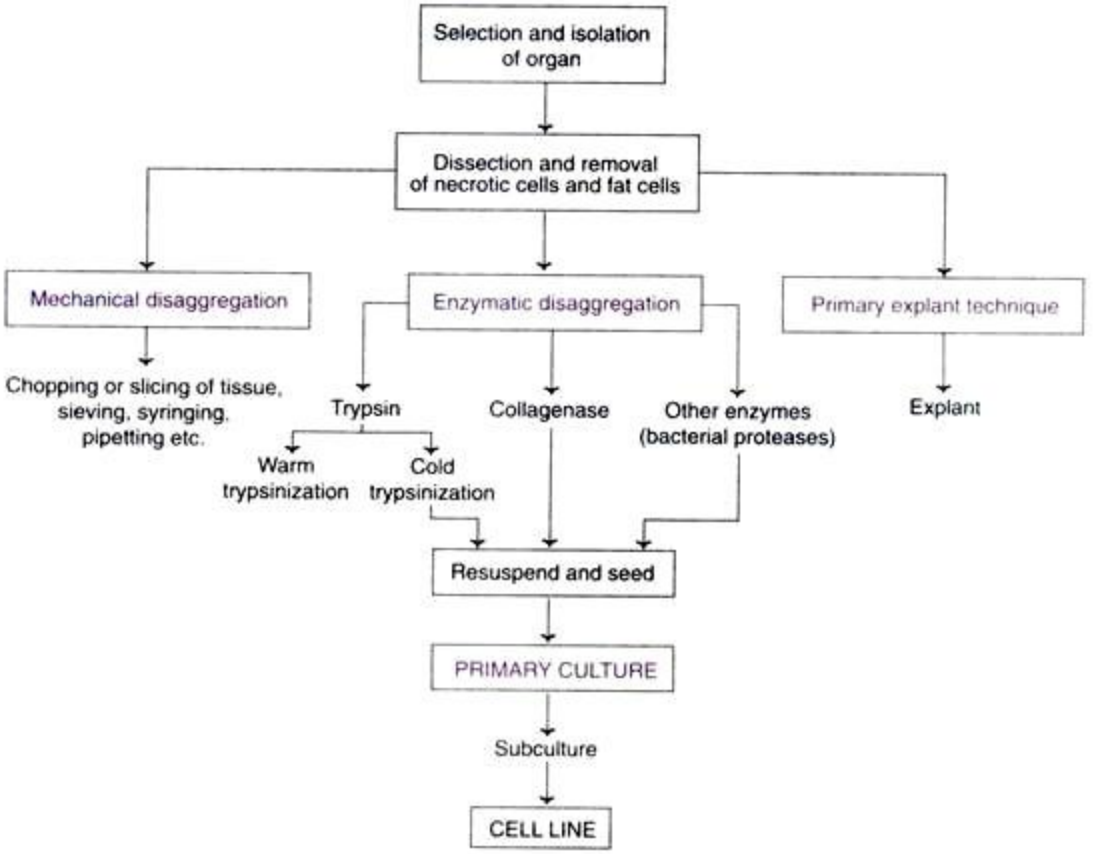

Fig. 36.1 : Different techniques used for primary culture.

Technique # 1. Mechanical Disaggregation:

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

The cells can be collected by two ways:

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

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

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

Technique # 2. Enzymatic Disaggregation:

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

Disaggregation by trypsin:

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

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

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i. The crude trypsin is more effective due to the presence of other proteases

ii. Cells can tolerate crude trypsin better.

iii. The residual activity of crude trypsin can be easily neutralized by the serum of the culture media (when serum-free media are used, a trypsin inhibitor can be used for neutralization).

Disaggregation of cells can also be carried out by using pure trypsin which is less toxic and more specific in its action. The desired tissue is chopped to 2-3 mm pieces and then subjected to disaggregation by trypsin. There are two techniques of trypsinization-warm trypsinization and cold trypsinization (Fig. 36.2).

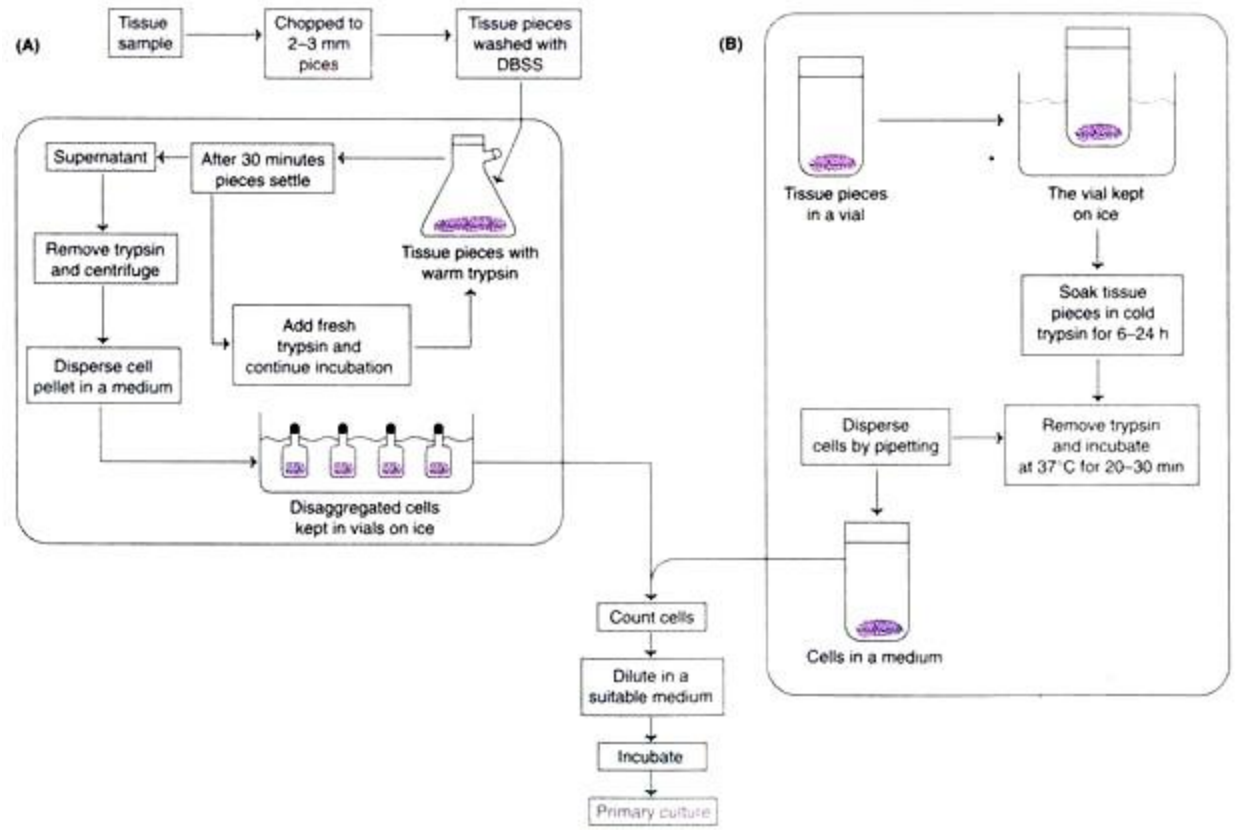

Fig. 36.2 : Preparation of primary culture by trypsin disaggregation (A) Warm trypsinization (B) Cold trypsinization (DBSS–Dissection basal salt solution).

Warm trypsinization (Fig. 36.2A):

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

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

Cold trypsinization (Fig. 36.2B):

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

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

The cold trypsinization method usually results in a higher yield of viable cells with an improved survival of cells after 24 hours of incubation. This method does not involve stirring or centrifugation, and can be conveniently adopted in a laboratory. The major limitation of cold trypsinization is that it is not suitable for disaggregation of cells from large quantities of tissues.

Limitations of trypsin disaggregation:

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

Disaggregation by collagenase:

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

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

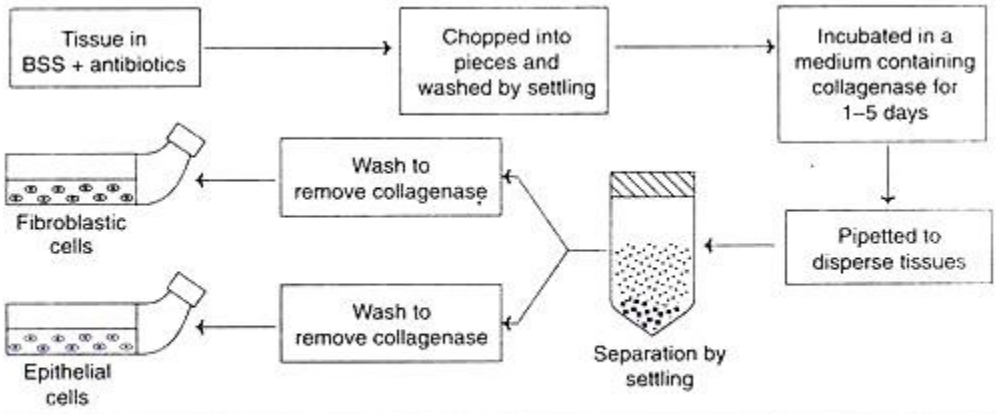

Fig. 36.3 : Important stages in collagenase disaggregation of tissue for primary culture (BSS–Basal salt solution).

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

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

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

Use of other enzymes in disaggregation:

Trypsin and collagenase are the most widely used enzymes for disaggregation. Certain bacterial proteases (e.g. pronase, dispase) have been used with limited success. Besides hyaluronidase, neuraminidase is also used in conjunction with collagenase for effective degradation of cell surface carbohydrates.

Technique # 3. Primary Explant Technique:

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

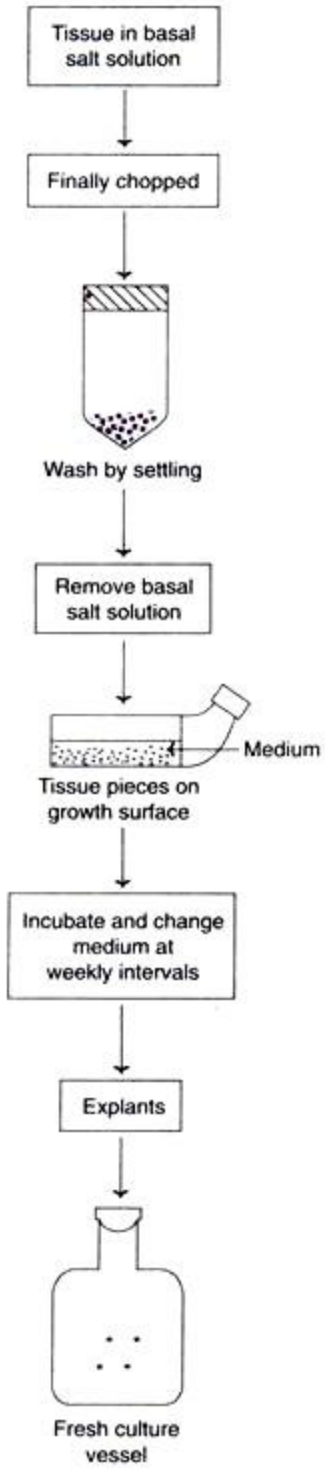

Fig. 36.4 : Primary explant technique for primary culture.

The tissue in basal salt solution is finely chopped, and washed by settlings. The basal salt solution is then removed. The tissue pieces are spread evenly over the growth surface. After addition of appropriate medium, incubation is carried out for 3-5 days. Then the medium is

changed at weekly intervals until a substantial outgrowth of cells is observed. Now, the explants are removed and transferred to a fresh culture vessel.

The primary explant technique is particularly useful for disaggregation of small quantities of tissues (e.g. skin biopsies). The other two techniques mechanical or enzymatic disaggregation however, are not suitable for small amounts of tissues, as there is a risk of losing the cells.

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

Separation of Viable and Non-Viable Cells:

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

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

Medical Ethics and Safety Measures in Culture Techniques:

Since the culture techniques involve the use of animal or human tissues, it is absolutely necessary to follow several safety measures and medical ethics. In fact, in some countries there are established legislation/norms for selection and use of tissues in cultures. For example, in United Kingdom, Animal Experiments (Scientific Procedures) Act of 1986 is followed.

The handling of human tissues poses several problems that are not usually encountered with animal tissues. While dealing with fetal materials and human biopsies, the consent of the patient and/his or her relatives, besides the consent of local ethical committee is required. Further, taking any tissue (even in minute quantities) from human donors requires the full consent of the donor in a prescribed format.

The following issues need to be fully considered while dealing with human tissues:

1. The consent of the patient and/or relatives for using tissues for research purposes.

2. Ownership of the cell lines developed and their derivatives.

3. Consent for genetic modification of the cell lines.

6. Patent rights for any commercial use of cell lines.

In the general practice of culture techniques using human tissues, the donor and/or relatives are asked to sign a disclaimer statement (in a prescribed pro-forma) before the tissue is taken. By this approach, the legal complications are minimized.

Safety measures:

Handling of human tissues is associated with a heavy risk of exposure for various infections. Therefore, it is absolutely necessary that the human materials are handled in a biohazard cabinet. The tissues should be screened for various infections such as hepatitis, tuberculosis, HIV, before their use. Further, the media and apparatus, after their use must be autoclaved or disinfected, so that the spread of infections is drastically reduced.

Manipulation of Animal cell line:

During the last three decades, animal cell culturing has been essential for biomedical research and biotechnological activities in general. Along with this increasing importance, biosafety concerns have pointed to the risks of manipulating animal cell cultures for human health and the environment. A maximal reduction of these risks necessitates a thorough risk assessment of the cell cultures used. It involves an evaluation of both the intrinsic properties of the cell culture, including subsequent properties acquired as a result of genetic modification, and the possibility that the cell culture may inadvertently or deliberately become contaminated with pathogens. The latter is a major hazard associated with the manipulation of animal cell cultures, as adventitious agents may be pathogenic and have a better capacity to survive in unfavorable conditions. Consequently, most of the containment measures primarily aim at protecting cells from adventitious contamination. Therefore, a comprehensive evaluation of the risks en-countered during the handling of cell cultures should include considerations regarding the type of manipulation as well. As a rule, cell cultures known to harbor an infectious etiologic agent should be manipulated in compliance with containment measures recommended for the etiologic agent. With the exception of very well-characterized cell cultures for which the use of a type II biosafety cabinet depends on the origin of the cells, work with cell cultures from human or primate origin should generally and minimally be performed under containment level 2 using a type II biosafety cabinet. In every case, containment measures should minimize adventitious

contamination of the cell cultures and offer a maximal protection of human health and the environment.

Cell Lines: Types, Nomenclature, Selection and Maintenance (With Statistics)

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Read this article to learn about the types, nomenclature, selection and maintenance of cell lines.

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

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

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

Types of Cell Lines:

Finite Cell Lines :

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

Continuous Cell Lines :

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

The continuous cell lines are transformed, immortal and tumorigenic. The transformed cells for continuous cell lines may be obtained from normal primary cell cultures (or cells strains) by treating them with chemical carcinogens or by infecting with oncogenic viruses. In the Table. 36.1, the different properties of finite cell lines and continuous cell lines are compared.

Table 36.1 Comparison of properties of finite and continuous cell lines

Property	*Finite cell line*	*Continuous cell line*
Growth rate	Slow	Fast
Mode of growth	Monolayer	Suspension or monolayer
Yield	Low	High
Transformation	Normal	Immortal, tumorigenic
Ploidy	Euploid (multiple of haploid chromosomes)	Aneuploid (not an exact multiple of haploid chromosomes)
Anchorage dependence	Yes	No
Contact inhibition	Yes	No
Cloning efficiency	Low	High
Serum requirement	High	Low
Markers	Tissue specific	Chromosomal, antigenic or enzymatic

The most commonly used terms while dealing with cell lines are explained below.

Split ratio:

The divisor of the dilution ratio of a cell culture at subculture. For instance, when each subculture divided the culture to half, the split ratio is 1: 2.

Passage number:

It is the number of times that the culture has been sub-cultured.

Generation number:

It refers to the number of doublings that a cell population has undergone. It must be noted that the passage number and generation number are not the same, and they are totally different.

Nomenclature of Cell Lines:

It is a common practice to give codes or designations to cell lines for their identification. For instance, the code NHB 2-1 represents the cell line from normal human brain, followed by cell strain (or cell line number) 2 and clone number 1. The usual practice in a culture laboratory is to maintain a log book or computer database file for each of the cell lines.

While naming the cell lines, it is absolutely necessary to ensure that each cell line designation is unique so that there occurs no confusion when reports are given in literature. Further, at the time

of publication, the-cell line should be prefixed with a code designating the laboratory from which it was obtained e.g. NCI for National Cancer Institute, Wl for Wistar Institute.

Commonly used cell lines:

ADVERTISEMENTS:

There are thousands of cell lines developed from different laboratories world over. A selected list of some commonly used cell lines along with their origin, morphology and other characters are given in Table. 36.2.

Table 36.2 A selected list of commonly used cell lines

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

Selection of Cell Lines:

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

Some of them are briefly described:

ADVERTISEMENTS:

1. Species:

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

2. Finite or continuous cell lines:

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

ADVERTISEMENTS:

3. Normal or transformed cells:
The transformed cells are preferred as they are immortalized and grow rapidly.

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

ADVERTISEMENTS:

5. Growth characteristics:
The following growth parameters need to be considered:
i. Population doubling time

ii. Ability to grow in suspension

iii. Saturation density (yield per flask)

iv. Cloning efficiency.

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

7. Phenotypic expression:
ADVERTISEMENTS:

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

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

Cell Morphology:

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

Replacement of Medium:

Periodic change of the medium is required for the maintenance of cell lines in culture, whether the cells are proliferating or non-proliferating. For the proliferating cells, the medium need to be changed more frequently when compared to non-proliferating cells. The time interval between medium changes depends on the rate of cell growth and metabolism.

For instance, for rapidly growing transformed cells (e.g. HeLa), the medium needs to be changed twice a week, while for slowly growing non-transformed cells (e.g. IMR-90) the medium may be changed once a week. Further, for rapidly proliferating cells, the sub-culturing has to be done more frequently than for the slowly growing cells.

The following factors need to be considered for the replacement of the medium:

1. Cell concentration:

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

2. A decrease in pH:

A fall in the pH of the medium is an indication for change of medium. Most of the cells can grow optimally at pH 7.0, and they almost stop growing when the pH falls to 6.5. A further drop in pH (between 6.5 and 6.0), the cells may lose their viability.

The rate of fall in pH is generally estimated for each cell line with a chosen medium. If the fall is less than 0.1 pH units per day, there is no harm even if the medium is not immediately changed. But when the fall is 0.4 pH units per day, medium should be changed immediately.

3. Cell type:

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

4. Morphological changes:

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

Since the first report of immobilized cells in 1966, the area has expanded very rapidly. However, only a few industrial processes are based on immobilized cells probably because profitable processes for large-scale cultivation of freely suspended procaryotic cells already exist. With animal cell culture, however, the advantages of immobilization are more profound and there already exist industrial processes based on immobilized cells. It is also very likely that immobilized animal cells will find an increasing importance in the correction of diseases.

IMMOBLILIZATIONOFANIMAL CELLS

Technical Field

The present invention relates to microcarriers. More particularly, the present invention relates to improved microcarriers and their use in the immobilization and culturing of anchorage-dependent animal cells, and to the ready removal of such cells from such micro- carriers. The surface-area limitations associated with culturing anchorage-dependent animal cells in, for example, roller bottles to a large degree have been overcome through the use of microcarriers Where the cells per se comprise the desired product, however, problems still remain relative to the efficient harvesting of such ceils.

Background Art

In the past, there has been considerable interest in the immobilization of cells, particularly those of microbial origin. K. Mosbach, Ed., "Methods in Enzym ology, " Vol. 44, Academic Press, New York, 1976; and

I. Chibata and T. Tosa in L. B. Wingard et al., "Applied Biochemistry and Bioengineering, " Vol. 1, Academic Press, New York, 1976, pp. 329-357. More recently, such interest has been extended by the reported immobilization of living plant cells in suspension. P. Brodelius et al., FEBS Letters, 103, 93-97 (1979).

The use of microcarriers for culturing mammalian anchorage-dependent cells in suspension has been given increasing attention in recent years. For example, a DEAE-Sephadexmicrocarrier was used in some early experiments as a support for the cultivation of fibroblast-like cells derived from embryonic rabbit skin (H ceils) and diploid human embryonic lung cells (HRL cells). A. L. van Wezel, Nature, 216, 64-65 (1967). vanWezel has continued to study the use of DEAE-Sephadexmicrocarriers for the homogeneous cultivation of primary cells and diploid cell strains. See, e.g., A. L. van Wezel, "Microcarrier Cultures of Animal Cells," in P. F. Kruse, Jr. and M. K. Patterson, Jr., Editors, "Tissue Culture: Methods and Applications, " Academic Press, New York, 1973, pp. 372-377; A- L. van Wezel et al., Develop, biol. Standard., 42, 65 (1979), presented at the 2nd General Meeting of ESACT, Paris 1973; and A. L. van Wezel et al., Process

Biochemistry, 13, 6 (1978). Interestingly, other workers also concentrated on the use of DEAE-Sephadexmicrocarriers. For example, Levine et al. proposed adding to the culture medium containing DEAE-Sephadexmicrocarrier beads a negatively charged non-nutritive component, e.g., carboxymethyl- cellulose, to compete with the positively charged sites on the microcarrier. They also screened various support (microcarrier) materials which appeared to include silica gel and several ion-exchange resins, as well as Sephadex per se. The DEAE-Sephadex beads were in the 90-105 μ range. D. W. Levine et al., "Optimizing Parameters for Growth of Anchorage-Dependent Mammalian Ceils in Microcarrier Culture," in "Cell Culture and Its Applications," Academic Press, New York, 1977, pp. 191-216. See also U.S. Patent No. 4,036,693 to D. W. Levine et al. Additional work by D. W. Levine et al. indicated that such microcarrier treatments as that described above can be eliminated if the charge capacity of the micro- carrier is adjusted or controlled within a certain range, thereby resulting in good growth of a wide variety of anchorage-dependent cells. Such range is from about 0.1 to about 4.5 milliequivalents per gram of dry material. The microcarriers, however, still appear to be positively charged, and the most suitable microcarrier apparently is DEAE-Sephadex. U.S. Patent No. 4,189,534 to D. W. Levine et al., or British Patent Specification No. 1,535,150 to Massachusetts Instititue of Technology. See also D. W. Levine, Ph.D. Thesis, "Production of Anchorage-Dependent Mammalian Cells on Microcarriers," Massachusetts Institute of Technology, 1977. Of course, materials other than DEAE-Sephadex have been employed as microcarriers. See, for example, U.S. Patent No. 3,717,551 to B. Bizzini et al., which discloses the cultivation of cells on porous silica spherules. Moreover, the immobilization of cells for cultivation purposes has been achieved by means other than through the use of microcarriers, such as by entrapping the cells within a polymeric or gel-like matrix. 3y way of illustration only, see P. Brodelius et al., FEBS Letters, 103, 93 (1979), which describes the entrapment of plant cells within alignate beads.

Finally, the use of gelatin, collagen, and related materials in the cultivation of cells is known. For example, U.S. Patent No. 4,169,761 to P. Precausta et al. discloses the cultivation of cells on collagen fibers which are dispersed or suspended in the nutrient medium. The use of collagen in the form of gel particles for the cultivation of cells on microcarrier surfaces also has been reported; the same report mentions similar uses for cellophane, cellulose sponge, and DEAE-Sephadex, among other materials, c-b. C. Horng, Ph.D. Dissertation,

"Primary Culture of Mammalian Cells on Microcarrier Surface," Graduate School, State University of New York at Buffalo, 1975, pp. 4, 5, 136, and 138. See also, J. Leighton, "Collagen-Coated Cellulose Sponge," in P. E. Kruse, Jr., Ed., "Tissue Culture: Methods and Applica tions," Academic Press, New York, 1973, pp. 367-371 [Chem. Abstr., 81, 23770n (1974)].

According to French Patent No. 2,419,321, DEAE- Sephadex beads can be pretreated with calf fetus serum to reduce the loss of the inoculum and the lack of reproducibility in cell cultivation procedures using DEAE- Sephadexmicrocarriers.

It perhaps should be noted that microbial cells have been incorporated in glutaraldehyde-crosslinked gelatin to give an immobilized glucose isomerase. U.S. Patent No. 4,191,810 to N. Yoshikazu et al. Also, the fractionation and manipulation of cells have been accomplished through the use of fiber fractionation techniques - the fiber typically is nylon, optionally coated

with, gelatin or a gelatin derivative which permits cell recovery by the simple expedient of melting the gelatin or gelatin derivative. See, e.g., G. M. Edelman and U. Rutishauser, "Specific Fractionation and Manipulation of Cells with Chemically Derivatized Fibers and Surfaces," in W. B. Jakoby and M. Wilchek, Editors, "Methods in

Enzymology, " Vol. 34, Academic Press, New York 1974, pp. 195-209.

With respect to harvesting or removing cells from microcarriers, the use of trypsin is well known. See, e.g., D. W. Levine et al. in "Cell Culture and Its Applications," Academic Press, New York, 1977, p. 207; U.S. Patent Nos. 4,036,693 and 3,717,551; and French Patent No. 2,419,321.

Disclosure of Invention It therefore is an object of the present invention to provide a microcarrier suitable for use in the immobilization and cultivation of anchorage-dependent animal cells and having a reduced cost.

It also is an object of the present invention to provide a microcarrier suitable for use in the immobilization and cultivation of anchorage-dependent animal cells, from which microcarrier such cells are readily removed if required. A further object of the present invention is to provide a magnetic microcarrier suitable for use in the immobilization and cultivation of anchorage-dependent animal cells, which microcarrier has a reduced cost and from which microcarrier such cells are readily removed if required.

Yet another object of the present invention is to provide a method for entrapping various animal cell types in alginate or agarose gels.

Still another object of the present invention is to provide a method for the ready removal of anchorage- dependent animal cells from the microcarriers of the present, invention.

These and other objects will be apparent to one having ordinary skill in the art from a consideration of the disclosure and claims which follow.

Accordingly, the present invention provides a method for the immobilization of animal cells, characterized in that anchorage-dependent cells are adsorbed on a micro- carrier which is enzymatically degradable without significant destruction of cell surfaces.

The present invention also provides a microcarrier for the immobilization of animal cells, characterized in that said microcarrier is enzymatically degradable without significant destruction of cell surfaces and that upon enzymatic degradation of said microcarrier having anchorage-dependent animal cells adsorbed thereon, said cells are released intact.

The present invention further provides a method for the immobilization of animal cells capable of cell division, characterized in that said cells are entrapped in a carrier comprising alginate or agarose. In a preferred embodiment, the microcarrier is a naturally occurring protein or polysaccharide or derivative thereof which is essentially water-insoluble at ambient temperature.

Brief Description of Drawings

Figure 1 illustrates the growth of two primary cell cultures on the gelatin beads of the present invention. Figure 2 illustrates the release of microcarrier- bound cells using trypsin, collagenase, and dispase, respectively.

Modes for Carrying Out the Invention

In the course of our studies to obtain a suitable gel matrix for entrapment of animal cells, we found, in analogy to T. Elsdale and J. Bard, J. Cell Biol., 54, 626-637 (1972), that collagen substrate adhered to ceils. On further investigation, a simple technique leading to the preparation of solid beads, 100-250 μm, of the closely related gelatin (the product obtained on boiling collagen) was developed. All the cells tested attached and proliferated. In this context, magnetic gelatin beads were also prepared and, as expected, no adverse effect on cell growth was observed. Potentially, such supports would permit facile recovery when used in media of viscous or particulate nature. Furthermore, it is conceivable to suspend the beads by applying an outer magnetic field, thereby minimizing the need for vigorous agitation when using carriers of high specific weight. Beads of ubiquitous chitosan, a partially deacetylated product of the polysaccharide chitin, were also prepared using a similar procedure and found to lead to attachment of cells. Their properties as supports for cell growth will be tested further. In our investigations to find supports for cell growth of anchorage- dependent cells, we coupled polylysine covalently to CNBr-activated agarose beads. Although nothing definite can be said at this point on the attachment capacity of such preparations for the cell types tested, it was found that agarose alone activated with high concentrations of CNBr did lead to attachment and growth of cells, probably due to the positive charge introduced on the matrix.

In our experiments involving detachment of cells, we attempted an alternative approach as there was the possibility with the new microcarriers used to enzymic ally dissolve the support directly. Both dispase and collagenase dissolved the beads, leading in the case of dispase to a clear solution. In contrast to trypsin treatment which involves destruction of the cell sur face to accomplish cell removal, the approach taken here should leave the entire cells intact and viable which is an advantage, e.g., when the cells are used for immunization or when analysis of surface antigens will be done. Summarizing, as gelatin is inexpensive and repre- sents a more 'natural' surface for cell attachment than those commercially available, it appears that such microcarriers will turn out to be valuable supports for cell growth as also strongly indicated by the spinner culture experiments reported here. The ease of cell release adds to the merits of such microcarriers. Furthermore, the microcarriers can be degraded substantially completely, thereby preventing the readsorption of the cells.

The studies reported here on the entrapment of animal cells in gels were initiated because such immobilized preparations should offer the same advantages as those observed with microorganisms or plant cells. For instance, isolated cells, when immobilized in the entrapped state, would allow convenient assay in analytical metabolic perfusion studies or could be used for the enzymic conversion of certain metabolites by analogy to steroid transformation using entrapped bacteria [P.-O. Larsson et al., Nature, 263, 796-797 (1976)] or be utilized for, e.g., hormone production. These data demonstrate that cell entrapment leaves at least the major part,

if not all, of their metabolic machinery intact. We applied particularly one of the more recently developed entrapping techniques, i.e., inclusion in calcium alginate [M. Kierstan and C. Bucke, Biotechnol. Bioeng., 19, 387-397 (1974); J. Klein and F. Wagner, DECHEMA-Monographien, 82, 142 (1979); and S. Ohlson et al., Eur. J. APPI. Microbiol.Biotechnol., 7, 103-110 (1979)].

It could be demonstrated with isolated rat hepatocytes that the cells remained at least partially intact on immobilization, as disruption of the cell membrane would have lead to leakage of the enzyme, lactate dehydrogenase. Subsequently, islets of Langerhans from mouse pancreas were immobilized by the same procedure, giving preparations still capable of insulin production/secretion. We also showed that adipocytes, entrapped in either calcium alginate or agarose, were still capable of metabolizing added radioactive glucose to fatty acids and could be stimulated further on addition of insulin. A drawback of the calcium alginate procedure is the requirement for Ca^{2+} or similar ions to keep the network intact and, following this, the necessity to operate in phosphate-free media. Entrapment in agarose particles, as described here and applied in the studies of free fatty acid release of adipocytes, may offer an alternative approach.

With regards to anchorage-dependent cells, good microcarriers including those described here are at hand, whereas for suspension cultures entrapment appears the best alternative for immobilization. Furthermore, anchorage-dependent cells might profit from entrapment within a three-dimensional network while adsorbed to their normal carrier as this would give some protection against, e.g., shear force. It may even be that anchor age-dependent cells for which no suitable microcarrier has yet been found may accept the polymer network as recipient attachment surface or at least remain viable as demonstrated here using trypan dye exclusion tests. Although no clear cell proliferation has yet been ob served with the various entrapped cell types, they remain viable for a considerable time and permit metabolic studies, enzymic transformations or syntheses to be carried out.

The present invention is further illustrated by the example which follows. Such example, however, is not be be construed as limiting the present invention in any manner.

Example A. MATERIALS AND METHOD A.1. Materials

Chitosan, agarose (type VII), alginic acid (sodium salt, type IV), collagenase (type I, 190 U/mg) were obtained from Sigma. Glutaraldehyde (25%) was from Merck AG. Cytodex and Sepharose CL-6B were products of Pharmacia, Sweden, and the magnetic particles, Ee-0_4 (5 μm), were a gift from Hδganas, Sweden. Arlacεl 83 was obtained from Atlas Chemical Co.,dispase (grade II, 0.5 U/mg) was from Boehringer. Gelatin (commercial grade) was from Kebo, Sweden. A.2. Cell Types

The following cells were kindly provided by Professor O. Sjδgren: the established cell lines HeLa and K562 (erythroleukemic), and the primary cell cultures S 157N (human skin fibroblasts), S 158A (human kidney carcinoma), DMH W49 (rat. colon carcinoma) and DME W1073 (rat colon carcinoma). All cells except K 562 were grown in Waymouth media supplemented with 20% fetal calf serum. X 562 were grown in SPMI medium with 10% fetal calf serum. All media were supplemented with gentamicin (50 mg/1).

A.3. Gelatin Microcarriers

A gelatin solution (10 ml; 20% (w/v)) obtained by heating to 50°C was dispersed under vigorous stirring in 100 ml of a mixture of toluene:chloroform (73/27, v/v) containing 2% (w/v) of Arlacel 83 at room temperature. After 10 min the mixture was filtered through a 100 μm nylon net whence, the collected microcarriers were transferred to acetone. After careful washing with acetone the microcarriers were evaporated to dryness. In order to obtain beads which would resist higher temperatures, cross-linking with glutaraldehyde was carried out. Dry beads (1.5 g) were reswollen in 100 ml water followed by the addition of 20 ml 25% glutaraldehyde. After gentle stirring for 30 min the beads were collected and washed with 0.15 M NaCl on a 100μm nylon net. The beads were dispersed in 0.15 M NaCl and autoclaved at 120°C for 15 min. In order to get a suitable size distribution of the beads, they were first filtered through a 250μm nylon net and the beads in the filtrate were then collected on a 100 μm net. They were then transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min. Magnetic microcarriers were obtained by the same procedure except that in addition to 9 ml 20% (w/v) gelatin solution, 1 g Fe_3O_4 particles were added prior to dispersion in the organic phase. A.4. Chitosan Microcarriers

A 2% (w/v) solution of chitosan in 1% formic acid was obtained by mixing chitosan with 1% formic acid and stirring overnight, followed by filtration of any undis solved material through a 250 μm nylon net. The chitosan solution (18 ml) was mixed with 2 ml 25% glutaraldehyde and dispersed under vigorous stirring in the same organic phase as described for gelatin microcarriers. After 15 min the mixture was transferred to methanol. The beads were then washed on a glass filter with methanol and subsequently with 0.15 M NaCl. After auto claving at 120°C for 15 min (to obtain more rigid beads), the preparation was first filtered through a 250 μm nylon net after which the beads in the filtrate were collected on a 100 μm nylon net. The beads were transferred to 0.15 M NaCl and sterilized through autoclaving at 120°C for 15 min. A.5. Test for Cell Growth Before use, all microcarriers were washed with 10 vol. medium. Gelatin and chitosan microcarriers (0.25 g wet wt) were mixed with 2.5 X 10^5 cells (S 157N, S 157A, DMH W49 and DMH W1073, respectively) in 5 ml media in 10 ml siliconized .test tubes. They were placed in a $C0_2$-incubator (5%) at 37°C and mixed in an end-over-end shaker. The cultures were. checked daily and the medium was replaced if necessary. The gelatin microcarriers containing Fe_3O_4 were tested with DMH W49. Sepharose CL-6B beads activated, with different amounts of CNBr were placed in 20 mm Petri dishes and mixed with 2.5 X 10 DMH W49 cells in 3 ml medium. The cells S 157N and DMH W1073 were allowed to grow on gelatin microcarriers (1 g beads, wet wt) suspended in 50 ml spinners. The starting cell concentrations were 35,000/ml and 22,000/ ml, respectively; 60% of the medium volume was replaced each day. For determination of the cell number, duplicate samples of 1 ml suspension were taken. After washing with phosphate-buffered saline (PBS) without

Ca 2+ and Mg2+, the beads were incubated with trypsin (0.1% in PBS with 0.02% EDTA and without Ca^{2+} and Mg^{2+}) for 15 min at 37°C and the released cells counted in a

Börker chamber.

A.6. An Alternative Method for Harvesting of Cells

After 6 days the spinner culture of DMH W1073 was divided into 3 equal fractions after washing as above. They were incubated with 3 ml enzyme solution, trypsin as described above and collagenase (2 mg/ml) in PBS with

Ca^{2+} but without Mg^{2+} and dispase (4 mg/ml) in Waymouth medium at room temperature. Samples were taken at intervals and the cell concentration determined. A.7. Alginate Immobilization

Alginate (1 part 4% (w/v)) dissolved in 25 mMHepes, 125 mMNaCl (pH 7.4) was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. The cell alginate suspension was extruded through a 0.8 mm nozzle into a solution of 25 mMHepes, 50 mM $CaCl_2$, 75 mMNaCl (pH 7.4), whereby beads with av. diam. 2 mm were formed. Immobilization was carried out at room temperature. After 5 min the beads were washed with medium and transferred to a spinner flask, which was placed in a CO_2-incubator at 37°C. At intervals beads were withdrawn and dissolved in 0.1 M EDTA (pH 7.4) and the cells were counted. A.8. Agarose Immobilization Agarose (1 part 4% (w/v)) dissolved in 25 mMHepes, 125 mMNaCl (pH 7.4) placed in a waterbath at 37°C was mixed with 1 part cell suspension in medium supplemented with twice the concentration of serum. This solution was made into beads by moulding it in a form made of Teflon. Solution was poured over a Teflon plate tightly covered with 3 mm holes. Another plate was used as support and the two were held together by clamps. Before moulding, the form was warmed to 37°C. After the agarose had solidified the form was taken apart and the 'cylindrical beads' were taken out. The beads were put in medium in a spinner flask and placed in a CO_2-incubator at 37°C. At intervals beads were taken out and dissolved by heating to 70°C and the cells were counted. A.9. Preparation of Entrapped β-Cells of Islets of Langerhans

Ninety isolated islets of Langerhans (av. diam. ~ 0.1 mm) were prepared from rat [I. Lundquist, Enzyme, 12, 647-657 (1971)] pancreas and washed with Krebs- Ringer buffer lacking phosphate [I. Lundquist, supra]. The cells were suspended in 0.125 ml buffer and mixed with 0.5 ml 2.5% (w/v) sodium alginate in the same buffer. The cell alginate suspension was extruded into a solution of the buffer containing 50 mM $CaCl_2$. After 15 min the beads were washed 3 X 10 ml with the buffer. Visual inspection showed that the beads contained an average of one islet/bead.

The scintillation vials were each filled with 10 beads and 2 ml buffer, gassed for 1 min with carbogen (95% O_2, 5% CO_2) and preincubated for 30 min at 37°C in a waterbath with shaking. After addition of 1 mg glucose to the vials, they were gassed and 0.1 ml samples taken after 0, 30, 60 and 120 min. The samples were frozen and stored until analyzed for insulin using radioimmunoassay. Visual inspection showed no release of the islets into the medium. A.10. Preparation of Entrapped Adipocytes

Rat adipocytes were prepared according to J. Gilemann, Diabetologia, 2 , 382-388 (1967). For entrapment in alginate the cells were transferred to 20 mMHepes, 130 mMNaCl, 2% (w/v) albumin (pH 7.4) mixed with an equal volume of 2% (w/v) alginate (autoclaved for 15 min at 120°C) and extruded into a solution of 25 mMHepes, 50 mM $CaCl_2$, 75 mMNaCl (pH 7.4). After 5 min the beads were washed with Krebs-Ringer buffer con taining 24 mMHepes, 0.55 mM glucose and 1% (w/v) albumin (storage medium).

With entrapment in agarose the cells were transferred to the above storage medium containing 2% (w/v) albumin and mixed with an equal volume of 5% (w/v) agarose. Beads were prepared as in section 2.8. After gelling the beads were transferred to storage medium. B. RESULTS B.1. Cell Attachment and Growth In a preliminary test, various cells were tested for their capacity to attach to different microcarriers (Table 1). All cells would attach to the various beads. Studies on cell growth were subsequently carried out with the most promising microcarrier, gelatin beads with the cells S 157N and DMH W1073. Upon plotting cell concentrations for the two primary cell cultures S 157N and DMH W1073 grown on gelatin beads versus culture time, it is seen that good growth occurred with no indication of cell death. Such plot is shown in Figure 1, which is based on the mean of two cell counts; curve A is for S 127N and curve B is for DME W1073.

Table 1

Attachment of primary cell cultures to different microcarriers

	S 157N	S 158A	DMH W49	DMH W1073
Gelatin	+	+	+	+
Gelatin (magnetic)	n.t.	n.t.	+	n.t.
Chitosan	+	+	+	+
CNBr-activated Sepharose CL-6B	n.t.	n.t.	+	n.t.

n.t., not tested

B.2. Harvesting of Cells

In Figure 2, the results of three different en zymic procedures leading to cell detachment from gelatin microcarriers are given. As seen, both collagenase (curve A) and dispase (curve B) gave rise to a far higher total cell concentration than normally applied trypsin treatment. B.3. Cell Entrapment

I . Isolated cells : In a preliminary study to test whether animal cells would survive entrapment in a gel matrix, hepatocytes from rats were isolated [C. H. Floren and A; Nilsson, Biochem. J. , 168, 483-494 (1977)] and entrapped in calcium alginate. The obtained beads were subsequently incubated in perfusion buffer and water, respectively. Release of lactate dehydrogenase was monitored. About 10- times lower leakage of the enzyme was found in the sample kept in the perfusion buffer compared to the sample in water. Subsequent incubation in water of the beads previously kept in perfusion buffer, however, led to considerable leakage of active lactate dehydrogenase. Subsequently, islets of Langerhans containing β-cells were isolated and entrapped following the same procedure to check whether the latter were capable of insulin production/secretion in the immobilized state. Over 0, 30, 60 and 120 min, samples were

withdrawn from the medium and assayed for insulin content using radioimmunoassay analysis. The amount of insulin secreted was 1, 23, 40 and 78 immunoreactive insulin units (μU/ml), respectively.

Finally adipocytes were isolated and entrapped in both Ca-alginate as well as agarose. They were subsequently tested for their capability to incorporate [3-^{3}H] glucose into lipids [A. J. Moody et al., Horm. Metab. Res., 6, 12-16 (1974)] following insulin stimulation as well as for their ability to release free fatty acids by noradrenaline stimulation [N. 0. Nilsson and P. Belfrage, J. Lipid Res., 20, 557-560 (1979)]. It was found that, at the outset, the immobilized adipocyte preparation showed a higher basal incorporation than free cells which was more pronounced with cells immobilized in Ca^{2+}-alginate. On stimulation with insulin, both preparations showed an increase in glucose incorporation (when higher concentrations of alginate were used, the cells did-not respond to insulin in the glucose test). The free fatty acid release was only studied with agarose-immobilized adipocytes as the alginate beads were too soft. The immobilized cells also showed a response to added noradrenaline by releasing free fatty acids after about twice the time required for free cells, and at ~1/3rd the rate for free cells.

II. Cell cultures: A number of cell types, i.e., fibroblasts, HeLa, DMH W49 and K 562, the latter growing in suspension, were entrapped in gels to test whether they would grow within a gel-matrix or remain viable. The gels prepared were beads of: (a) 2% alginate; (b) 2% agarose; (c) a mix ture of 2% agarose and 0.5% alginate; and (d) 2% agarose containing simultaneously entrapped Cytodex particles to which cells already had been attached. In all cases serum was entrapped simultaneously with the cells as in-diffusion of some of the components of serum was likely to be hindered. No proliferation of cells tested was observed under the conditions used; however, on average, 10-30% of the cells remained viable as judged by the trypan blue exclusion test, after 1 week of incubation. In controls run with HeLa cells, non-entrapped but kept free in solution in siliconized tubes, they had disappeared after

1 day.

It is to be understood that the foregoing de tailed description is given merely by way of illustration and that many variations may be made thereon without departing from the spirit and scope of the invention.

Immobilisation of Cells: Meaning and Applications | Biotechnology

Immobilisation of whole cells has been defined as the physical confinement or localisation of intact cells to a certain defined region of space with preservation of some, or most, catalytic activity.

The increased stability under extreme conditions of pH and temperature, as well as the re-use and applicability in continuous processing systems that enclose immobilised cells instead of soluble enzymes make the cells a preferred, versatile tool in both food industry and medicine.

There are several different approaches to the classification of immobilised biocatalysts, but the most frequently employed classification is based upon the method of immobilisation selected for a specific application.

The selection of immobilisation method depends, therefore, upon the application, the nature of the microorganism being immobilised, as well as the resources available. Table 1 shows several, possible immobilisation methods that available for whole microbial cells. Most immobilisation methods can be applied either to whole cells or to enzymes.

Some of the advantages of whole-cell immobilisation in comparison with enzyme immobilisation are: the higher stability and enzyme activity, multivariate enzyme applications, and the lower cost.

On the other hand, disadvantages of using whole cell immobilisation in comparison with enzyme immobilisation are linked to the increased diffusional barriers caused by the much larger sizes of cells in comparison with enzymes.

Table 1. Immobilisation Methods for Whole Microbial Cells.

Binding		Flocculation
	Weak bonds	Adsorption
		Ionic
	Strong bonds	Covalent
		Cross-linking
		Thermal Gelation
	Entrapment	Ionotropic Gelation
		Polymerization
Physical retention	Membrane Retention	Dialysis Culture
		Ultrafilters

Adsorption is the least expensive and mildest immobilisation method. It uses weak interaction forces such as hydrogen bonds, hydrophobic interactions and Van der Waal forces to immobilise cells or enzymes.

However, the sensitivity of this interaction to pH makes the leakage of cells immobilised by this technique quite common. Important applications of this technique are related to the production of fructose and vinegar, and also waste water treatments.

Ionic binding uses the properties of negatively charged microbial cells to interact with positively charged ion exchangers. The results obtained with this technique are also sensitive to extreme pH values, and the binding strength is greater in comparison with adsorption. However, the mild conditions employed by this technique make it suitable for use for immobilisation of both enzymes and whole cells.

Covalent binding and cross- linking offer better strength than the previous techniques; however, there is an encountered toxicity in the reagents that are used to produce immobilisation. Entrapment techniques are, however, the most commonly encountered in the industry and they are based on the formation of thermally reversible gels, ionotropic gels and polymerisation.

Application of Immobilisation of Cells:

Entrapment of cells in a gel-like matrix by ionotropic gelation using alginates and carrageenans is certainly the most useful method for industrial purposes. The properties of the gel-like matrix allow the cell to remain viable and with its catalytic ability for a long period of time.
For example, an increment in the yeast concentration obtained through immobilisation techniques has helped the brewing industry to reduce fermentation process times and the size of their storage facilities. Unfortunately, because of the high concentration of diacetyl, and the low concentration of higher alcohols and esters, the flavour of the fast fermented beer has been compromised.

Even the amino acid profile has been altered. The main factor causing this uncommon imbalance is the insufficient mass transfer in the older designs of fermentation reactors; thus, the use of new reactor designs, and combining technologies could improve the quality of the products obtained through such fermentation reactions. Nutraceuticals are defined as food components that have health benefits beyond traditional nutritional value.

Novel biotechnology tools like immobilisation were also applied for the isolation and incorporation of such food components in ordinary foods. The synthesis of nutraceuticals was reported to be successful by employing immobilised lipases, such as those from Candida antartica and Lactobacillus ruteri. The introduction of conjugated linoleic acid (CLA) in dairy foods has been made possible through the immobilisation of lipases.

There is a quite extensive list of immobilisation technique applications in medicine. A very important group of such applications is concerned with the regulation of equilibrium between coagulation and dissolution of coagulated blood (fibrinolysis) through the use of immobilised enzymes.

The high probability of death caused by thrombosis (involving the formation of clots in the blood vessels), has committed physicians to the use of fibrinolytic therapy for the treatment of occlusions in those parts of the body where a surgical intervention would be too risky.

Amongst the most important enzymes that have been immobilised for use in such therapy are Plasmin and Heparin. The use of biotechnology as well as microscopic techniques has helped refine and greatly improve such therapeutical means.

Current regulations for the disposal of toxic chemicals in the environment as well as the detoxification of water used in any agricultural and industrial process brings the need for novel biotechnology tools to be developed in order to solve such problems in a cost-efficient manner.

Enzymes have been isolated from genetically manipulated microorganism strains with the purpose of accelerating the rate of degradation of organic and some inorganic compounds in wastewater as well as in soils.

Synchronization of cell

Mammalian cells are amenable to study the regulation of cell cycle progression in vitro by shifting them into the same phase of the cycle. Procedures to arrest cultured cells in specific phases of the cell cycle may be termed in vitro synchronization. The procedure described here was developed for the study of primary astrocytes and a glioma cell line, but is applicable to other mammalian cells. Its application allows astrocytes to reenter the cell cycle from a state of quiescence (G(0)), and then, under carefully defined experimental conditions, to move together into subsequent phases such as the G(1) and S phases. A number of methods have been established to synchronize mammalian cell cultures, which include physical separation by centrifugal elutriation and mitotic shake off or chemically induced cell cycle arrest. Yet, there are intrinsic limitations associated with these methods. In the present protocol, we describe a simple, reliable, and reversible procedure to synchronize astrocyte and glioma cultures from newborn rat brain by serum deprivation. The procedure is similar, and generally applicable, to other mammalian cells. This protocol consists essentially of two parts: (1) proliferation of astrocytes under optimal conditions in vitro until reaching desired confluence; and (2) synchronization of cultures by serum downshift and arrested in the G(0) phase of the cell cycle. This procedure has been extended to the examination of cell cycle control in astroglioma cells and astrocytes from

injured adult brain. It has also been employed in precursor cloning studies in developmental biology, suggesting wide applicability.

Cell Synchronization Types: Microbial and Mammalian Cell Synchronization

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ADVERTISEMENTS:

Cell Synchronization Types: Microbial and Mammalian Cell Synchronization!

Cells remaining in same physiological phase giving balanced growth are called synchronous culture.

Then, synchrony, no matter how achieved, will last for many generations in unlimited growth medium under conditions met by at least certain prokaryotes.

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Efforts to produce the desired synchronization of cell division have been made in both microbial as well as in mammalian cell cultures.

In both systems cells synchronization is carried out for various purposes. Also, in both systems synchronous cultures can be divided into two broad procedures. They are: physical method and chemical method. These are achieved by induction synchrony and selection synchrony. Induction synchrony makes use of inducing cells by some treatment to divide synchronously.

But selection synchrony uses a fraction of cells of a growing culture at a particular phase of the growth cycle. This fraction of cells is then grown separately as a continuous culture. Each of the methods can have several techniques. Cells which could be synchronized are E. coli, C. utilis, S. cerevisiae and CHO (mammalian).

A. Microbial Cell Synchronization:

1. Concepts:

The induction synchrony used in microbial system can be carried out by three techniques e.g., inhibitor block, starvation and growth and multiple changes of temperature or light. Likewise the selection synchrony may also be achieved by three techniques. These are gradient separation,

membrane elution and filtration. Besides this growth limitation by cell surface has also been used.

ADVERTISEMENTS:

The dependency of specific growth rate (μ) on substrate concentration (s) followed Monod's law. Growth is first order at low and zero order at high concentration. One may consider μ_m to be independent of surface to volume (A/V = S) because at high substrate concentration the transport capability is high and un-limiting. However, the first order constant is dependent on S_0.
This is because the rate of uptake of a cell suspended in a medium of low substrate concentration is proportional to the cell surface area. For augmentation of this concept K_s in Monod's Law is replaced by KVS_0. The validity of this substitution is demonstrated by considering cells growing at very low substrate concentration or under starvation where cell surface area determines the growth rate.
Thus, the method of synchronizing which has been used widely with microorganisms is to starve a culture. It allows the culture to run into a stationary phase and then to add fresh medium, the culture usually grows synchronously for one or more divisions. This starvations synchrony has been best utilized in developing synchronous budding yeasts. The purpose of such synchronization has been for regulation of cell proliferation, biochemical and/or biological events in such phases.

2. Theoretical aspects:

(i) Surface to Volume Ratio for Rods with Hemispherical Poles:

In order to fix ideas and to indicate computational method, it is necessary to consider simplest case for an organism whose shape is a right cylinder with hemispherical poles. The volume is given by,

$$V = \frac{4}{3}\pi R^3 + \pi R^2 (L - 2R)$$

or
$$V = \pi R^2 \left[L - \left(\frac{2}{3} R \right) \right] \quad (6.16)$$

and the area

$$A = 4\pi R^2 + 2\pi R (L - 2R)$$

$$A = 2\pi RL \quad (6.17)$$

where R = radius, L = end to end distance. The length of a cell of a particular volume can be found from a rearrangement of equation 6.16.

$$L = \frac{V}{\pi R^2} + \frac{2}{3} R \quad (6.18)$$

Then substituting L in equation 6.16, 6.17 and 6.17 the surface area to volume ratio becomes

$$S_0 = \frac{A}{V} = \frac{2\pi R \left[\frac{V}{\pi R^2} + \frac{2}{3} R \right]}{\pi R^2 \left[L - \frac{2R}{3} \right]}$$

$$S_0 = \frac{2}{R} + \frac{4}{3} \frac{\pi R^2}{V} \quad (6.19)$$

Using surface area to volume ratio through cell cycle as a parameter it may be possible to maintain synchrony of such cell growth.

(ii) Growth Limited by Cell Surface : The dependency of specific growth rate (μ) on substrate concentration may be expressed by Monod's model

$$\mu = \frac{\mu_m s}{k_s + s} \quad (6.20)$$

In analogy to Fick's diffusion law

$$\frac{dq}{dt} = D.A. \frac{dc}{dx} \quad (6.21)$$

one can unite
$$\frac{dV}{dt} = YPAs \quad (6.22)$$

In equation 6.21, q is the quantity transported, D is the diffusion constant, A is the surface area, and dc/dx is the concentration gradient. In equation 6.22, Y is the yield coefficient (corresponding to the cellular volume produced by a unit amount of the limiting nutrient), P is the permeability constant (which is the diffusion constant divided by the thickness of the membrane, i.e. D/dx) and s is the external substrate concentration (since the internal concentration is presumed to be maintained at zero). For uptake always limited by an active

transport system, P would not have the meaning of a permeability constant, but would have the significance of

$$\frac{n V_{max}}{k_m + V}$$

for the n permease assemblies on a unit surface area (V_{max} and K_m are the kinetic constants of the permease assembly). Dividing both sides of equation 6.22 by V and substituting S_0, with A/V, equation 6.22 becomes

$$\frac{dV}{Vdt} = YP\, s_0 \,.\, s \qquad (6.23)$$

If the dilution cycle results in a high concentration for part of the cycle then for that part, this relationship would be

$$\frac{dV}{Vdt} = \mu_{max} \qquad (6.24)$$

Equation (6.24) can be written in the form

$$m = \frac{dV}{Vdt} = m_{max} \frac{x}{(s'/s_0)} \qquad (6.25)$$

Where μ_{max}/k' has been substituted for YP. These two equations are the high and low substrate limits where diffusion through the medium is never limiting. The Monod relation of bacterial growth is a relationship analogous to the Michaelis-Menton enzyme law covering both extremes. In the present purpose it would be given by

$$\frac{dV}{V \cdot dt} = \frac{\mu_m s}{[(k'/s_0) + s]} \qquad (6.26)$$

The Blackman "Law of the Minimum" formulation of growth is probably more generally pertinent instead of the hyperbola. Equations (6.24) and (6.25) apply as well in the intermediary concentration and growth as synchrony is determined by

$$\frac{dV}{V \cdot dt} = \frac{s}{(k'/s_0)}, s \leq s_c \qquad (6.27)$$

and

$$\frac{dV}{V \cdot dt} = \mu_{max}\, s > s_c \qquad (6.28)$$

where s_c is the critical concentration such that uptake of the critical substrate just becomes nonlimiting i.e.

$$s_c = \frac{\mu_{max}\, k'}{s_0} \qquad (6.29)$$

indicating transfer from starvation medium to growth medium.

(iii) Importance of Cell Synchronization:

ADVERTISEMENTS:

In starvation stress synchrony, the cells (S. cerevisiae) subjected to starvation induction degrade its existing proteins at considerable rate. This leads to the generation of an amino acid pool that is utilized to synthesize new proteins whose induction is unique to the maintenance of synchrony in starvation state.

This method of synchronizing has been used widely with microorganism culture. The principle is to run the culture into the stationary phase and then add fresh synthetic medium. The starvation synchrony as used for budding yeasts has been depicted in Fig. 6.5.

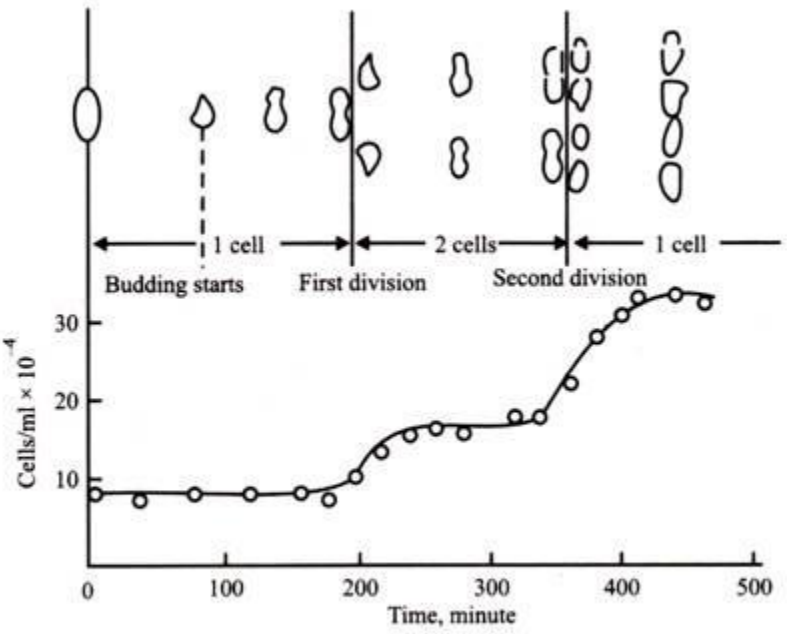


Fig. 6.5 Growth of a synchronised yeast culture (*Saccharomyces cerevisiae*) on inoculation into synthetic medium.

It shows in developing synchronized yeast growth the first cycle is very long, because of the long lag phase of about 75 minutes, which precedes the start of building. Using membrane elution technique its characteristics in comparison to other relevant types of culture can be realized from the summarized form given in Fig. 6.6. Interesting implications of phased or synchronous culture may concern growth rate, cell composition, variability in the cell cycle of growing and non-growing cells.

ADVERTISEMENTS:

In developing starvation synchrony in bacterial cultures like E. coli it has been observed that at the onset of starvation it undergoes a temporarily ordered program of starvation gene expression involving 40-80 genes which some four hours later yields cells possessing an enhanced general resistance.

Two classes of genes are induced upon carbon starvation stress, the cst genes requiring cyclic AMP and pex genes not requiring this nucleotide for induction. On careful analysis of carbon starved synchronous culture of E. coli by 2D gel autoradiographs, it was revealed that up to 50 proteins are induced upon carbon starvation.

As depicted in Fig. 6.6 in membrane elution technique, a random growing culture (E. coli) is collected on a membrane filter. The filter is then inverted allowing fresh medium to run through and thus excess cells are washed off. The new born cells which are released from the filter surface are suitable for growing as a synchronous culture.

The distribution of cell ages in a random culture appears as given in Fig. 6.7(a) and the relative concentration level of cells in the elute after loading and eluting shows the profile as in Fig. 6.7(b). If one follows the rate of synthesis of macromolcules and the elution pattern of radioactivity per cell from the membrane culture, which was radio pulse-labeled with a radioactive precursor of the macromolecules, the profile that appears to be as in Fig. 6.8 a and b.

A fairly clear picture of the synthetic pattern of DNA in different media can be observed in this synchronization mode. Phased culture in comparison to other strategies is performed at steady repeated condition state as shown in Fig. 6.9. From this figure it can be observed that in batch and continuous cultures the individual cells are at different points of development in their replication cycles. However, in phased culture cells grow approximately in unison during the cell cycle. This enables cell size amplification.

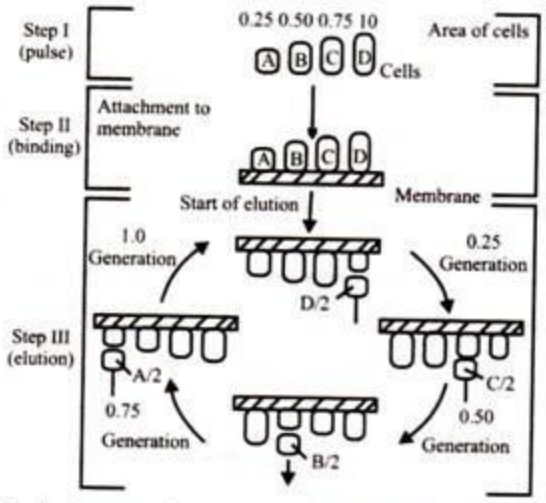


Fig. 6.6 Pulse-labelling before membrane elution with *Escherichia coli*. Outline of the procedure for determining the rate of incorporation of a labelled molecule into cells of different ages in an exponential unsynchronised culture

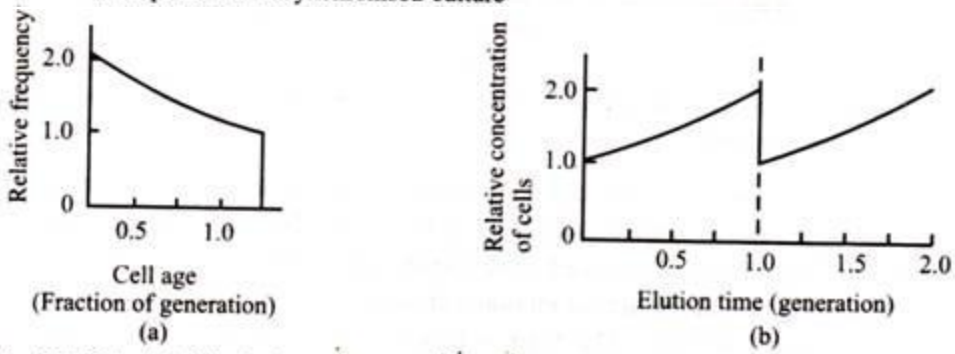


Fig. 6.7 Pulse-labelling before membrane elution with *Escherichia coli*, (a) idealised age distribution in an exponential phase culture containing no dispersion of generation times of individual cells. (b) Theoretical concentration of cells in the eluate from a membrane-bound culture with an age distribution as shown in Fig 6.7 (a)

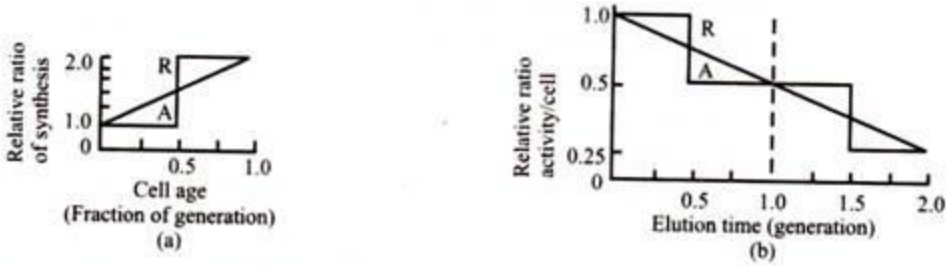


Fig. 6.8 Pulse labelling before membrane elution with *Escherichia coli*. (a) Rate of synthesis of two hypothetical macromolecules through the cell cycle. (b) Theoretical radioactivity per cell in the eluate from a membrane-bound culture if it has been pulse-labelled with the radioactive precursors of the macromolecules in (a)

B. Mammalian Cell Synchronization

1. Purposes and methods:

Like in microbial cells the methods of synchronizing mammalian cells in culture serve various purposes.

The important purposes of mammalian cell synchronization relate to the regulation of the following:

1. Cell proliferation, biochemical and/or biological events in each phase.

2. Cell metabolism and other cell cycle-dependent events (protein, MAb synthesis)

3. Cell attachment

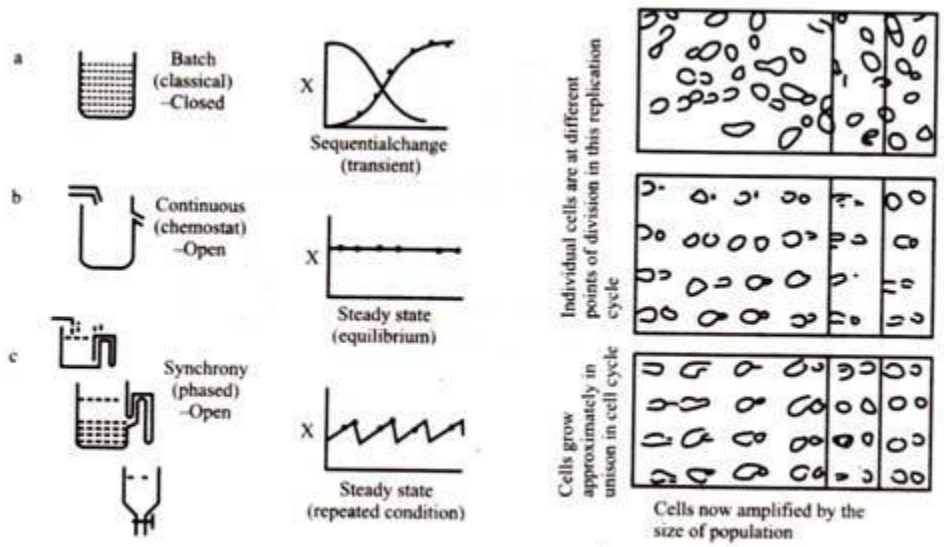


Fig. 6.9 Cell amplification[20]

In this system also method of synchronization can be divided into two broad classes.

1. Physical methods

i. mitotic attachment

ii. ficoll gradient centrifugation

2. Chemical methods

i. double thymidine block (DTB)

ii. isoleucine deprivation (ID)

iii. serum deprivation (SD) and hydroxy urea (HU)

By double thymidine block synchronous DNA synthesis in mammalian cells (CHO) could be achieved. However, the degree of synchrony has been observed to be cell line dependent (Fig. 6.10 a and b)

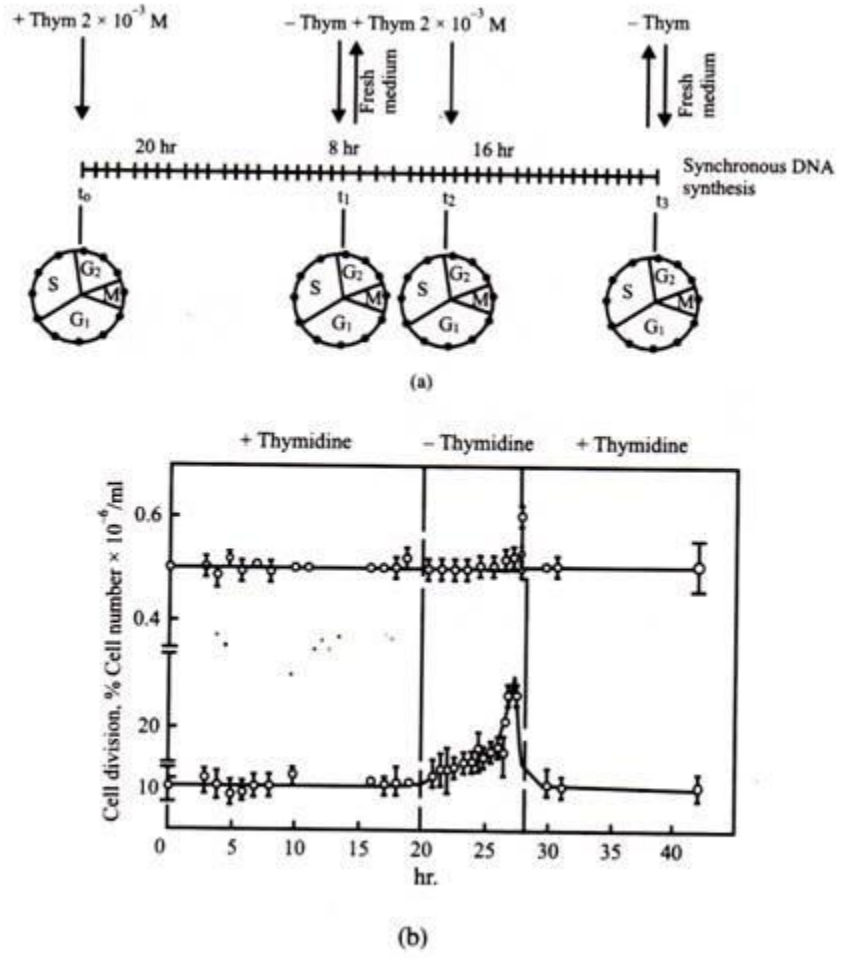


Fig. 6.10 Double thymidine block

In isoleucine deprivation cells (CHO) are incubated in F10 medium without isoleucine and glutamine plus 15% serum for 48 hrs, then isoleucine and glutamine are added to the normal concentration. The degree of synchrony is fair (Fig. 6.11).

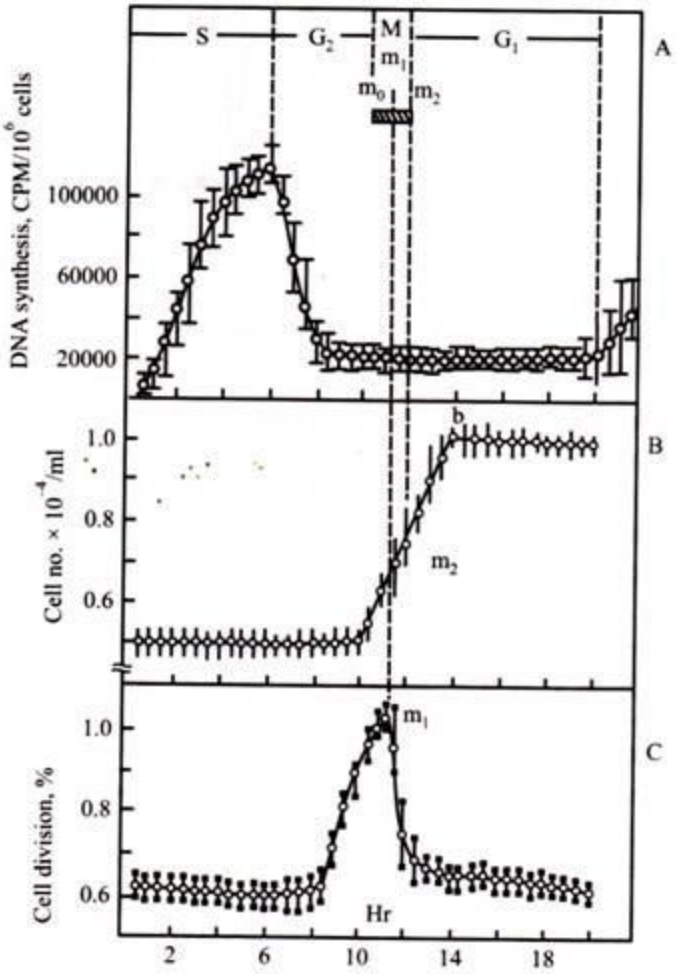


Fig. 6.11 Isoleucine deprivation to develop synchrony

In SD and HU method cells are incubated with low serum (1% or 0.5%) medium for 48 hrs, then serum-deficient medium is replaced by medium containing 10% serum, six hrs later HU is added to a final concentration of 1.5 mM and incubated for another 14 hrs. After that cells are re-suspended in fresh medium. The degree of synchrony has been stated to be excellent for obtaining cells synchronized at G_x/S boundary (Fig. 6.12).

2. Comparison of the methods:

Examination of figures 6.10 through 6.12 may reveal an inter-comparison of the methods as given in table 6.3.

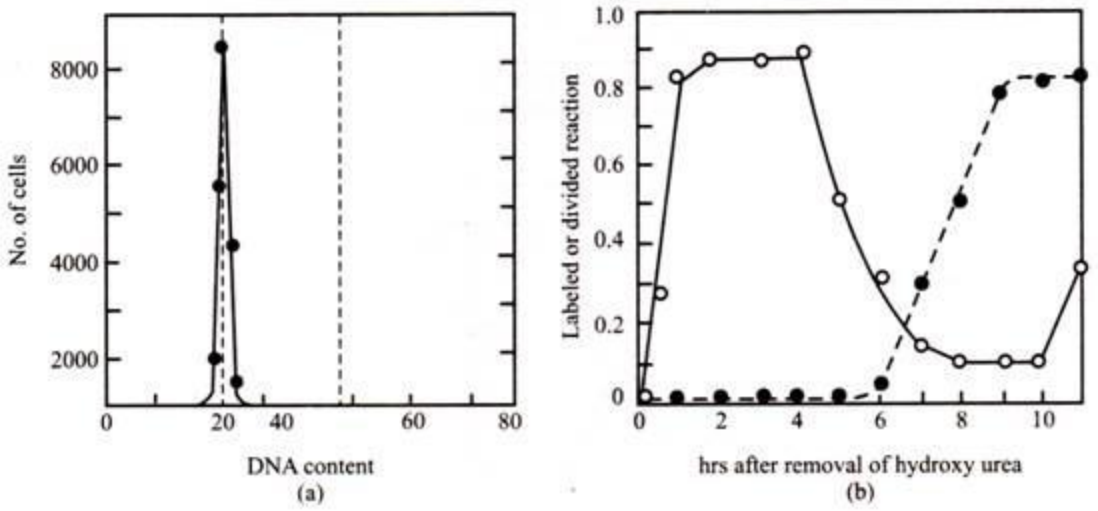


Fig. 6.12 Chemical methods of synchronization in mammalian cells (a) serum deprivation (SD), (b) hydroxy urea (HU) deprivation

Table 6.3

Method	*Comparative*	
	Advantage	*Disadvantage*
DTB	Gives good synchrony in certain cell lines e.g. CHO	Cell line dependent
ID	Synchronizing large quantities of cells in suspension	Not applicable to all cell lines
SD/HU	Degree of synchrony is excellent for obtaining cells synchronized at G_1/S boundry	Needs longer time

The cells synchronized by above methods are characterized in terms of the following:

1. DNA synthesis (incorporating [^{3}H] thymidine into DNA.
2. Cell density and
3. Cell division (percent of double cells in population).

Mycotoxins are structurally diverse secondary metabolites produced by many microfungi. Hitherto, 300 to 400 mycotoxins have been identified. They contaminate most cereals and feedstuffs, which heavily threaten human and animal health with acute, sub-acute and chronic toxicological effects, especially concerned in carcinogenesis in human. Many mycotoxins at low concentrations are able to induce the expression of cytochrome P450 and other enzymes in biotransforming and metabolizing mycotoxins in vivo and in vitro. Mycotoxins and their metabolites elicit different cellular disorders and adverse effects such as oxidative stress, translation inhibition, DNA damage and apoptosis in host cells, and therefore cause the different

but related cytotoxicity. In this review, we summarize biotransformations of mycotoxins in animal and human cells by CYP450 isoforms and other enzymes, the expression alterations of these enzymes under mycotoxins exposure, and the recent progress in mycotoxins cytotoxicity in different cell lines. Furthermore, we try to generalize the molecular mechanisms of mycotoxins effects in human and animal cells.

Cryopreservation of Cell Lines

OPS Diagnostics has several products used for cryogenically storing cell lines, such as Cryogenic Vials and Cryogenic Storage Boxes. Please visit our website to see these and other cryogenic products.

Introduction

The preservation of cells is an extremely important aspect of cell culture. The only effective means of preservation of animal cells is by freezing, which can be accomplished with either liquid nitrogen or by employing cryogenic freezers. The freezing process involves slowly reducing the temperature of prepared cells to -30 to -60°C followed by a transfer to temperatures less than -130°C. Once at ultralow temperatures, the cells are biologically inert and can be preserved for years.

Preparation

Cryopreserving cultured cells differs from preserving bacteria and fungi in that higher viability is required. Where a 1% survival rate of a microbial culture can be practical, such low viability is unacceptable with cultured cells. High survival rates are clearly important for cell lines due to the expense and difficulty in preparation , slow relative rate of growth, and tendency to change with repeated passage in culture. Consequently, methods used for cell culture cryopreservation must ensure high viability (i.e., >90%).

Factors that can affect the viability of cryopreserved cells include growth conditions prior to harvesting, the physiological state of the cells, the cell density, choice of cyroprotectant, and handling techniques. Actively growing cells harvested from late-logarithmic to early-stationary phase cells usually yield the highest number of viable cells following freezing. Once harvested, the desirable final concentration of cells should be between 10^6 to 10^7 cells/ml. Higher densities are often useful with adherent cells since thawed cells can be diluted and plated at a desired density. Cryoprotectants such as DMSO and glycerol are valuable to prevent cell dehydration during the freezing process. The cell suspension is generally prepared at a concentration twice that desired for freezing so that an equal volume of 2X cyroprotectant can be added. Gentle handling techniques during harvest and concentration will improve viability of the recovered cells. Excessive enzymatic treatment, vigorous pipetting, and high-speed centrifugation should be avoided.

Cryoprotectants

The diffusion of cryoprotective agents such as glycerol or dimethylsulfoxide (DMSO) into a cell will result in a partial replacement of intracellular water and help to prevent dehydration (from ice formation) during freezing. Glycerol is also known to stabilize proteins in their native states and to assist in the maintenance of critical macromolecular interactions at subzero temperatures. The cryoprotectant should be prepared separately by combining the cryoprotective agent and the

growth medium for the cells. Cryoprotective agents are usually used individually in concentrations ranging from 5-15% (v/v) with the optimum varying with the cell type. It is important that the cryoprotective agents be of highest possible quality and sterilized prior to use. Glycerol may be sterilized by autoclaving for 15 minutes and should be stored in small aliquots to prevent introduction of contaminants. DMSO should be sterilized by filtration with a 0.2 µm nylon syringe filter and stored at -20°C in small, single-use sealed aliquots. Air oxidation of DMSO is relatively rapid and these products are toxic to cells. DMSO should not be allowed to come into contact with the skin as it is rapidly absorbed and is a reported neurotoxin. Preformulatedcryoprotective media can also be purchased.

Equilibration

Cells mixed with the cryoprotectant require an equilibration time at room temperature prior to the onset of the cooling process. This time generally ranges from 15 to 45 minutes and allows penetration of the cell by the cryoprotectant for maximum protective effect.

Cooling:

The rate of cooling controls the size of the ice crystals and the rate at which they are formed, both of which may affect cell recovery. In most cases a slow, uniform cooling rate of -1°C per minute from ambient is effective. Since programmable-rate freezing units are not generally available to the cell culturist, alternative methods have been developed. Placement of the cryovials in a Styrofoam rack (from 15 ml centrifuge tubes) on the shelf of a -80°C freezer for 2-3 hours will result in a non-uniform cooling rate but is close to -1°C per minute and satisfactory for a range of cell types. Transfer should then be made to the storage temperature.

Storage

The temperature at which frozen cells are stored will affect their viability. Storage at -80°C may permit slow chemical reactions (due to small amounts of unfrozen water), which will eventually result in cell death. A temperature of less than -130°C is required to completely stabilize cell preparations. This is usually achieved by storage in liquid nitrogen (-196°C), liquid nitrogen vapor, or in an cryogenic freezer (-150°C). All three methods are used with each presenting its own strengths.

Liquid nitrogen is a non-mechanical method of cryopreserving cells. A large thermos-like container is used to house either racks or sleeves that hold cryogenic vials. Cells stored in nitrogen can be placed above the liquid in a cold vapor phase or in the liquid nitrogen itself (-196°C). Simple systems rely on a cycle of filling the tank and allowing the nitrogen to evaporate followed by refilling. Liquid nitrogen storage systems do not require electricity, but rather a ready source of liquid nitrogen. A small 50 liter tank will require filling every 5-6 weeks at a yearly cost of $900 to $1000. Though liquid nitrogen is widely used for cell preservation, two problems exist with this storage method. Cells stored in vapor phase can experience wide temperature fluctuations (i.e., -120 to -195°C), which can be potentially damaging to cells. Secondly, capped vials submersed in the liquid can leak and pick up contaminants and also pose a risk of exploding when removed from the liquid (see **Recovery** below).

Cryogenic freezers are an alternative to the traditional methods of cryopreserving animal cells. Cryogenic freezers use high efficiency compressors to reach temperatures as low as -150°C. No filling is necessary with freezers although back-up non-mechanical refrigerants are available for added security. Additionally, freezers are generally easier to catalog than many liquid nitrogen systems.

Recovery

Unlike the freezing process, rapid thawing of frozen cells is necessary to maintain viability. Certain precautions should be exercised when thawing cells. Vials stored in liquid nitrogen, especially screw capped tubes, often fill with liquid nitrogen while submersed. When these tubes are removed from the tank, the tubes may pressurize and burst. Thus, a face shield or goggles should be worn while thawing cells. Vials stored in cryogenic freezers are a reduced risk of bursting. Directly after removal from storage, vials should be thawed with agitation (but not for fragile hybridoma cells) in a 37°C water bath. As the last ice crystals are melting, the vial is removed from the water. Wipe, spray, or submerse the vial with 70% ethanol before opening it in a biosafety hood.

It is prudent when working with an unfamiliar cell line to determine the percentage of viable cells recovered by Trypan Blue staining. This may serve to uncover any deficiencies in the cryopreservation process. Note that safety precautions must be taken when recovering vials from the liquid nitrogen. Insulated gloves should be worn to protect against burns from the low temperatures. Though specially-designed cryovials are used to store cells, a face shield and laboratory coat serve to protect against fragments of exploding vials caused by introduction of liquid nitrogen (an all too common occurrence with leaky vials).

Cryopreservation Protocol

Materials

- cultured cells
- hemocytometer and cover slip
- 2X cryoprotective medium (e.g., DMEM with serum and 15% DMSO or glycerol)
- cryovials or glass ampoules
- propane torch (for glass ampoules)
- cryogenic freezer (-150°C)
- -80°C freezer

Protocol (Freezing)

1. If cells are a monolayer culture, gently trypsinize to detach.
2. Count an aliquot of cells in hemocytometer.

3. Adjust the density of cells in culture medium to 1 X 10^7 cells/ml. Add an equal volume of 2X cryoprotective medium. Allow the cells to sit at room temperature 15 min. so that the cryoprotectant can diffuse into the cell.

4. Transfer 1 ml of cells to a plastic or glass vial. For plastic vials it is necessary to use caps with O-rings that will allow for a tight fit. Most plastic cryogenic tubes placed in liquid nitrogen do not form tight seals and will allow liquid nitrogen to seep into the tube if submersed. This can be a concern since cultured cells, viruses, and bacteria, which may be present in the liquid phase of the nitrogen, can potentially contaminate a culture stored in a screw capped tube. This is not a concern if cells are stored in only the vapor phase or in a cryogenic freezer. To completely enclose the cells, glass ampoules can be used and sealed with a flame by rolling the neck of the ampoule in a flame until it becomes soft and pliable. Using forceps, slowly pull the neck of the ampoule while continuing to roll the tube. As the neck separates from the vial, roll the end of the vial in the flame to seal. Once the vial is cooled, it can be submersed in a solution of Methylene Blue or Trypan Blue in order to ensure the vials are closed. Wash the vials to remove the stain and examine the cell suspension for the dye. Any dye on the inside of the vial means the vial was not sealed and should be discarded.

5. Clearly label the vials using permanent ink. Include information on cell type and date. This should be cross referenced to additional information on the cell line. Cool the vials at a rate of 1°/min until they have reached -80°C. Glass vials should be slanted while freezing so that the liquid can expand without cracking the glass. This can be done by placing the vials in a Styrofoam box and placing in a -80°C freezer overnight.

6. Transfer to a cryogenic freezer for permanent storage.

Protocol (Thawing)

1. Remove vials from the cryogenic freezer. If cells are stored in liquid nitrogen, use tongs and insulated gloves, keeping in mind that pressure will build up inside the vials as the nitrogen expands into a gas. The vial may shatter, thus wear goggles and a lab coat.

2. Thaw in a 37°C water bath with constant gentle shaking until completely thawed (<1 minute). Carefully observe whether the glass vials have cracked during freezing or thawing.

3. Wash the vial with 70% ethanol. Open the cryogenic tube or snap the ampoule aseptically. Plate cells immediately into pre-warmed medium.

4. After attachment of monolayer cells, usually 1 to 10 hours, change the medium to remove the cryoprotectant. If the cells are non-adherent, allow a sufficient recovery time (about 6 hours), then gently pellet (5 minutes at 400 X g) and resuspend in fresh medium.

1	For culturing, plasma from the adult chicken is preferred to mammalian plasma because	it forms a clear and solid coagulum even after dilution	it is too opaque	it doesn't produce solid clots	it forms a semi solid coagulum	it forms a clear and solid coagulum even after dilution2
2	Toxicity due to accumulation of ammonia can be overcome	a. by substituting glutamine by glutamate	b. by controlled addition of glutamine at low level	c.by removal of ammonia or ammonium from culture medium	d. all of the above	d. all of the above
3	Range of osmolarity tolerated/accepted in mOsm/Kg of H2O by mammalian cells is	a. 150-300	b. 280-360	c.300-325	d. 360-400	b. 280-360
4	Disaggregating of cells can be achieved by	a. physical disruption	b. enzymatic digestion	c. treating with chelating agents	d.all of the above	d.all of the above
5	Accumulation of lactate leads to	a. increase in pH	b. no change in pH	c. reduction in the pH of culture hence loss of cell viability	d. no loss of cell viability	c. reduction in the pH of culture hence loss of cell viability
6	When dissolved oxygen is lower than the critical concentration, viable cell concentration declines because of	a.incomplete glutamine oxidation	b.increase in specific lactate production from glucose	c. both (a) and (b)	d. accumulation of ammonia	c. both (a) and (b)
7	According to Eagle, the growth of L-strain and Hela-strain cultures require to have mandatory presence of	a. 6 amino acids	b. 8 amino acids	c. 10 amino acids	d. 12 amino acids	d. 12 amino acids
8	Excess CO2 suppress cell growth and productivity by	a.inhibiting respiration	b. altering intracellular pH by diffusing across cell membrane	c. both (a) and (b)	d.altering pH of the medium	c. both (a) and (b)
9	Which of the following is incorrect?	a.Established cell lines (ECL) have short doubling time	b. ECL are invariably aneuploid	c. ECL grow in higher density	d.ECL do not show much evidence of spatial orientation	c. ECL grow in higher density
10	What is the effect of excess accumulation of metabolite products (lactate and ammonium) on cells?	a. They act as growth promoters	b. They act as growth inhibitors	c. Have no effect on cells	d. Lactate helps in the growth while ammonium inhibits the growth	Lactate helps in the growth while ammonium inhibits the growth
11	What is the concentration of CO2 required for culturing animal cells?	2-5%	1-10%	10-15%	15-20%	1-10%
12	Which of the following is correct?	ECL can be established in suspension cultures whereas it is exceptional for primary cell lines (PCL)	ECL and PCL can be established in suspension cultures	c.PCL can be established in suspension cultures	none of the above	ECL can be established in suspension cultures whereas it is exceptional for primary cell lines (PCL)
13	Which of the followings are the metabolic products of glucose and glutamine?	CO2 and NH3	CO2 and lactate	Lactate and ammonium	Lactate only	Lactate and ammonium
14	To prevent the accumulation of lactate	low glutamine concentration is rec	high glutamine concentration is required	low glucose concentration is required	high glucose concentration is required	low glucose concentration is required
15	What are the main constituents of culture for animal cell growth?	Glucose and Glutamine	Growth factors	Cytokines	All of these	Glucose and Glutamine
16	During the growth of animal cells in culture, it is noticed that the cells do not look very healthy. After an investigation, this is found that there is a lot of lactic acid in the culture fluid. What is probably wrong with this culture?	Ethyl alcohol is being produced in excess	The cells have too much oxygen	Glycolysis is being inhibited	The cells do not have enough oxygen	The cells do not have enough oxygen
17	Range of optimum glutamine concentration present in the culture media is	1-2 mmol/litre	2-7 mmol/litre	7-15 mmol/litre	15 - 20 mmol/litre	2-7 mmol/litre
18	The human fibroplast is a classical example of	stable primary cell lines	established cell lines	cell transformations	d.none of these	stable primary cell lines
19	pH of culture medium is initially controlled by	Presence of co2	b.presence of bicarbonate buffer	addition of bases	none of these	
20	Which of the following abnormality, resulted from the inheritance of an unbalanced complement of chromosomes can be diagnosed through karotyping?	Down's syndrome	Turner's syndrome	Klinefelter's syndrome	all of these	all of these
21	In animal cell culture, particularly mammalian cell culture, transformation means	uptake of new genetic material	phenotypic modifications of cells in culture	both (a) and (b)	release of genetic information	phenotypic modifications of cells in culture
22	How does CO2 help in the cell metabolism during cell culture?	It participates in the de novo synthesis of purines and pyrimidines	Helps in the cells respiration	For monitoring pH of the culture	All of the above	It participates in the de novo synthesis of purines and pyrimidines
23	Which of the following is not the explantation technique?	Slide culture	Carrel flask culture	Roller test tube culture	Adherent primary culture	Adherent primary culture
24	Cells which have undergone transformation frequently become	anchorage independent	anchorage dependent	stable	unstable	anchorage independent
25	Range of optimum glucose concentration present in the	**5.5 - 55 mmol/litre**	55 - 75 mmol/litre	75-105 mmol/litre	105-150 mmol/litre	5.5 - 55 mmol/litre
26	The technique of organ culture may be divided on the basis of employing	solid medium	liquid medium	both (a) and (b)	semi-solid medium	both (a) and (b)
27	At low glucose concentration, below 0.25 mmol/litre, large portions of glucose and glutamine is shunted via	oxidative pathway	anaerobic pathway	both (a) and (b)	none of these	oxidative pathway
28	An established cell line can be called where it has been subcultured at least	70 times at an interval of 3 days between subcultures	40 times at an interval of 3 days between subcultures	70 times at an interval of 1 day between subcultures	50 times at an interval of 3 days between subcultures	70 times at an interval of 3 days between subcultures
29						
30						
31						
32						
33						

Animal Cell Culture: Scale-Up Process

The scale-up process is divided into two categories. The two categories are: (1) Scale-up in Suspension and (2) Scale-up in Monolayer and also about the monitoring of cell growth in the scale-up process.

Scale-Up Process:

Scale-up involves the development of culture systems in stages from (small scale) laboratory to (large scale) industry. The methodology adopted to increase the scale of a culture depends on the proliferation of cells and is broadly divided into two categories.

1. Scale-up in suspension.

2. Scale-up in monolayer.

Scale-Up in Suspension:

Scale-up in suspension is the preferred method as it is simpler. Scale-up of suspension culture primarily involves an increase in the volume of the culture. Small scale generally means the culture capacity less than 2 litres volume (or sometimes 5 litres).

Stirred suspension cultures:

It is usually necessary to maintain cell strains in stirred suspension cultures, by agitation (or stirring) of the medium. The stirring of the culture medium is achieved by a magnet encased in a glass pendulum or by a large surface area paddle. The stirring is usually done at a speed of 30-100 rpm. This is sufficient to prevent sedimentation of cells without creating shear forces that would damage cells.

Static suspension cultures:

Some cells can grow in suspension cultures, without stirring or agitation of the medium, and form monolayer cells. However, static suspension cultures are unsuitable for scale-up.

Factors in Scaling-Up:

For appropriate scale-up, the physical and chemical requirements of cells have to be satisfied.

Physical parameters:

i. Configuration of the bioreactor.

ii. Supply of power.

iii. Stirring of the medium.

ADVERTISEMENTS:

Chemical parameters:

i. Medium and nutrients.

ii. Oxygen.

iii. pH and buffer systems.

ADVERTISEMENTS:

iv. Removal of waste products.

Some of the relevant aspects of the factors in scale-up have already been described under cell culture-general considerations The most commonly used techniques for stirred suspension cultures are briefly described hereunder.

Stirrer Culture:

A diagrammatic representation of a stirrer flask is shown in Fig 37.2. The size of the stirrer flask is in the range of 2-10 litres. It is fitted with a magnetized rotating pendulum, and two side arms — one for the addition of cells and medium, and the other for the supply of CO_2.

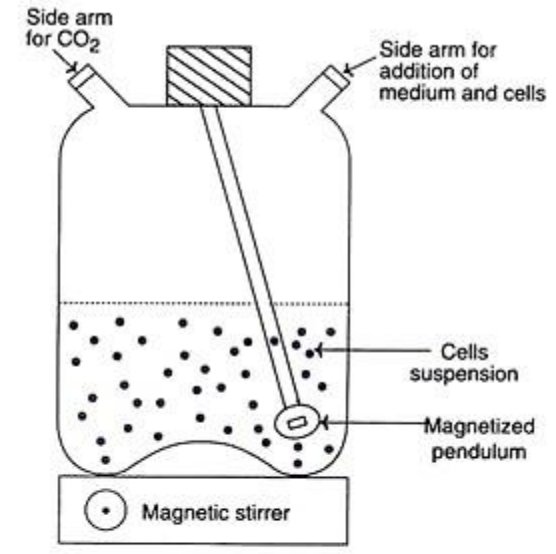


Fig. 37.2 : Diagrammatic representation of a stirrer flask.

The stirrer culture vessel is autoclaved (at 15 lb/ in^2 for 15 minutes), and is then set up as in Fig. 37.2. The flask is seeded with the culture. Then medium along with an antifoam agent is added. The flask is connected to CO_2 and stirred at a speed of 60 rpm. The flask is incubated for about 2 hours.

The contents of the small stirrer flask are transferred to a large flask and the entire set up is restarted. Incubation at 37°C is carried out for 4-7 days. The growth of the cells is monitored daily, and the cells are counted. There is a tendency of the cells to enter apoptosis, if the concentration exceeds 1×10^6 cells/ml.

Continuous Flow Culture:

In a continuous flow culture, it is possible to keep the cells at a desired and set concentration, and maintain. This is carried out by a bio-stat or chemo-stat (Fig 37.3).

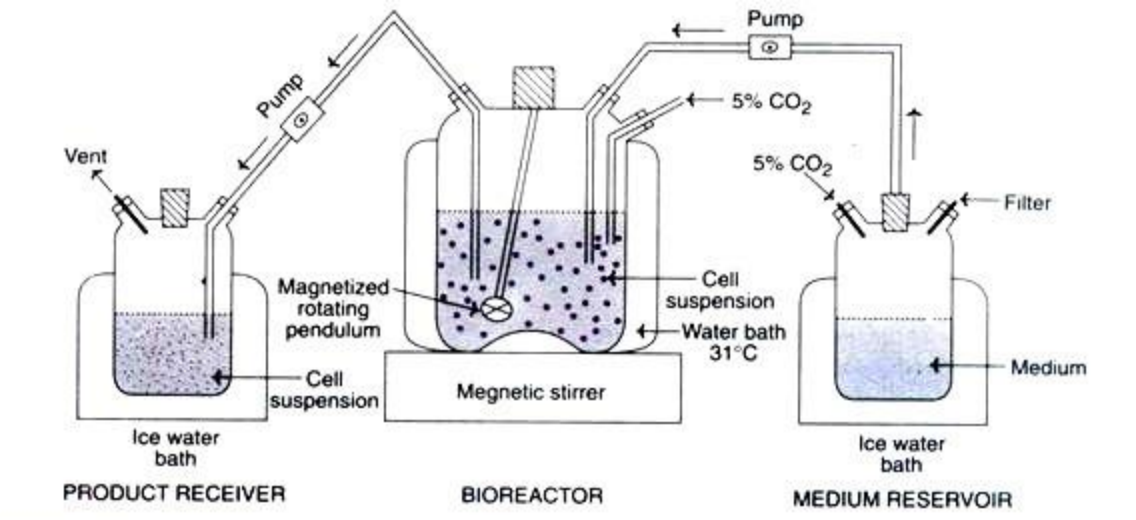


Fig. 37.3 : Suspension culture in a biostat (chemostat).

Continuous flow culture consists of growing the cells at the mid-log phase, removal of a measured volume of cells, and replacement by an equal volume of medium. The equipment, specially designed for this purpose has the facility for removal of the cells and addition of medium.

The flow rate of the medium addition can be determined from the growth rate of the culture. The medium flow can be regulated by a peristaltic pump. By this technique, it is possible to keep the culture conditions constant rather than to produce large number of cells. The continuous flow cultures are useful for monitoring metabolic changes in relation to cell density. However, these cultures are more susceptible to contamination.

Air-Lift Fermenter Culture:

The major limitation of scale-up in suspension culture is inadequate mixing and gas exchange. For small cultures, stirring of the medium is easy, but the problem is with large cultures. The design of fermenter should be such that maximum movement of liquid is achieved with minimum shear to damage the cells.

A diagrammatic representation of an air-lift fermenter is depicted in Fig. 37.4. A 5% CO_2 in air is pumped through the bottom of the fermenter. The bubbles formed move up to agitate and aerate the culture. These bubbles carry a flow of liquid along with them and release at the top which goes to the bottom for recycling.

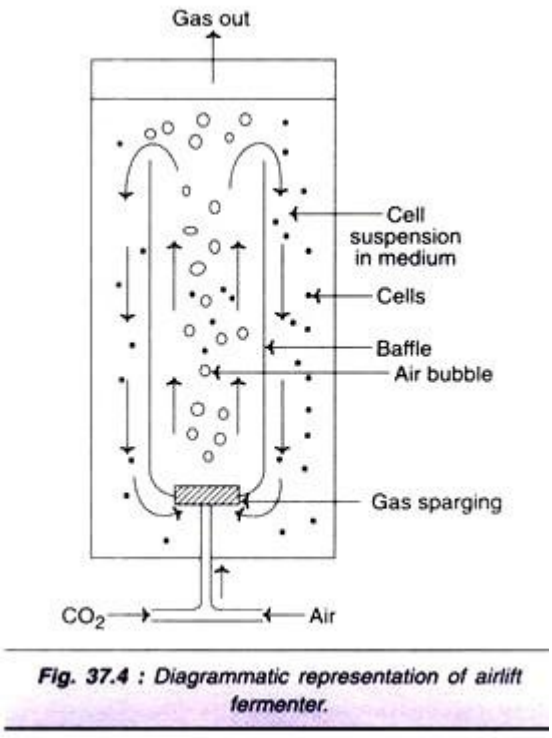


Fig. 37.4 : Diagrammatic representation of airlift fermenter.

It is possible to continuously supply O_2 to the culture in this technique. Air-lift fermenter culture technique is suitable for fragile animal as well as plant cells. This fermenter is extensively used in the biotechnology industry for culture capacities up to 20,000 litres.

NASA bioreactor:

NASA (National Aeronautics Space Administration, USA) constructed a bioreactor to grow the cells at zero gravity by slowly rotating the chamber (Fig. 37.5). The cells remain stationary and form three-dimensional aggregates and this enhances the product formation. In the NASA bioreactor, there is almost no shear force; hence the cells are not damaged.

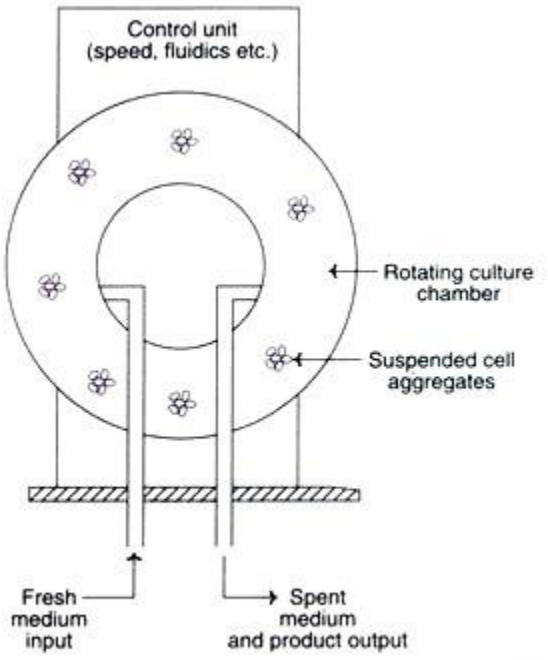


Fig. 37.5 : A bioreactor developed by NASA.

As the culture chamber stops its rotations, the cell aggregates sediment and the medium can be replaced.

Other Systems for Suspension Culture:

Rotating chambers:

The mixing and aeration of the culture medium can be achieved by 2 or 3 rotating chambers. The chambers are so designed that the cell suspension and mixing are high in one chamber while the product and spent medium remain in the other chamber. These chambers are separated by semipermeable membrane.

Perfused suspension culture:

This also has two compartments. The cells are kept in a low-volume compartment at high concentration, while the medium is perfused in adjacent compartment. The product can be collected in a third compartment.

Scale-Up in Monolayer:

The monolayer culture are anchorage- dependent. Therefore, for the scale-up of monolayer cultures, it is necessary to increase the surface area of the substrate in proportion to the number of cells and volume of the medium. Suspension cultures are preferred as they are simple. The advantages and disadvantages of monolayer cultures are listed.

Advantages:

i. Change of medium and washing of cells easy.

ii. It is easy to perfuse immobilized monolayer cells.

iii. The cell product formation (pharmaceutically important compounds e.g. interferon, antibodies) is much higher.

iv. The same set up and apparatus can be repeatedly used with different media and cells.

Disadvantages:

i. Tedious and costly.

ii. Require more space.

iii. Growth of cells cannot be monitored effectively.

iv. Difficult to measure control parameters (O_2 pH, CO_2 etc.)

For scale-up of monolayer cultures, a wide range of tissue cultures and system have been developed. A selected few of them are briefly described.

Roller Bottle Culture:

A round bottle or tube is rolled around its axis (by rollers) as the medium along with the cells runs around inside of the bottle (Fig. 37.6). As the cells are adhesive, they attach to inner surface of the bottle and grow forming a monolayer.

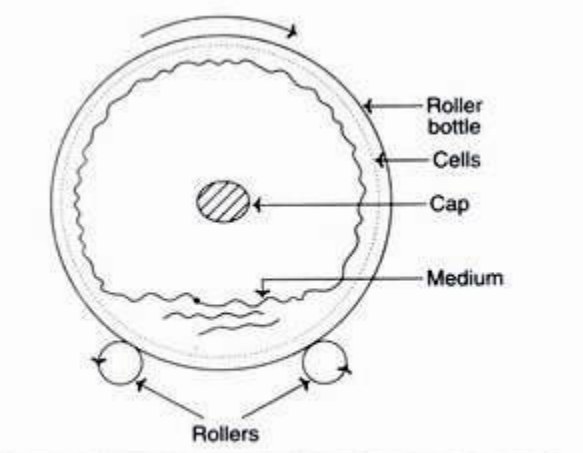


Fig. 37.6 : Roller bottle culture.

Roller bottle culture has certain advantages.

i. The medium is gently and constantly agitated.

ii. The surface area is high for cell growth.

iii. Collection of the supernatant medium is easy.

There are limitations in roller culture.

i. Monitoring of cells is very difficult.

ii. Investment is rather high.

Multi-surface Culture:

The most commonly used multi-surface propagator of monolayer is Slunclon cell factory (in short Nunc cell factory). It is composed of rectangular petri dish-like units with huge surface area (1,000-25,000 cm^2). The units are interconnected at two adjacent corners by vertical tubes (Fig. 37.7). The medium can flow between the compartments from one end.

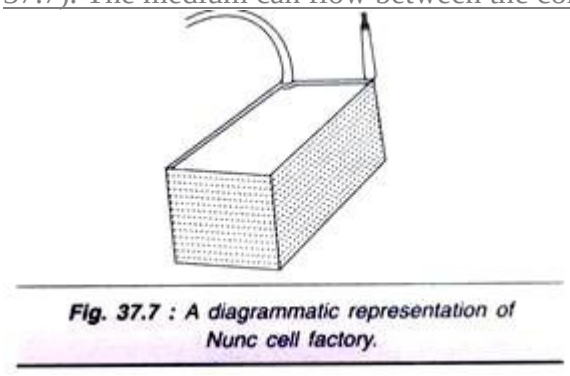


Fig. 37.7 : A diagrammatic representation of Nunc cell factory.

The cell factory is almost like a conventional petridish or a flask with multiplayer units. The main limitation of cell factory is that it is very difficult to monitor the growth of cells. The major advantage however, is its simple operation to produce large number of cells.

Multi-array Disks, Spirals and Tubes:

The surface area for growth of monolayer cultures can be increased by using disks, spirals or tubes. They are however, not in common use as their commercial importance is limited.

Micro-carrier Culture:

Monolayers can be grown on small spherical carriers or micro-beads (80-300 pm diameter) referred to as micro-carriers. The micro-carriers are made up of any one the following materials (trade names given in brackets).

i. Plastic (acrobeads, bioplas).

ii. Glass (bioglass, ventreglas).

iii. Gelatin (ventregel, cytodex-3).

iv. Collagen (biospex, biospheres)

v. Cellulose (DE-52/53).

vi. DEAE Dextran (cytodex I, dormacell).

The micro-beads provide maximum surface area for monolayer cultures. This actually depends on the size and density of the beads. The cells can grow well on the smooth surface at the solid-liquid interface. However, micro-carriers need efficient stirring without grinding the beads. The main advantage with micro-carrier culture is that it can be treated as a suspension culture for all practical purposes.

Micro-carriers can be cultured in stirrer flask (See Fig. 37.2) or in continuous suspension (See Fig. 37.3). In fact, the suppliers of micro-carriers provide the technical literature and other relevant information for setting up a micro-carrier culture.

Factors affecting micro-carrier culture:

i. Composition and coating of beads (gelatin and collagen beads are preferred as they can be solubilized by proteases).

ii. Higher stirring speed is usually required.

iii. Glass beads are used when the micro-carriers need to be recycled.

Analysis of micro-carrier culture:

The cell counting techniques are difficult to be used for micro-carrier cultures. The growth rate can be detected by analyzing DNA or protein.

Perfused Monolayer Culture:

The growth surface areas of the monolayer cultures can be perfused to facilitate medium replacement and improved product formation and recovery. The perfusion can be carried out with pumps, oxygenator and other controllers. Perfusion of fixed and fluid-bed reactor is briefly described.

Fixed-bed reactors:

The fixed-bed reactor has a bed of glass beads (Fig. 37.8A). The medium is perfused upwards through the bed. The cells are grown on the surfaces of the beads. The products can be collected from the top along with the spent medium.

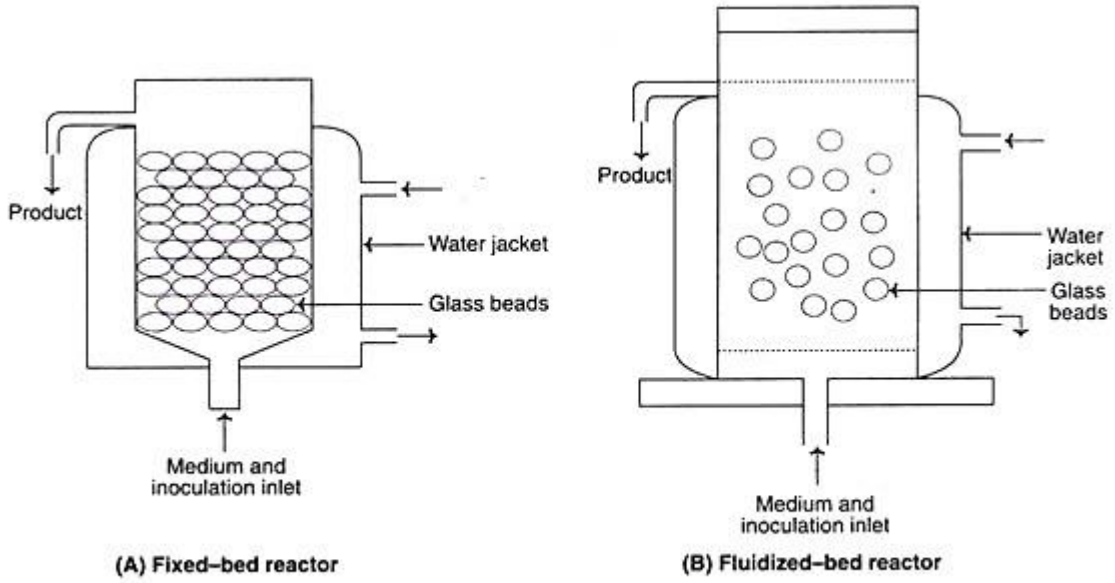


Fig. 37.8 : Fixed and fluidized–bed reactors.

Instead of glass beads, porous ceramic matrix with micro-channels can also be used in fixed-bed reactors.

Fluidized-bed reactors:

In a fluidized-bed reactor, the beads are suspended in a stream of medium (Fig. 37.8B). These beads are porous in nature, and are made up of ceramics or a mixture of ceramics mixed with natural products such as collagen. They are of low density and float in the medium. The flow rate of the perfused medium is equal to the sedimentation rate of the beads. The cells can grow as monolayers on the outer surfaces and inside of the porous beads.

Other perfused monolayer cultures:

Membrane perfusion, hollow-fiber perfusion, matrix perfusion and microencapsulation are among the other techniques for perfusion of monolayer cultures.

Monitoring of Cell Growth in Scale-Up:

Monitoring of the progress of cell growth and the culture systems are very important in scale-up.

Monitoring of Suspension Cultures:

The progress of suspension cultures can be monitored in situ by measuring glucose, O_2, CO_2, pH or metabolites produced (lactate, ammonia) or specialized products formed (e.g. immunoglobulin's by hybridoma cells).

The cell proliferation and rate of biomass formation can also be determined by estimating DNA, protein and ATP. The different parameters for monitoring of a bioreactor for suspension culture are depicted in Fig 37.9.

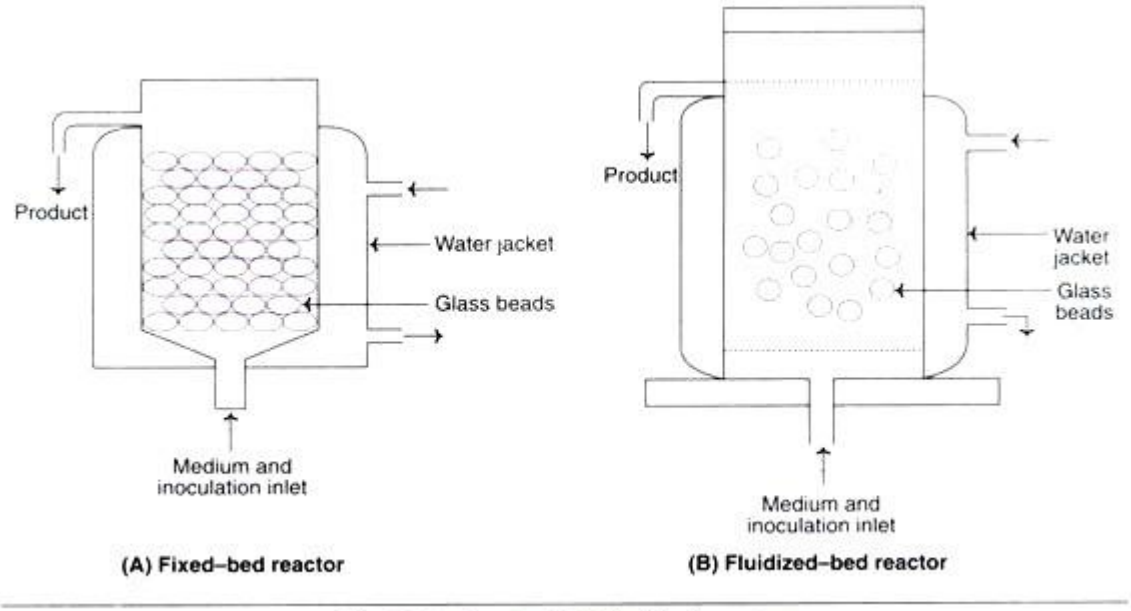


Fig. 37.8 : Fixed and fluidized–bed reactors.

Monitoring of Monolayer Cultures:

It is rather difficult to monitor monolayer cell cultures for scale-up. This is due to the fact that in most of the techniques employed for monolayer cultures, the cells cannot be observed directly to monitor the progress of the culture.

In recent years, nuclear magnetic resonance (NMR) technique is used to assay the contents of culture. The characteristic NMR spectra generated by specific metabolites enables the identification and quantitation of metabolites, besides detecting the progress of cell growth.

1. The virus commonly used to infect cell cultures for the production of interferon is	Corona virus	Sendai virus	Polio virus	Small pox virus	Sendai virus
2.the production of complete animal from somatic cell of an animal is called as	gene cloning	animal cloning	cell cloning	all of these	animal cloning
3. the culture fluid of 1000 to 5000 colonies of hybridoma are screened for monoclonal antibody by p antigen capture by analysis q-western blot analysis r norther blot analysis s- antibody capture analysis	pq	rs	ps	rs	ps
4. Reverse vaccinology indicates	from antibody to vaccine development	from genome sequence to vaccine development	from antigenic polysaccharide to vaccine development	from antigenic protein to vaccine development	from antigenic protein to vaccine development
5. Antibody diversity is generated by	allelic exclusion	protein splicing	Somatic mutation	interchromosomal recombination	allelic exclusion
6. cdc mutant useful for the study of	various stages of cell cycle	homeodomain	apoptosis only	chromosome break point	various stages of cell cycle

Tissue engineering

The classic tissue engineering (TE) paradigm consists in the isolation of primary cell from the patient and the further seeding on a three-dimensional (3D) porous scaffold, specifically designed to induce cell proliferation and differentiation. The bioartificial system is developed in a specific environment, where the metabolic, mechanical and electrical stimuli are provided by a bioreactor. In this tissue-specific designed system, cells start to produce extracellular matrix, leading to *in vitro* tissue maturation. The tissue-engineered construct is then implanted in the patient, undergoing to a remodeling process *in vivo*, which allows the tissue restoration. **(B)** The TE approach for the development of *in vitro* models. The first step is to understand what part of the system considered should be represented and which specific conditions should be met. The second step is the design of the appropriate scaffold, i.e., the design of a 3D porous construct recapitulating the architecture and the mechanical and surface properties of the tissue/organ to be modeled. The third step is the selection of the cells to be seeded in the designed scaffold. The subsequent steps should be the design and fabrication of a bioreactor and the selection of the appropriate chemical signals (e.g., cocktail of growth factors) to be included in the model. Once a model is successfully developed, it can be improved by increasing its complexity, by introducing additional cell type, chemical factor or coupling different mechanical stimuli. The final step is the validation, aiming to demonstrate the relevance of the model for the intended purpose. The validation procedure is fundamental and this step should be carefully designed.

In the tissue engineering (TE) paradigm, engineering and life sciences tools are combined to develop bioartificial substitutes for organs and tissues, which can in turn be applied in regenerative medicine, pharmaceutical, diagnostic, and basic research to elucidate fundamental aspects of cell functions *in vivo* or to identify mechanisms involved in aging processes and disease onset and progression. The complex three-dimensional (3D) microenvironment in which cells are organized *in vivo* allows the interaction between different cell types and between cells and the extracellular matrix, the composition of which varies as a function of the tissue, the degree of maturation, and health conditions. In this context, 3D *in vitro* models can more realistically reproduce a tissue or organ than two-dimensional (2D) models. Moreover, they can overcome the limitations of animal models and reduce the need for *in vivo* tests, according to the “3Rs” guiding principles for a more ethical research. The design of 3D engineered tissue models is currently in its development stage, showing high potential in overcoming the limitations of already available models. However, many issues are still opened, concerning the identification of the optimal scaffold-forming materials, cell source and biofabrication technology, and the best cell culture conditions (biochemical and physical cues) to finely replicate the native tissue and the surrounding environment. In the near future, 3D tissue-engineered models are expected to become useful tools in the preliminary testing and screening of drugs and therapies and in the investigation of the molecular mechanisms underpinning disease onset and progression. In this review, the

application of TE principles to the design of *in vitro* 3D models will be surveyed, with a focus on the strengths and weaknesses of this emerging approach. In addition, a brief overview on the development of *in vitro* models of healthy and pathological bone, heart, pancreas, and liver will be presented.

Introduction

Tissue engineering (TE) was defined by Langer and Vacanti in early 90s as "an interdisciplinary field which applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function". TE aims to induce tissue-specific regeneration processes, thus overcoming the well-known drawbacks of organ transplantation (i.e., donor shortage, need of immunosuppressive therapy). TE approaches have been recently proposed for the design of reliable *in vitro* models of healthy or pathological tissues and organs, which can be employed for drug screening and the evaluation of new therapies, as well as the investigation of the complex phenomena regulating disease onset and progression. Besides their high scientific potential, these models also bring some advantages in terms of ethical and economic issues.

From the ethical point of view, the employment of animals for biomedical research purposes has been thoroughly debated and the topic still opens the door to discussion. The principle of 3Rs (Replacement, Reduction, and Refinement) introduced, which encourages the research community to recognize the importance of welfare for animals used in science, is currently embedded in national and international legislation. In view of this, a large amount of resources have been invested to develop methods to replace animals in research. Moreover, although animal models have significantly contributed to both our understanding of human biology and the development of modern medicine, they often show limits in the reproduction of specific human conditions. Even though some human pathologies can be induced in animal models, the molecular mechanisms driving their onset and progression are often significantly different. The increasing number of existing animal models and the inefficacy on humans of some drugs successfully tested on animals are symptoms of animal model inability to effectively recapitulate human physiology.

Economic aspects should be also considered: the actual costs for successfully transforming a drug candidate from a new molecular entity (NME) to a clinical product are between $800 million and $2.2 billion, with development timelines spanning 8–12 years. Moreover, there is a high failure rate for NMEs in lead development, especially those in expensive late-stage clinical trials. It has long been recognized that two-dimensional (2D) cell monocultures used in preclinical studies lack many of the requisite phenotypic characteristic often necessary for their utility in predictive drug assays. The three-dimensional (3D) environment in which cells grow *in vivo*, in fact, allows them to actively interact with the surrounding extracellular matrix (ECM) and cells, thus providing stimuli (e.g., soluble factors and physical forces) that strongly influence their functions and gene expression profile. Moreover, the accumulation of waste products in the culture medium, the limited nutrient supply, and the lack of cell-specific mechanostimulation often result in cell death or loss of functions, as a consequence of the non-physiological cell culture conditions.

In order to overcome the above mentioned limitations of current drug-screening methodologies and reduce the use of animals, 3D models have been investigated and a number of studies are in progress, with the aim of making them more and more reliable and sophisticated. Furthermore, unlike animal models, 3D *in vitro* models give the possibility to independently identify and modulate cellular and molecular factors responsible for disease onset and progression, allowing the investigation of the contribution of each of them on the development of a specific disease and thus changing the way to study tissue physiology and pathophysiology. The introduction of these models in the biomedical research practice may lead to numerous advantages, such as the reduction of animal use as well as the overcoming of the limits associated with traditionally employed models (i.e., animal and 2D cell culture models), and the achievement of more reproducible data, thanks to the possibility to tightly control the experimental parameters, with lower cost and less time. A 3D *in vitro* model allows the cells to grow and interact with each other and with the ECM in the all spatial dimensions. The 3D structure can be achieved through a 3D matrix support (scaffold) or by using scaffold-free organoids cultures. have recently published a comprehensive review on 3D *in vitro* models based on organoids. In this review, we first give a brief overview on the

main components of an *in vitro* 3D tissue-engineered model that should recreate *in vitro* the *in vivo* surrounding environment and stimuli, i.e., the 3D porous matrix (scaffold), the cells, and the applied cues (biochemical or physical). Then, we discuss how the TE approaches have been employed in the modeling of two of the most studied engineered tissues, bone and cardiac tissue, as well as some less engineered organs, which are of considerable interest due to the incidence of their pathologies, namely pancreas and liver.

Scaffolds, Cells, and Stimuli for *In Vitro* Modeling

The classic TE paradigm involves the combination of living cells with a natural, synthetic, or bioartificial support to develop a biological substitute or a 3D living construct that is structurally, mechanically, and functionally equal to a tissue. Moving from the first definition of TE in the early 90s through the huge amount of work carried out and published by many research groups all over the world, researchers have gained high expertise in cell manipulation, materials science, and bioengineering for the design of highly complex biomimetic tissue substitutes for reparative/regenerative purposes. These tools and specific competences have been transferred in the last decade to the development of 3D engineered *in vitro* models, as schematized. In its current definition, modeling does not mean to finely replicate *in vitro* the native tissue/organ, but that the model should be properly designed to recapitulate the particular conditions that are intended to be mimicked. Effectively mimicking a tissue is extremely complex since several aspects must be taken into account and each single tissue has different features. In this context, the fundamental elements that should be considered are (I) scaffolds, (II) cell sources, and (III) chemical and physical stimuli.

FIGURE 1

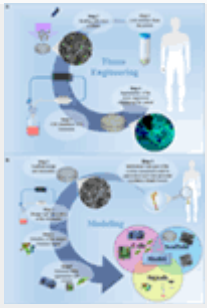


Figure 1. **(A)** The classic tissue engineering (TE) paradigm consists in the isolation of primary cell from the patient and the further seeding on a three-dimensional (3D) porous

scaffold, specifically designed to induce cell proliferation and differentiation. The bioartificial system is developed in a specific environment, where the metabolic, mechanical and electrical stimuli are provided by a bioreactor. In this tissue-specific designed system, cells start to produce extracellular matrix, leading to *in vitro* tissue maturation. The tissue-engineered construct is then implanted in the patient, undergoing to a remodeling process *in vivo*, which allows the tissue restoration. **(B)** The TE approach for the development of *in vitro* models. The first step is to understand what part of the system considered should be represented and which specific conditions should be met. The second step is the design of the appropriate scaffold, i.e., the design of a 3D porous construct recapitulating the architecture and the mechanical and surface properties of the tissue/organ to be modeled. The third step is the selection of the cells to be seeded in the designed scaffold. The subsequent steps should be the design and fabrication of a bioreactor and the selection of the appropriate chemical signals (e.g., cocktail of growth factors) to be included in the model. Once a model is successfully developed, it can be improved by increasing its complexity, by introducing additional cell type, chemical factor or coupling different mechanical stimuli. The final step is the validation, aiming to demonstrate the relevance of the model for the intended purpose. The validation procedure is fundamental and this step should be carefully designed.

Scaffolds

The complexity of *in vivo* tissue organization allows cells to interact with each other and with the surrounding ECM. In an engineered *in vitro* model, the scaffold must be designed to finely replicate *in vitro* the architecture of the native tissue, i.e., its ECM framework to let cells to adhere, spread, proliferate, differentiate, maturate, and produce ECM, similarly to what they do *in vivo*.

The combination of competences among materials science, biomedical engineering, and molecular biology has allowed the complex interaction between cell and materials to be better understood. The choice of the most suitable biomaterial for scaffold fabrication is a key element for the model design, since it strongly influences cellular functions. Biomaterials should be carefully selected depending on the modeled tissue/organ, acting as a synthetic ECM that interacts with cells at the molecular level, influencing cell functions and driving the

complex cellular processes that lead to the development of a valid *in vitro* engineered tissue model. Material selection strongly depends on tissue mechanical properties, since the scaffold's mechanical properties should match those of the tissue to be modeled, in healthy and pathological conditions. In fact, a pathologic tissue presents altered ECM properties, e.g., the different architecture and mechanical properties of osteoporotic bone compared to its healthy counterpart, and tissues stiffening under inflammatory conditions and aging processes, as discussed in more detail for pancreas and cardiac tissue in the following paragraphs. Thus, to model a pathologic system, the scaffold should be designed in order to reproduce these altered ECM features. A straightforward approach could consists in mimicking the dynamic of disease progression through the employment of scaffolds with time-varying properties. For instance, hydrogels can be properly designed so as to present time-dependent stiffening by directing their crosslinking reaction.

Mechanobiology studies have highlighted the importance of scaffold mechanical properties to properly direct cell behavior. Due to their high stiffness and load-bearing properties, ceramics and their composites are generally used in TE of hard tissues, while polymers are mainly employed in the engineering of soft tissues.

Material surface also plays a key role in guiding cell behavior and fate, being the primary interface for cell interaction. In order to obtain the desired cell response, it is possible to modify the biomaterial surface with bioactive molecules, such as specific proteins or peptide sequences (e.g., the fibronectin-derived arginylglycylaspartic acid peptide sequence -RGD-), that are recognized by cells as integrin-binding domains. Typically, surface modification takes place after scaffold fabrication, without affecting its structure or mechanical properties. The characteristics of the final model strongly depend on the selected fabrication technique and the possibility to tailor the processing parameters in order to fulfill the requirements for the intended application. Thus, TE scaffolding techniques developed for regenerative medicine can be exploited for modeling applications. Both the top-down and the bottom-up approaches have been employed for the design of tissue-engineered *in vitro* models. In top-down strategies, cells are cultured on scaffolds specifically designed to mimic the tissue to be modeled in terms of structure, composition, and mechanical properties. On the other hand,

the bottom-up approach aims to mimic and replicate the functional unit of a tissue and to create a more biomimetic scaffold. These modular scaffolds can be obtained through both microencapsulation and microfabrication techniques as well as employing traditional cell culture strategies.

The scaffold should present a high degree of porosity, with interconnected pores and pore dimension adequate for the specific application. This porous architecture allows cells migration also in the inner part of the 3D construct and permits nutrient/oxygen diffusion and waste removal. Several research works demonstrated that the morphology of cells cultured on 3D scaffolds significantly differs from that of cells cultured on 2D surfaces. Most cells are able to differentiate and develop a physiologically relevant tissue *in vitro* only if cultured in a 3D environment. Moreover, 3D patterned scaffolds have shown to promote greater cell aggregation, proliferation, and differentiation than 2D substrates. The pattern size influences cell morphology, proliferation, and migration. It was also demonstrated that cell response to the substrate morphology strongly depends on the cell type. Topography can also enhance the differentiation of progenitor cells into their programmed pathway. All of these observations show the importance of creating appropriate microstructures able to mimic the native tissue. To replicate the spatial gradient of properties, composition, and functions that is typical of many biological tissues (e.g., bone and cartilage), scaffolds with functional spatially distributed gradients have been developed. The design of these scaffolds is complex and requires the employment of computer-aided tools and computational modeling to guarantee a biomimetic environment for *in vitro* tissue development.

Cell Sources

The choice of the most appropriate cell source is a challenge in the design and further development of a tissue-engineered model. In fact, the development of representative *in vitro* tissue/organ models depends on the availability of tissue-specific cellular phenotypes, able to recapitulate *in vitro* the characteristics of normal or pathological natural tissues. Moreover, the number of cells to be included in the model should be carefully considered to guarantee a physiologically relevant 3D replica of the tissue functional unit. To bridge the gap between animal models and clinical trials, *in vitro* models should include human cells.

Most of the human cells employed in TE are adult primary cells isolated from patients. These cells are representative of the functional unit of a tissue, since they can be isolated from tissue biopsies harvested from healthy or pathological patients. On the other hand, adult primary cells have limited life span and low proliferation rate, and their isolation procedure is complex. To overcome these limits, stem cells have been employed. Stem cells are undifferentiated cells able to both self-renew and differentiate to one or more types of specialized cells, which can be isolated from different sources, such as embryos, fetuses, or adult tissues, and their differentiation capability depends on the cell source. The main critical issues in stem cell employment concern (i) the ability to control cell differentiation pathways toward the desired lineages and (ii) the immature phenotype of the cells derived from stem cells, which have a gene expression profile similar to fetal cells. Nevertheless, in the design of new *in vitro* systems for pharmacological and toxicological tests, the use of human stem cells represents a fundamental resource. As an example, cardiomyocytes differentiated from human embryonic stem cells (ESCs) have been successfully employed as pharmacological model for the evaluation of different cardioactive drugs. Induced pluripotent stem cells (iPSCs) are engineered stem cells derived from differentiated somatic cells by overexpression of specific transcription factors. These cells have characteristics similar to pluripotent ESCs and, under certain conditions, can differentiate toward various phenotypes. Moreover, iPSCs can be isolated from patients affected from a specific pathology, thus allowing the *in vitro* modeling of the disease and the study of the mechanisms involved in its onset and progression. Patient-derived iPSCs lines can be also exploited as cellular assays for new drug testing and safety assessments, opening the way to a personalized method that can vary in function of the patient/pathology. The relevance of iPSCs *in vitro* disease modeling has been recently reviewed.

Physicochemical Stimuli

The *in vivo* environment guarantees the presence of fundamental molecular cues that direct cell behavior, while the vascularization provides nutrient supply and waste removal. Thus, the presence of molecular factors influencing cellular division, shape, spreading,

proliferation, death, and secretion of ECM components is necessary to successfully model morphogenetic events.

In the design of a 3D model, it should be considered that cells in the middle of the construct could behave differently from cells growing on the surface, depending on nutrients concentration gradient. The development of a successful 3D engineered tissue could be hindered by a limited diffusive transport of nutrients through its thickness. To avoid local concentrations and overcome the diffusion limits, that affect cell behavior, chemical, and mechanical signals should be coupled. Moreover, cells are subjected to extracellular and intracellular mechanical forces *in vivo* that determine their fate. In particular, cells respond to dynamic cues, such as electric fields, osmotic and hydrostatic pressure, stress, strain, fluid flow, and streaming potential, by modifying the surrounding ECM. Mechanical stimuli are usually provided to tissue-engineered constructs by bioreactors specifically designed to reproduce the *in vivo* conditions.

In particular, bioreactors provide mechanical or electrical stimuli and allow a fine modulation of culture conditions to reach tissue maturation. Microscale technologies, such as novel platforms based on microfabrication and microfluidics, have shown to be another important tool, allowing real-time monitoring and high-throughput results, with the possibility to test a single parameter in an independent way. These technological devices could be a valid support for the development of 3D models with relevant functional characteristics, assuring a good reproducibility. The reviews published by Ghaemmaghami et al., Huh et al., and Inamdar and Borenstein provide some insights on this topic.

Bone *In Vitro* Models

Bone is a connective tissue in which cells are surrounded by an ECM constituted by an inorganic phase (~70% w/w) and an organic phase (~30% w/w). The inorganic phase is mainly composed by hydroxyapatite, while the organic one is constituted of type I collagen and other non-collagenous proteins. Bone ECM 3D organization allows the transmission of mechanical stresses, which have shown to be fundamental for bone development. Bone homeostasis is normally guaranteed by the synergic action of osteoblasts and osteoclasts,

which respectively secret and resorb bone ECM in the bone remodeling process. More than 90% of the bone cells population is constituted by osteocytes that are responsible for sensing and transducing the mechanical forces transmitted through the bone and consequently orchestrating the signals of bone resorption and deposition.

Cell cultures on classic plastic 2D supports and animal models have been largely employed to study the basic mechanisms driving bone physiological and pathological processes, but they have been shown to be limited in many applications. Traditional 2D culture lacks the capability to imitate the 3D microenvironment of the native bone, which is fundamental for the regulation of cell–cell and cell–ECM interactions. One of the main limitations of animal models employed for orthopedic research is the interspecies variation of bone tissue. It has been demonstrated that bone composition, density, and mechanical properties of commonly employed animal models (i.e., cow, sheep, pig, dog, chicken, and rat) are significantly different from those of humans. In particular, the rat, which is the most commonly employed animal model, presents the most significant differences from human bone. Moreover, animal models employed to study bone physiopathology present limitations due to differences in bone healing and remodeling processes. One of the most common human bone diseases is osteoporosis: it has been recently estimated that one in five men and one in three women over 50 will experience an osteoporotic fracture in their lifetime. This pathology derives from an imbalance in the remodeling process in which bone resorption prevails on bone deposition and it has high prevalence in postmenopausal women. Animal models for the study of osteoporosis have been thoroughly reviewed in the literature in the last decades. It has to be noted that only human and non-human primates may naturally be affected by osteoporosis. However, acquisition of aged female primates is difficult, expensive, and risky in term of zoonotic diseases transmission. The most common approach is the induction of osteoporosis in animals through ovariectomy. However, none of the already available animal models is able to accurately reproduce the conditions of postmenopausal osteoporosis. Therefore, several different models are usually employed at the same time, with an increase in costs, without the assurance of reaching predictable results of what occurs in humans.

Alternative 3D models have been proposed to reproduce *in vitro* the bone environment. The first example of *in vitro* bone model is represented by explants of bone tissue cultured *ex vivo*, closely mimicking the *in vivo* situation, with the main advantage of isolating local effects from systemic factors. *Ex vivo* cultured bone models have been employed for the study of bone cell biology and the interactions of cells with each other and with the surrounding bone ECM. Nevertheless, the limited nutrient and oxygen supply to the central portion of the explanted bone cultured under static conditions leads to cell necrosis, constituting a big limitation of *ex vivo* models. The introduction of bioreactors allowed an increase in cell viability and the development of long-term *ex vivo* cultured models, demonstrating the importance of mechanical stress on osteoblast behavior. However, the use of explants of human bone tissue cultured *ex vivo* has shown a large variability due to the different sex, age, health conditions, and life style of the donor patients.

In the context of modeling, where reproducibility plays a key role, the employment of TE models based on cells cultured on 3D porous scaffolds looks like a valid alternative.

To the best of our knowledge, the first *in vitro* TE-based 3D model of bone turnover was developed. The molecular mechanisms driving bone turnover was investigated by coculturing murine primary osteoblast and osteoclast precursors for 40 days on a porous ceramic scaffold (Skelite®). In this 3D model, osteoclasts showed the tendency to differentiate when osteoblasts were already differentiated, but the ECM was not completely organized. Under osteogenic stimulation, enhanced bone deposition and reduced resorption were observed, because of the increased expression from osteoblasts of osteoprotegerin, a natural inhibitor of osteoclast formation and functions. This model demonstrated that the 3D environment is able to stimulate osteoprogenitor differentiation in osteoblasts, which in turn promotes osteoclast precursor differentiation in osteoclasts. However, the use of animal cells in this model did not permit to overcome the limits associated with the use of animal-based models. Thus, the use of human cells is required to develop a relevant alternative model.

Few years later, a bone tissue model for the study of bone turnover based on the coculture of osteoblastic/osteoclastic human cells and endothelial cells. A 3D porous ceramic scaffold

composed of hydroxyapatite and beta-tricalcium phosphate was first seeded with osteoprogenitors and endothelial cells, isolated from the stromal vascular fraction of human adipose tissue and then with osteoclast progenitors derived from human peripheral blood. The cells were cultured in a perfusion bioreactor in the presence of osteoclastogenic factors (κB factor ligand, dihydroxyvitamin D_3). This study demonstrated that the osteoclastic resorption activity is strictly correlated to the presence of pre-seeded osteoprogenitors that synthetize ECM components. This 3D multi-culture construct was the first model coupling osteoclastic matrix resorption to active matrix deposition by osteoblastic cells. Once validated, such a model may find application in the testing of drugs regulating bone turnover, or in the evaluation of new bone substitute biomaterials, reducing the use of laboratory animals, according to the 3Rs principle.

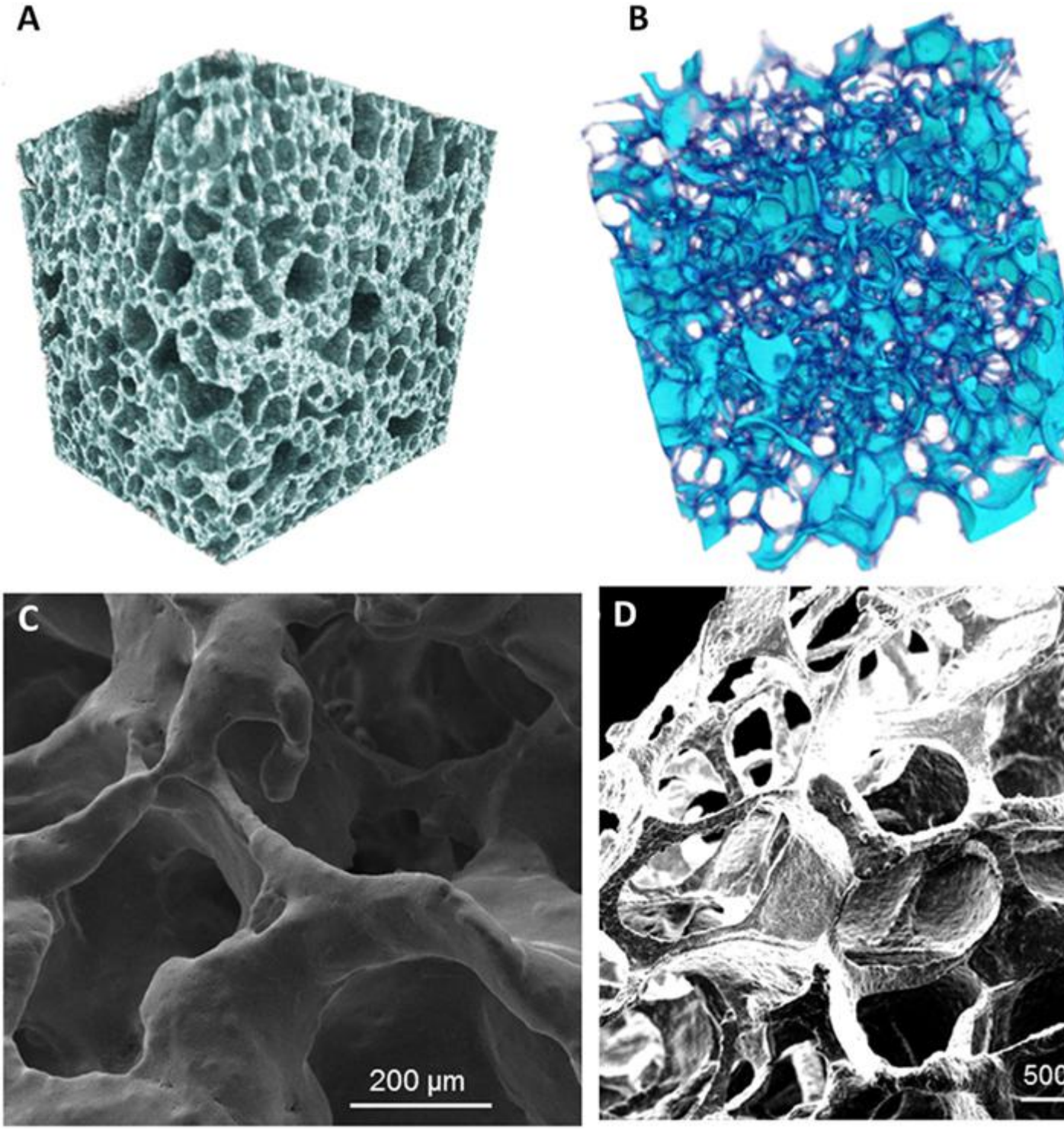
A more complex *in vitro* model of the bone remodeling process was recently developed by , culturing human osteoblast and osteoclast precursors with human umbilical endothelial cells (HUVECs) and including human bone marrow-derived mesenchymal stem cells (HBMSCs). In fact, HBMSCs cocultured with endothelial cells can differentiate in mural cells (pericytes and vascular smooth muscle cells), which have a key role in the development and maintenance of the vascular network. The cells were tetra-cultured in a collagen/fibrinogen hydrogel enriched with calcium phosphate nanoparticles for 10 days. The developed 3D construct showed a good *in vitro* vascularization and improved osteoclast and osteoblast differentiation compared to the respective monocultures. HUVEC and HBMSCs positively influenced the differentiation processes, with enhanced differentiation of both osteoclasts and osteoblasts in the tetra-culture compared to their coculture. This research provides another example of successfully coupling *in vitro* the osteoblast-mediated mineralization (bone deposition) and osteoclast digestion of calcium phosphate (bone resorption). The authors also anticipated that the developed model could be finely tailored to mimic the remodeling process in the presence of pathologies correlated to bone homeostasis dysregulation (e.g., osteoporosis and sclerosteosis). However, it has to be demonstrated first that the cell functions can be preserved in the construct for long-term cultures in order to constitute a valid *in vitro* model. The main limitation of this model is the lack of imitation of bone ECM

characteristics, being scaffold stiffness and architecture (i.e., porosity, pore size, distribution, and interconnection degree) important cues to direct osteoblast precursor differentiation toward the osteogenic lineage and mimic the native bone tissue and its health state.

Although osteocytes have been shown to play an important role in regulating osteoblast activity, the aforementioned studies did not include these cells in the models. Since human primary osteocytes do not proliferate *in vitro*, most of the research around this cell type is performed employing murine osteocyte-like cell lines (MLO-Y4, MLO-A5, IDG-SW3, and Ocy454). The large majority of the *in vitro* models employed to study osteocyte functions are surprisingly 2D, not mimicking the *in vivo* physiological conditions where osteocytes are embedded in the ECM. In a 3D model developed by , MLO-Y4 cells showed an osteocyte-like morphology and the formation of a 3D network. In this study, MLO-Y4 were embedded in a type I collagen-based matrix and cocultured for 1 week with human osteoblast-like cells (MC3T3-E1 or MG63) to investigate cell response under mechanical stimulation. This model could constitute a valid instrument to study the interactions between osteoblasts and osteocytes, but its main drawback concerns the use of cell lines instead of human primary cells.

The importance to develop tissue-engineered pathology-specific bone models has been recently highlighted by . Actually, they developed 3D bioceramic scaffolds mimicking the morphology and architecture of normal and osteoporotic bone trabecular tissues, with the aim to overcome the lack of suitable models to study osteoporosis. The scaffolds were fabricated using a bioactive glass, CEL2, previously designed by . They successfully employed the sponge replication technique to finely tailor the structure of the resulting 3D scaffold. In particular, they studied the effect that the processing parameters have on the final scaffold properties by varying the sponge template porosity, the slurry composition (made mainly from water, polyvinyl alcohol and CEL2 powder), the number of impregnation steps of the template in the slurry, and the final compression step to remove the excess of slurry. The obtained scaffolds showed total porosity, pore size, and strut thickness in the range of those of healthy and pathological trabecular human bone of the femoral head. This research could open the door to new modeling approaches for the *in vitro* investigation of osteoporosis, once

the scaffold will be seeded with relevant cells in order to obtain a complete tissue-engineered model.



The TE approach was also exploited by for the development of an *in vitro* model of the early phase of bone fracture healing, which aimed to support preclinical testing of novel therapeutic approaches. In order to mimic the neighboring bone fragments at the interface with the fracture hematoma, the scaffold was made from two discs of lyophilized human

cancellous bone with a fibrin suspension in the middle. The cells selected for the model were rabbit periosteal osteoprogenitor cells and the final construct was cultured for 2 weeks under dynamic conditions in a bioreactor, with the aim to simulate the *in vivo* mechanical stimulation. In particular, the bioreactor was specifically designed to allow the mimesis of different conditions, such as partial or full weight-bearing and normal walking, by applying cyclic pneumatically driven compression stresses on silicone membranes. The major limitation of the model was the absence of immunocompetent cells, such as platelets, polymorphonuclear neutrophils, monocytes, and macrophages (Bueno and Glowacki, 2011), responsible for the inflammatory response that constitutes the initial phase of fracture healing (McKibbin, 1978). Moreover, the model lacked growth factors and cytokines that are commonly released after traumatic events. Periosteal cells embedded in the fibrin matrix responded to the applied stimulation by producing type IX collagen, thus demonstrating endochondral ossification and showing similarities to the early phase of fracture repair.

As an alternative to human bone primary cells that are highly sensitive to culture conditions, source, patient age, and isolation/purification techniques, and lost differentiation ability after a low number of passages in culture (Bouet et al., 2014), primary mesenchymal stem cells (MSCs) can be isolated from bone marrow, adipose tissue or periosteum, and can be induced to differentiate toward the osteogenic lineage. Another alternative can be represented by the use of human iPSCs (hiPSCs), which have been recently employed in bone TE for regenerative applications (TheinHan et al., 2013; Kang et al., 2014; Tang et al., 2014; Jeon et al., 2016; Ji et al., 2016). These cells may constitute an interesting cell source for the design of *in vitro* models of healthy and pathologic bone tissue. A complete 3D *in vitro* bone model including human osteoblasts, osteoclasts and osteocytes has not been developed yet. The combination of all bone cells in an *in vitro* bone model is challenging and will be of utmost importance to better understand bone cell biology, i.e., to elucidate the role of osteocytes in bone remodeling.

In Vitro Cardiac Tissue Models

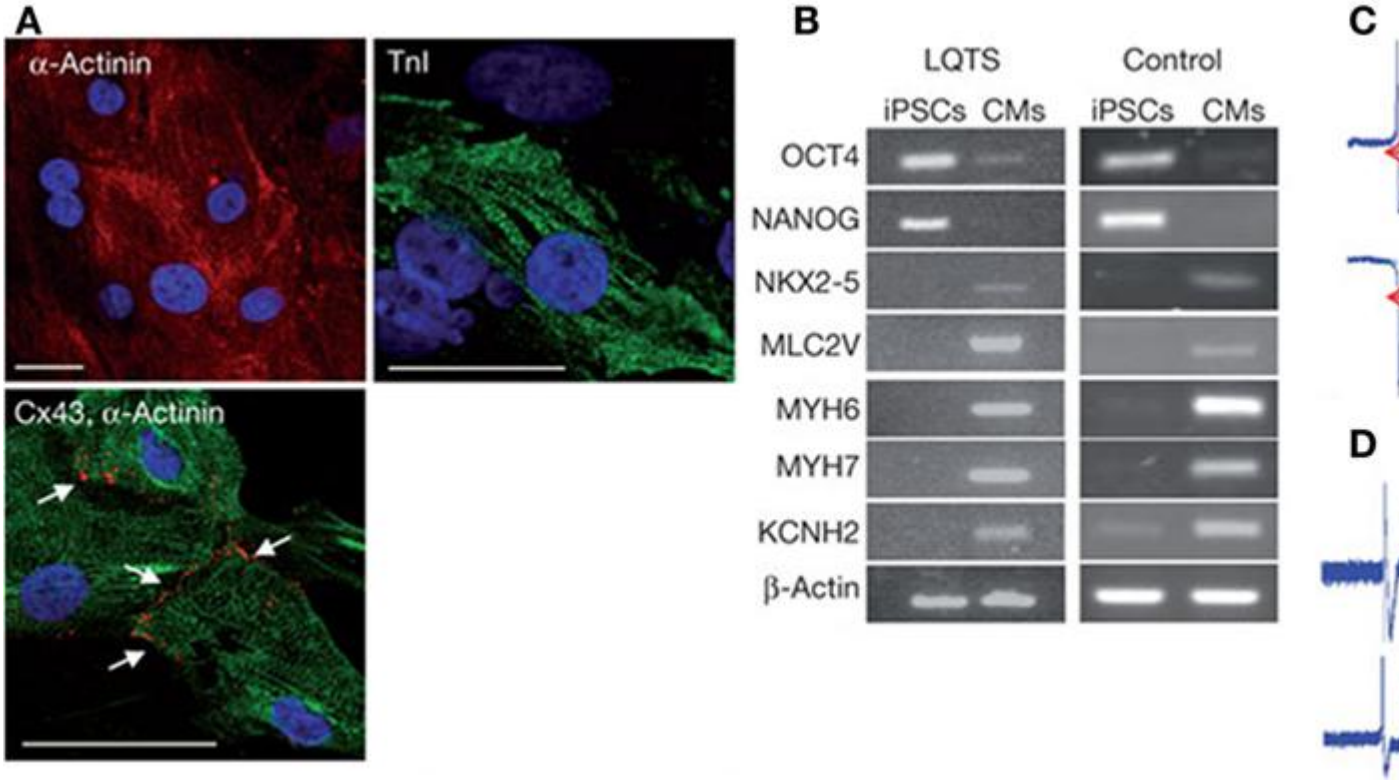
The heart is a complex organ containing a wide variety of cell types (e.g., cardiomyocytes, Purkinje cells, fibroblasts) embedded in an anisotropic and hierarchical architecture. The

activity of heart cells is regulated by an internal control system, sensitive to both external and systemic cues (Benam et al., 2015). Such a complex architecture is supported by a well-defined 3D framework based on fibrous proteins (e.g., collagen, elastin), adhesive glycoproteins (e.g., laminin, fibronectin), and proteoglycans (Parker and Ingber, 2007). This 3D ECM network is responsible for the typical organization of cardiac muscle fibers along a preferred direction, which results in the peculiar mechanical properties and cell functions characterizing the myocardium.

As a consequence of the key role of ECM architecture on heart development and function, the ideal *in vitro* cardiac tissue model should accurately reproduce heart 3D anisotropic structure and vasculature in both healthy and pathological state, control and properly guide cell–cell and cell–ECM interactions and regulate cell fate and functions (Mathur et al., 2016). In pathological conditions, after a myocardial infarction or an ischemia, as well as with aging, the myocardium undergoes a remodeling process characterized by loss of functional cardiomyocytes, hypertrophy of the remaining cells and fibrosis, that result in a progressive alteration of heart mechanical properties (i.e., stiffness increase) and loss of function (Brower et al., 2006; Kwak, 2013). Consequently, an *in vitro* model of pathological cardiac tissue should accurately recapitulate cardiac changes in terms of structure, mechanical properties, cell density, and functions. Such an approach is essential in the design of suitable *in vitro* models for (i) drug development and testing (cardiotoxicity is a major cause of withdrawal of newly commercialized drugs) (Ferri et al., 2013) and (ii) the investigation of disease onset and progression. Both animal models and 2D cell cultures fail to accurately and fully reproduce human physiology in healthy and pathological conditions, as well as in young and aged states. For example, human CMs show significantly different electrophysiological properties with respect to the murine ones: mice heart rate at rest is about 10 times that of humans, while QT segment in humans is about four times that of mice (Passier et al., 2008; Vunjak Novakovic et al., 2014; Benam et al., 2015). As a consequence of the multifactorial nature of cardiovascular diseases, a different animal model should be used depending on the investigated pathology [e.g., porcine is a valid model of atherosclerosis and restenosis since it develops lesions more similar to human disease (Zaragoza et al.,

2011; Leong et al., 2015)]. An appropriate selection of the model would be essential to avoid failing of many research findings upon translation to humans. On the other hand, traditional 2D *in vitro* cell cultures do not properly replicate the complexity of the *in vivo* environment and fail to maintain cardiac cells in culture for a long time, thus limiting the possibility of long-term studies. In this context, bioengineered healthy or pathologic heart tissue constructs can (i) exhibit genotypic and phenotypic properties more similar to native environment, (ii) reproduce cardiac tissue architecture, (iii) promote cell–cell and cell–ECM interactions, stimulating the formation of gap junctions, (iv) favor tissue maturation and the expression of a phenotypic profile similar to that of mature cardiomyocytes, and (v) allow accurate functional parameters measurements (Benam et al., 2015). In the design of a suitable and functional *in vitro* cardiac model, the first issue researcher's interface with is the selection of the optimal source of beating CMs. Early developed heart models were based on immortalized or primary cells extracted from several species, such as chickens, mice, and rats, that did not accurately reproduce the physiology of the human heart, as previously mentioned, and failed in the reproduction of several pathological conditions, such as reduced conductivity, fibrosis, and scar formation (Harding et al., 2007). For these reasons, they were soon replaced by CMs derived from cardiac progenitor cells (CPCs), human ESCs, and hiPSCs that better recapitulate human physiology and pathophysiology (Benam et al., 2015; Mathur et al., 2016). Recent advancements in genome-editing technologies have opened the way to the possibility to engineer hiPSC genome by introducing the gene mutations associated with a specific heart disease, thus allowing the generation of pathological CMs to be used for disease modeling (Wang et al., 2012; Musunuru, 2013; Miyaoka et al., 2014). Do date, hiPSC-derived CMs have been successfully employed to model several rhythm-associated diseases [e.g., long QT syndrome (LQTS) and polymorphic ventricular tachycardia] and dilated and hypertrophic cardiomyopathies, which were thus thoroughly investigated at the cellular level (Figure 3). Moreover, hiPSCs open the way to the development of patient-specific *in vitro* models that could be exploited in the future to better understand disease onset and progression, and specially to test the effects of drugs and therapies on each patient. Nevertheless, an important issue still needs to be

addressed: CMs derived from stem cells are usually small in size and immature, showing a gene expression profile more similar to fetal CMs than adult cardiac muscle cells, and exhibit reduced contractility (Itzhaki et al., 2011). Since several topographical, electrical, mechanical, biochemical, and cellular cues actively contribute to heart development and CM maturation *in vivo*, efforts are currently directed to the exploitation of engineering methods (e.g., morphological and superficial cues, electrical, and mechanical stimulation) to induce stem cell-derived CMs maturation. As an example, properly surface-patterned substrates have been recently coupled with biochemical cues to enhance maturation of hiPSC-derived CMs and design a 3D beating human cardiac micro-chamber mimicking the developing human heart.



Two-dimensional *in vitro* models based on cell seeding on substrate surfaces functionalized with cardiac ECM proteins (e.g., laminin, fibronectin), or microfabricated according to well-defined anisotropic patterns successfully mimic CMs organization in the native cardiac tissue and recapitulate essential physiological and pathological properties of cardiac muscle cells. For instance, aligned murine CMs showed calcium handling, action potentials and conductivity more similar to adult mouse heart, with respect to the same cells cultivated on

randomly oriented substrates. The capability of such models to recapitulate the spatial heterogeneity and conductivity of cardiac tissue was successfully exploited to study pathologies of the electrical conduction system of the myocardium.

The same approach was exploited to design a model of the border zone (the interface between healthy cardiac tissue and an infarcted area). *In vivo*, the border zone shows a non-uniform anisotropic structure resulting from the remodeling cascade activated by a myocardial infarction, which makes this tissue easily susceptible to arrhythmias. The developed model was based on human skeletal myotubes (simulating the typical fibrosis of the border zone) cocultured with neonatal rat ventricular CMs (recapitulating the non-uniform anisotropic architecture of the border zone) on fibronectin-coated coverslips. This system successfully modeled the onset of reentrant arrhythmias in the border zone, allowing the study of the effects of several drugs on this pathology and the explanation of the scarce effects of sodium channel blockers. However, *in vitro* 2D models fail to completely reproduce the mechanical contraction and architecture typical of the heart, thus hindering the ability of cells to interact and exert forces on each other. With the final aim of overcoming these drawbacks, several research groups designed 3D models recapitulating both healthy and pathological cardiac tissue. Scaffolds designed and fabricated for such applications should be biocompatible, exhibit a 3D structure with interconnected pores, which promote cell homing, nutrient and oxygen supply and waste removal, and reproduce both the structural and mechanical properties of the native cardiac tissue. The literature reports on the fabrication of 3D scaffolds based on both natural and synthetic polymers and their blends by either conventional or advanced technologies. A widely investigated technology consists in cardiomyocyte loading into biodegradable natural polymers, e.g., collagen, Matrigel and fibrin, subjected to polymerization according to well-defined geometries (rings, cylinders, plates). Matrigel was often used to enhance cell viability and adhesion due to its composition rich in growth factors and ECM components. Moreover, it was successfully blended with other natural polymers, such as fibrinogen and thrombin, or collagen type I, and seeded with neonatal rat CMs or stem cell-derived CMs to design models for drug screening or for the investigation of dilated cardiomyopathy and arrhythmogenesis. 3D cardiac tissue models were also successfully

developed by culturing CMs derived from ESCs and CPCs on a fibrin-based hydrogel. The diabetic myocardium was successfully recapitulated by seeding neonatal rat CMs on a collagen-based scaffold (Gelfoam®). The developed substrates were cultured in four different situations: normal glucose without or with the addition of insulin (N and NI, respectively), and high glucose without or with the addition of insulin (H and HI, respectively). Results demonstrated that, in diabetic conditions (i.e., H), the gene expression in the bioengineered constructs was similar to that observed in animal models, and accompanied by contractile dysfunctions and reduced electrical excitability. Insulin administration enhanced cell viability, contractility and normalized gene expression in both NI and HI models. On the other hand, administration of anti-diabetic drugs showed anti-apoptotic effects and enhanced excitability in bioengineered constructs cultured according to H conditions, but did not affect gene expression. Ring-shaped bioengineered cardiac tissues were developed to study ischemia/reperfusion conditions *in vitro*. Recapitulation of ischemic conditions (1% O_2 for 6 h) induced the onset of conductive system defects, connexin-43 (the main cardiac connexin found in the gap junctions) dephosphorylation and the down-regulation of cell survival associated proteins, similarly to *in vivo* observations in humans. Such ischemic conditions turned out inhibited by treating the models with cardioprotective drugs, e.g., cyclosporine and acetylcholine. Synthetic polymers are promising alternatives to ECM-derived ones, due to their high versatility, repeatability and controlled composition. The literature reports the exploitation of several synthetic polymers for the design of both cardiac patches and *in vitro* models. The most suitable synthetic polymers for such applications are elastomers, e.g., poly(ester urethane)s poly(glycerol sebacate) , and poly[(1,8-octanediol)-co-(citric acid)] (POC) , which can be easily processed by both conventional and non-conventional techniques. Bursacet al. successfully combined a 3D polyester-based porous scaffold seeded with neonatal rat-derived cardiac muscle cells with dynamic cell culture in a bioreactor to design a bioengineered tissue showing cardiac-specific structure and electrophysiology that make it suitable for *in vitro* studies of impulse propagation. A model of LQTS was successfully developed by Ma and colleagues by seeding CMs derived from iPSCs from pathological patients on anisotropic scaffolds produced by two-photon initiated

polymerization. Healthy cardiac tissue models were also fabricated by seeding iPSC-derived CMs isolated from healthy individuals. The models were validated by assessing their differences in terms of contractility, QT segment duration, tissue structure and response to several molecules, e.g., caffeine, nifedipine, and propranolol.

Aratyn-Schaus et al. recently developed an *in vitro* model of cardiac cell therapy to test the hypothesis that newly formed cardiomyocytes show a weak contractile strength that hampers stress transmission at the junction with native myocytes. To this aim, two cell microtissues were designed by seeding mouse CMs, recapitulating native myocardium, and CMs derived from iPSCs and ESCs, modeling newly formed cells, on soft gels coated with fibronectin, according to a well-defined pattern, mimicking the striated structure and mechanical properties of the heart. The mechanical coupling between the two microtissues was thoroughly studied, demonstrating that newly formed CMs couple with native cells to support synchronous contraction, but the reduced force transmission between them may hamper the complete recovery of contractility. In 2017, the design of cardiac microtissues has been further enhanced by Giacomelli and coworkers that have recently developed a human-derived model recapitulating the cardiomyocyte–endothelium crosstalk, which is responsible for the regulation of heart dimension, oxygen supply, and growth factor secretion and has poorly been considered so far in both cardiac patch and engineered model design.

A 3D scaffold-free cardiac tissue model based on multiple cell type coculture has been recently created for the first time by Rogozhnikov et al. by developing a strategy based on the combination of liposome fusion, bio-orthogonal chemistry, and cell surface engineering, with the ability to trigger and guide the self-assembly of three different cell types (cardiomyocytes, endothelial cells and cardiac fibroblasts) into a functional 3D cardiac tissue. Such an approach could open the way to a new era in cardiac TE, allowing the development of high cell density constructs showing spontaneous synchronous beating throughout the entire matrix (without the application of electrical cues) and efficient cell–cell and cell–ECM crosstalk.

Pancreas *In Vitro* Models

The pancreas is an organ characterized by two distinct functional portions: the exocrine and the endocrine pancreas. The functional unit of the exocrine pancreas is the pancreatic acinar cell, which has the main functions of synthesis, storage and secretion of digestive enzymes. These enzymes are normally activated in the duodenum, meanwhile acute pancreatitis occurs when they are prematurely activated within the pancreatic acinar cell. The endocrine portion of the pancreas is constituted by small clusters of cells called islets of Langerhans. Pancreatic islets contain five cell types: alpha cells (α cells) secrete the hormone glucagon; beta cells (β cells), the most abundant cells in the islets, produce insulin; delta cells (δ cells) secrete the hormone somatostatin; epsilon cells (ε cells) secrete ghrelin, and PP cells secrete pancreatic polypeptides. Moreover, several minor hormones are synthetized from pancreatic islet cells. Hormone secretion is modulated by nervous signals and, thanks to a high vascularization, hormones secreted by islets have ready access to the circulation.

Type I diabetes is a chronic pathological condition in which a cell-mediated autoimmune destruction of pancreatic β cells usually leads to a deficiency in insulin release. Type II diabetes is the most diffused form of diabetes, characterized by disorders of insulin action, due to insulin resistance, and abnormalities in insulin secretion by β cells. Due to the high relevance of these diseases, pancreas studies have been mostly focused on the islets of Langerhans. Moreover, islets are a key focus of type II diabetic drug efficacy testing. Mice constitute the most investigated animal model in pancreas research. However, there are differences in Langerhans islets cell population of mice and human: the ratio β cells/α cells is higher in mice than in humans, meanwhile δ and PP cells are double in humans with respect to mice. Also the architectures of human and mice pancreas present some differences, promoting heterologous contacts between α and β cells in humans, which result in a higher sensitivity to glucose compared to mouse islets. In 2009, Kim and colleagues studies showed that mice present human-type islets when subjected to pathologic conditions characterized by an increased demand for insulin, such as inflammation, obesity, diabetes, and pregnancy. These species differences have raised concerns regarding the use of mice prototypic islets, highlighting the need to develop more realistic islet models.

The first challenge in engineering pancreatic islets is the isolation and purification of viable and functional islets. It has been shown that following islet isolation and purification, apoptotic cell death occurs, involving mainly β cells. This response is mainly due to the loss of cell–cell communication cell–matrix interactions and the disruption of the peri-insular basement membrane, a critical component of the pancreatic ECM, mainly composed of type IV collagen, laminin, and fibronectin. Moreover, when cultured *in vitro*, islet cells undergo necrotic cell death predominantly in the islet core, as a consequence of inadequate oxygen supply due to the high metabolic demands and islet size.

In order to reach a successful strategy to preserve islet cell survival and functions, several studies have focused on restoring the ECM environment. In particular, it has been demonstrated the positive response of pancreatic islets to collagen I/collagen IV mixture, laminin, and peptide epitopes (e.g., RGD sequence of fibronectin), mimicking fundamentals components of the peri-insular basement membrane of pancreatic ECM.

Three-dimensional cultures are preferred to 2D, since β cells easily lose viability and the ability to secrete insulin when cultured on 2D traditional systems. From this point of view, tissue-engineered scaffolds represent a valid alternative to effectively support pancreatic cell culture and secretion of hormones and polypeptides, while bioreactors are useful tools to provide the required perfusion conditions to prevent *in vitro* cell necrosis.

Synthetic polymeric scaffolds have been extensively studied for islet culture and transplantation. In particular, polyester scaffolds were fabricated from lactide and glycolide monomers and copolymers, using different fabrication techniques.

Blomeier and colleagues fabricated macroporous biodegradable scaffolds (pore size in the range of 250 to 400 μm) from copolymers of lactide and glycolide (PLGA) using a gas foaming/particulate leaching process, which improved islet function *in vivo* . Mao et al. developed a construct constituted of PLGA scaffolds (pore size between 100 and 300 μm) seeded with islet-like cells, which transplanted *in vivo* in diabetic immunodeficient mice led to correction of blood glucose levels . In another study, rat islet cells were seeded on porous polyglycolic acid (PGA) fiber scaffold (Synthecon Inc.) previously coated with poly-L-lysine

(PLL) in order to improve cell adhesion. In contrast to the scaffold-free control group, islet cells cultured on scaffolds exhibited improved morphology, viability, and increased insulin secretion.

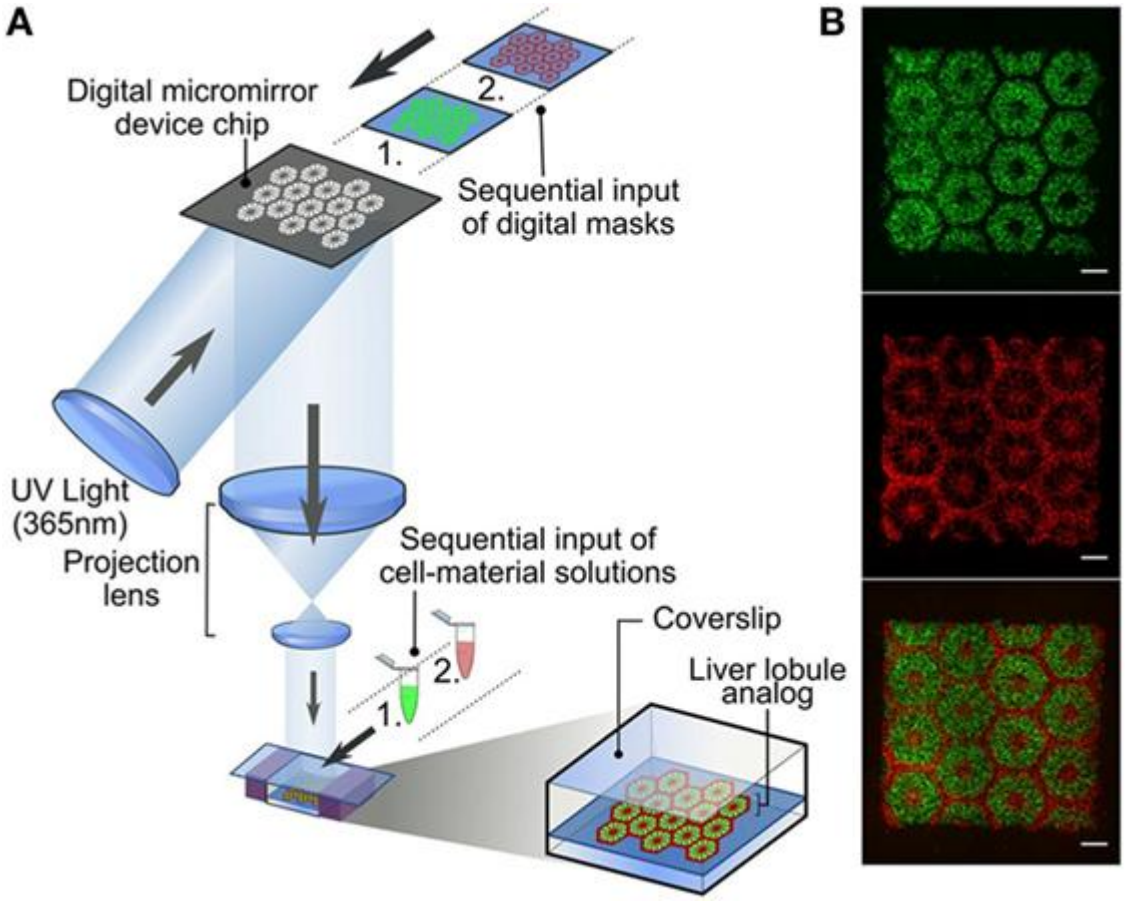
Functionalization of synthetic polymeric scaffolds with bioactive molecules, such as ECM components, has been proposed to mimic the ECM environment. The successful use of PLGA scaffolds produced by gas foaming process and coated with collagen IV, fibronectin, and laminin for culturing and transplant mouse islets. Those scaffolds showed good viability, insulin production and vessel density within the transplanted islets.

Although synthetic polymeric scaffolds have been successfully employed according to the classic TE approach, they are not adequate to replicate pancreas mechanical properties, which play a key role in the design of valuable tissue and organ models. In fact, the pancreas is a non-linear viscoelastic soft tissue. The shear stiffness of healthy pancreatic tissue was found to be in the range of 1–2 kPa, whereas the stiffness of polyesters is higher, namely elastic modulus is generally in the range of 6–7 GPa for polyglycolide) and 2–3 GPa in the case of polylactic acid and poly(lactic-co-glycolic) acid. On the other hand, porous scaffolds used in the previously mentioned studies did not resemble pancreas morphology and a justification for the architecture selection is not generally reported.

It should be observed that further investigations are necessary to understand how pathologic conditions affect the mechanical properties of pancreatic tissue, such as in chronic pancreatitis. In the diagnosis of chronic pancreatitis, it is presumed that pancreatic shear stiffness is proportional to the degree of fibrosis. There is a lack of studies regarding the mechanical characterization of the scaffolds proposed, which is particularly important to elucidate the different behavior of healthy and pathological tissues. Therefore, pathological and physiological models may be achieved taking into account this consideration.

Synthetic and natural-derived hydrogels have been demonstrated to be good candidates as scaffold materials for pancreatic TE because of their high water content, structural and mechanical similarities with the native ECM and high permeability for low molecular weight nutrients, metabolites and hormones, which is a key point in β cell survival.

developed an *in vitro* model of 3D pancreatic islet microenvironment to study the stability and functions of β cells. In this research work, microbeads made from cross-linked poly(ethylene glycol)-co-poly-L-lysine (PEG-co-PLL) hydrogels were developed to act as "synthetic neighbors" of the β cells, aiming to reproduce the high cellular density environment of pancreatic islets. The copolymerization of PLL with PEG was designed to introduce positive charges on the gel surface, allowing the absorption of ECM components derived from rat pancreatic decellularized matrix and inducing cell–ECM interactions. In order to mimic the *in vivo* cell–cell interactions, microbead surface was modified with the cell surface receptor and its membrane-bound ligand pair EphA/EphrinA. In fact, in the pancreatic islets, β cells communicate via EphA receptors and EphrinA ligands. This 3D microenvironment was able to mimic both native cell–cell and cell–ECM interactions, enhancing β cell viability (up to 21 days) and consequently supporting insulin production.



Three-dimensional (3D) printed hepatic lobule models. **(A)** 3D bioprinting of human iPSCs (hiPSC)-derived hepatocytes and supporting cells encapsulated in photocurable hydrogels

[5%w/v gelatin methacrylate (GelMa) for hiPSC-derived hepatocytes to obtain, after photopolymerization a matrix with stiffness similar to the healthy liver tissue, a blend of glycidal methacrylate-hyaluronic acid and GelMa for supporting endothelial and mesenchymal cells to favor endothelial cell proliferation and support vascularization processes] was carried out in two steps according to well-defined patterns **(B)** to finely reproduce their localization in native lobules. In detail, hiPSC-derived hepatocytes were first patterned according to a digital mask with proper geometry, followed by the patterning of the supporting cells using a second digital mask (scale bars 500 µm, supporting cells and hiPSC-hepatocytes were fluorescently marked in red and green, respectively).

Among natural-derived hydrogels, alginate has been successfully used in the classic approach of pancreatic TE, where microencapsulation of the islets is performed to preserve them from immune mediated destruction after transplantation. Thus, alginate hydrogels could represent a valid scaffold for the development of pancreatic *in vitro* models. However, the major limitation of alginate microbeads is the high permeability to a range of small molecules, which can damage or destroy the encapsulated islets. To overcome these problems a perm-selective coating with PLL or Poly-L-Ornithine has been proposed, since these polyamino acids firmly bind to alginate, thereby restricting the permeability of alginate-based microcapsules. In contrast, the capsule must be permeable to nutrients, metabolites and hormones to allow islet survival. It was observed that a reduction in capsule size improves the diffusion of nutrients to the islets. Omer et al. demonstrated that alginate capsules with a diameter of 600 ± 100 µm showed improved stability *in vivo* over larger capsules with diameters of 1000 ± 100 µm. Nevertheless, with reduction in capsule size an increase in the number of capsules containing protruding islets was observed, leading to a higher number of capsules affected by an inflammatory response. Decreasing the islet density in alginate can solve this problem. Not only the size but also the morphology of the microcapsules is an important parameter: spherical microcapsules are necessary for long-term functions.

In order to overcome problems related to the supply of oxygen and nutrients, Li et al. proposed an *in vitro* perfused 3D model for diabetic drug efficacy tests, able to mimic the *in vivo* perfusion conditions of pancreatic islets. Specifically, rat islets were encapsulated in

ultrapure alginate and cultured *in vitro* in a commercial micro-bioreactor system (TissueFlex®). The system supported islet viability and functions *in vitro* over a 7-day culture period. The model displayed a high sensitivity in responding to two typical anti-diabetic drugs (tolbutamide and GLP-1) and drug dosages over conventional 2D and 3D static models.

Bioreactors have shown to be important in the modeling of pancreatic islets environment and in the understanding of the effects of dynamic culture conditions on β cell performance. Hou et al. cultured rat pancreatic islets in stasis and simulated microgravity, in the presence or absence of a PGA fibrous scaffold . The simulated microgravity was achieved through a rotary cell culture system, which allowed high mass transfer of nutrients by maintaining normal morphology of the islets. After 5 days in culture in this bioreactor, the islet grafts were transplanted into leg muscles of diabetic rats to observe their functions and morphology. The results demonstrated that islets cultured under dynamic conditions in the scaffolds exhibited better viability and insulin production compared to those cultured in static condition, confirming the importance of both scaffolds and dynamic culture in the mimesis of the native environment and the maintenance of cell viability and functions. This approach can be translated to the design of an *in vitro* functional islet model.

Pancreas TE is mainly focused on the encapsulation of Langerhans islets for further transplantation. The requisites that should be met in that case are different from those required in the development of an *in vitro* model of the islets themselves. However, there are some common requisites, such as the challenging maintenance of the islet cell viability and functions after isolation. The research works surveyed in this review reported a progress in the design of biomimetic constructs able to support both cell survival and functions in culture; however, more engineered systems are needed to develop valuable pancreas models. Such a goal is challenging, due to the complexity of this organ. Forthcoming pancreatic models need to mimic the native tissue in all the mechanical, topographical and chemical aspects, as well as in the set of physiological cues that characterize the complex pancreatic environment. The models developed for the pancreas are few, indicating that the design of a model for such complicate organ requires further efforts and a closer collaboration between different fields of research.

In Vitro Liver Models

The liver is the largest visceral organ in humans, playing a wide and complex array of vital functions, ranging from metabolic and regulatory activities to protein synthesis and organism defense processes. The liver is characterized by a complex array of vasculature, endothelial cells and parenchymal cells (hepatocytes, hepatocyte precursors, stellate cells, epithelial cells and fibroblasts). Hepatocytes are the leading hepatic parenchymal cell type in terms of both mass (about 60% of liver cells) and number of functions carried out. Liver ECM plays a pivotal role on hepatocyte viability, proliferation, migration and functions. In fact, as a consequence of their structural and functional polarization, hepatocytes need well-defined cell–cell and cell–ECM interactions to remain viable, proliferate and exert their activities. From a structural point of view, the liver is characterized by a complex, highly organized architecture composed of a tessellating system of hexagonal constructs (lobules), fundamental for maintaining hepatic functions .

In pathological conditions of fibrosis, liver architecture is distorted because of ECM proteins accumulation and the formation of fibrous scar tissue, which induce an increase in tissue stiffness. Advanced fibrosis results in cirrhosis that causes dysfunctions and is responsible for hepatic insufficiency.

At present, the procedure leading to the clinical application of newly developed drugs requires more than 10 years and a high economical investment (about one billion euros). Moreover, the majority of the drugs reaching phase III clinical trials shows hepatotoxicity, which hampers their approval for administration in humans, and about one third of approved drugs are withdrawn from the market for unpredicted liver toxicity through currently used experimental setups. Currently, no animal model fully recapitulates all the hepatic and extrahepatic features of healthy and pathological liver: first, some hepatic diseases do not exist in rodents, and second, animals can show higher or lower susceptibility to drugs compared to humans. A wide variability is usually observed between humans and animal models in terms of drug pathogenicity, time of action and effects. In this context, there is increasing interest in developing more reliable *in vitro* liver models, able to imitate liver functions in pharmacokinetics, thus allowing a more accurate drug testing as well as

compliance to 3Rs principle. At the same time, a thorough investigation of hepatic functions and pathologies (e.g., fibrosis, cirrhosis, fatty liver) and the molecular mechanisms at their basis could contribute to a better characterization and understanding of the role of this organ in human homeostasis and metabolism, as well as disease onset and progression.

The simplest *in vitro* liver models consisting of hepatic sub-cellular fractions (e.g., single enzymes, cytosolic factions, mixed fractions) are easy to use and suitable for the investigation of single metabolic functions. However, they cannot be exploited to assess the influence of other parameters/functions on the modeled metabolic pathway and fail in the accurate reproduction of liver complex structure, which strongly influences its functions. 2D liver tissue models comprising monolayer and collagen-sandwich cultures are more reliable than sub-cellular fractions and allow long-term cell culture. Nevertheless, they fail in the mimesis of cell–cell and cell–ECM interactions, essential for hepatocyte viability and functions, resulting in cell dedifferentiation and loss of the majority of their phenotypic properties, including their drug-metabolizing capacity, thus hampering their application in drug testing. Primary human hepatocytes are the gold standard in the design of *in vitro* models reproducing human liver functions for the investigation of drug metabolism and toxicity, as in the study of disease onset, progression and response to new therapies. However, the capability to maintain liver native functions for only few days (about three days) under conventional cell culture conditions, together with tissue shortage and its high variability, have limited the establishment of 2D primary hepatocyte cell culture and consequently the use of these cells in the development of liver models. Primary rodent hepatocytes have been widely exploited in drug testing and drug-drug interaction studies, but the translation of the obtained results to humans is made difficult by differences in metabolism among the two species. On the other hand, human immortalized hepatic cancer cell lines, such as HepG2, show unlimited availability and maintain certain liver functions, e.g., albumin production. However, they do not exhibit drug-metabolizing capacities, which can cause inaccurate drug toxicity testing. In several studies, immortalized hepatic cells turned out suitable models for parent compound toxicity studies and in the assessment of cell polarity and chemotherapy resistance. Hepatocytes derived from stem cells (embryonic- and adult tissue-derived stem cells) exhibit

promising liver-specific features, such as the expression of cytochrome P450 (PYP450) enzymes (enzymes involved in the metabolism of many molecules) and the formation of structures similar to the bile canaliculi. Moreover, they provide a highly available hepatocyte source from different donors, thus enhancing reproducibility and allowing the study of individual-specific drug toxicity. However, the secretion of liver-specific proteins and enzymes, as well as the expression of CYP450 enzymes and membrane transporters, need to be comparable to those of native hepatocytes. 3D liver models accurately replicating liver tissue in terms of architecture and functions are gaining more interest as efficient systems ensuring long-term cell viability and functions and allowing the study of drug metabolism, liver adverse effects and repeated dose testing. Perfused livers and liver slices show the advantage of retaining the 3D architecture, cell–cell and cell–ECM interactions of the native organ, but they do not allow long-term studies since necrosis occurs within 48–72 h and metabolic enzymes levels decrease in 6–72 hours. Therefore, the use of biomaterial-based 3D liver tissue models is a rapidly expanding field, devoted to the design of novel smart constructs, accurately mimicking liver tissue in its healthy and pathological state, from a morphological, mechanical and biochemical point of view. The literature reports several *in vitro* 3D liver models based on native or stem cell-derived liver cells, cultured under static or dynamic conditions in ECM-based scaffolds (decellularized liver), 3D porous scaffolds/hydrogels based on natural or synthetic polymers. For instance, Mazza et al. have recently reported a successful human liver decellularization protocol, with preservation of the native honeycomb architecture of connective tissue fibers and essential ECM proteins (collagen type I and IV, fibronectin). The procedure was followed by repopulation with human liver-derived cells, thus confirming that the optimized decellularization procedure did not damage the liver tissue features, essential for cell homing, migration and proliferation. Similar results were also reported by Uygun and colleagues, which also demonstrated that the preservation of the native vasculature results in enhanced cell engraftment, survival and maintenance of hepatocyte functions (albumin secretion, urea synthesis and cytochrome P450 similar to normal liver). The 3D structure resulting from decellularization is also an efficient

substrate for stem cell differentiation: Wang et al. reported hepatic stem cell differentiation into parenchymal cells, showing mature and stable phenotype for more than 2 months.

Among synthetic polymers, biocompatible and biodegradable polyesters, e.g., PLGA, poly(L-lactic acid) (PLLA), PGA and poly(ε-caprolactone) (PCL), have been widely investigated in hepatic TE. PLGA- and PLLA-based 3D porous scaffolds showed the ability to favor primary hepatocyte adhesion, proliferation and maturation, as well as ESC differentiation toward the hepatocyte lineage. 3D nanofibrous scaffolds produced by electrospinning were identified as promising substrates for stem cell differentiation toward hepatocytes. For instance, several studies reported the hepatic differentiation of bone marrow-derived MSCs, human ESCs and cord blood-derived stem cells cultivated on electrospun matrices. Moreover, Vinci et al. have recently designed an *in vitro* liver model based on a multi-layer PLGA-based scaffold fabricated by pressure assisted microsyringe using a hexagonal repetitive unit mimicking hepatic lobules. This study demonstrated that cell density and cell–cell interactions are strongly influenced by substrate architecture, while cell metabolism is mainly regulated by nutrient supply and interstitial-like flow. Indeed, albumin and urea production rates turned out greatly augmented during dynamic cell culture in a low-shear, high-flow bioreactor. The presence of integrin-binding sites and specific sequences in natural polymers can be exploited to activate the desired cell behavior and signaling cascades. Dvir-Ginzberg and colleagues reported the capability of alginate scaffolds to maintain hepatocyte functions during 1-week culture time and allow liver cell reorganization into spheroids and their development into hepatic functional tissue after 6 weeks. Furthermore, alginate scaffolds showed ability to induce hepatic differentiation of bone marrow-derived stem cells in the presence of specific growth factors. Hyaluronate-based scaffolds promoted hepatic functions of liver cells as a consequence of the signaling pathways activated by cell binding to the material, since hepatocytes have specific binding sites for hyaluronate. Similarly, chitosan properly modified with specific polysaccharide residues can bind liver cells, thus improving their functions and metabolic activity. Moreover, doping natural polymer-based scaffolds with conducting polymers seems to positively influence hepatocyte adhesion and proliferation, enhancing the electrical communication among cells.

Induced pluripotent stem cells have recently arisen as promising candidates for the design of patient-specific hepatic models. In detail, Ma et al. encapsulated hiPSC-derived hepatic cells and supporting endothelial and mesenchymal cells in gelatin-based hydrogels and 3D printed the resulting cellularized biomaterials according to predefined biomimetic patterns mimicking hepatic lobule structure.

From a morphological point of view, hiPSC-derived hepatocytes, human vein endothelial cells and adipose tissue-derived stem cells were embedded in a 3D hexagonal structure, according to well-defined patterns finely mimicking their localization in the native lobules. On the other hand, from a functional point of view, high expression of liver-specific genes, increased metabolic activity, and enhanced cytochrome P450 induction were observed. Therefore, the designed 3D tri-culture model showed improved morphological, phenotypic and functional properties, thus opening the way to the clinical introduction of *in vitro* models for personalized *in vitro* drug screening and disease study.

Discussion and Future Directions

Nowadays, TE approaches are widely investigated for the development of 3D *in vitro* models of healthy and pathological tissues and organs. The results summarized in this review demonstrate that the TE approach can be successfully employed in the development of 3D models of many human tissues and organs, such as bone, heart, pancreas, and liver. This interdisciplinary field is rapidly developing and advancing. However, despite the already published exciting results in the design, fabrication, and validation of organ/tissue models, there are still challenges that need to be addressed.

The main limitation deals with the identification of the proper cell sources for model design, and in particular with the difficulty to isolate human primary cells and culture them *in vitro* for long-term experiments, since primary cells show high sensitivity to culture conditions and progressive loss of differentiation potential after a low number of passages in culture. In the last decade, the most promising novelty in the cell biology field is the discovery of iPSCs. Reprogramming adult cells to embryonic-like states has innumerable potential applications in regenerative medicine and drug development. iPSC-related research

fields are highly active and rapidly developing. iPSCs are interesting cell sources and represent a breakthrough that will ultimately open many new avenues, although many technical and basic science issues remain.

The immature phenotype of differentiated cells derived from progenitor cells (induced, embryonic and adult stem cells) makes them appropriate for neonatal tissue/organs or early-stages diseases models. Moreover, the properties of human-derived cells strongly depend on the tissue source, the patient age and health condition, the adopted isolation/purification technique as well as the applied differentiation protocol.

Three-dimensional cell surrounding environment exerts a synergistic role in guiding cell fate and behavior; therefore, a fine replication *in vitro* of the *in vivo* environment in terms of both architecture and mechanical properties is mandatory. Furthermore, the development of a biomimetic environment is a key aspect in the long-term culture of any type of cells. Such a goal is challenging, due to the complexity of human organs/tissues and the difficulty to mimic them at different aging and health stages in all the mechanical, topographical and chemical aspects, as well as in the set of physiological cues characteristic of their environment. In this scenario, bringing together new advances in material engineering, microfabrication techniques and microfluidics is gaining more and more importance.

The advancement in biomaterials science, including the design and development of new synthetic copolymers, ceramic and glass–ceramics, bioartificial blends of natural and synthetic materials, can be exploited to finely tune the chemical, thermal, mechanical and surface properties of the scaffold-forming materials. The progress of these custom-made materials allows to accurately recapitulate the bulk properties of the native tissue at different health levels.

Furthermore, emergent advanced scaffold fabrication methods are gaining more and more interest as they allow the fabrication of more reproducible scaffolds with a highly controlled process. These include a good control of pore size and interconnection, which facilitates gas diffusion, nutrient supply and waste removal, leading to a degree of vascularization of the constructs similar to native tissues.

Specifically, techniques based on rapid prototyping, bioprinting, organ printing and bottom-up approaches are emerging as promising tools, with the potential to overcome the drawbacks of conventional approaches, and providing a step forward the clinical validation of 3D *in vitro* engineered tissue models as a consequence of their high versatility, temporal and spatial control. Finally, to increase the industrial scalability of the models and allow high-throughput applications, recent developments in cell biology, TE, microfluidics and biomaterials are now being integrated in microfluidically perfused organ-on-chip models.

Some relevant *in vitro* tissue models have recently started to appear in the literature, improving the confidence that, in the future, the design of 3D models of high quality and relevance can significantly reduce the number of animals used in research as well as the failure of drug-screening methodologies.

Traditional approaches to restoring tissue function involve organ donation. Despite attempts to encourage organ donations, there is a shortage of many transplantable human tissues. Currently, more than 256,000 patients in the United States are awaiting organ transplantation, and less than 28,000 people receive transplants annually (HRSA/OPTN, April 2013).

Tissue engineering is an interdisciplinary science that involves the use of biological sciences and engineering to develop tissues that restore, maintain, or enhance tissue function. However, to realize the dream of creating off-the-shelf organs for transplantation, a renewable source of cells is required. Embryonic stem (ES) cells have potential as a source of cells for tissue engineering applications because they can differentiate into all somatic cells and proliferate indefinitely. However, several challenges must be addressed before ES cells can be used safely and effectively in clinics. This chapter introduces tissue engineering approaches, and the role of ES cells in various tissue engineering applications.

Two main approaches are utilized in this area to produce **engineered tissue**. First, scaffolding can be **used** as a cell support device upon which **cells** are seeded in vitro; **cells** are then encouraged to lay down matrix to produce the foundations of a **tissue** for transplantation.

What stages of early embryonic development are important for generating embryonic stem cells?

Embryonic stem cells, as their name suggests, are derived from embryos. Most embryonic stem cells are derived from embryos that develop from eggs that have been fertilized *in vitro*—in an *in vitro* fertilization clinic—and then donated for research purposes with informed consent of the donors. They are *not* derived from eggs fertilized in a woman's body.

B. How are embryonic stem cells grown in the laboratory?

Growing cells in the laboratory is known as cell culture. Human embryonic stem cells (hESCs) are generated by transferring cells from a **preimplantation**-stage embryo into a

plastic laboratory culture dish that contains a nutrient broth known as culture medium. The cells divide and spread over the surface of the dish. In the original protocol, the inner surface of the culture dish was coated with mouse embryonic skin cells specially treated so they will not divide. This coating layer of cells is called a feeder layer. The mouse cells in the bottom of the culture dish provide the cells a sticky surface to which they can attach. Also, the feeder cells release nutrients into the culture medium. Researchers have now devised ways to grow embryonic stem cells without mouse feeder cells. This is a significant scientific advance because of the risk that viruses or other macromolecules in the mouse cells may be transmitted to the human cells.

The process of generating an embryonic stem cell line is somewhat inefficient, so lines are not produced each time cells from the preimplantation-stage embryo are placed into a culture dish. However, if the plated cells survive, divide and multiply enough to crowd the dish, they are removed gently and plated into several fresh culture dishes. The process of re-plating or subculturing the cells is repeated many times and for many months. Each cycle of subculturing the cells is referred to as a passage. Once the cell line is established, the original cells yield millions of embryonic stem cells. Embryonic stem cells that have proliferated in cell culture for six or more months without differentiating, are pluripotent, and appear genetically normal are referred to as an embryonic stem cell line. At any stage in the process, batches of cells can be frozen and shipped to other laboratories for further culture and experimentation.

C. What laboratory tests are used to identify embryonic stem cells?

At various points during the process of generating embryonic stem cell lines, scientists test the cells to see whether they exhibit the fundamental properties that make them embryonic stem cells. This process is called characterization.

Scientists who study human embryonic stem cells have not yet agreed on a standard battery of tests that measure the cells' fundamental properties. However, laboratories that grow human embryonic stem cell lines use several kinds of tests, including:

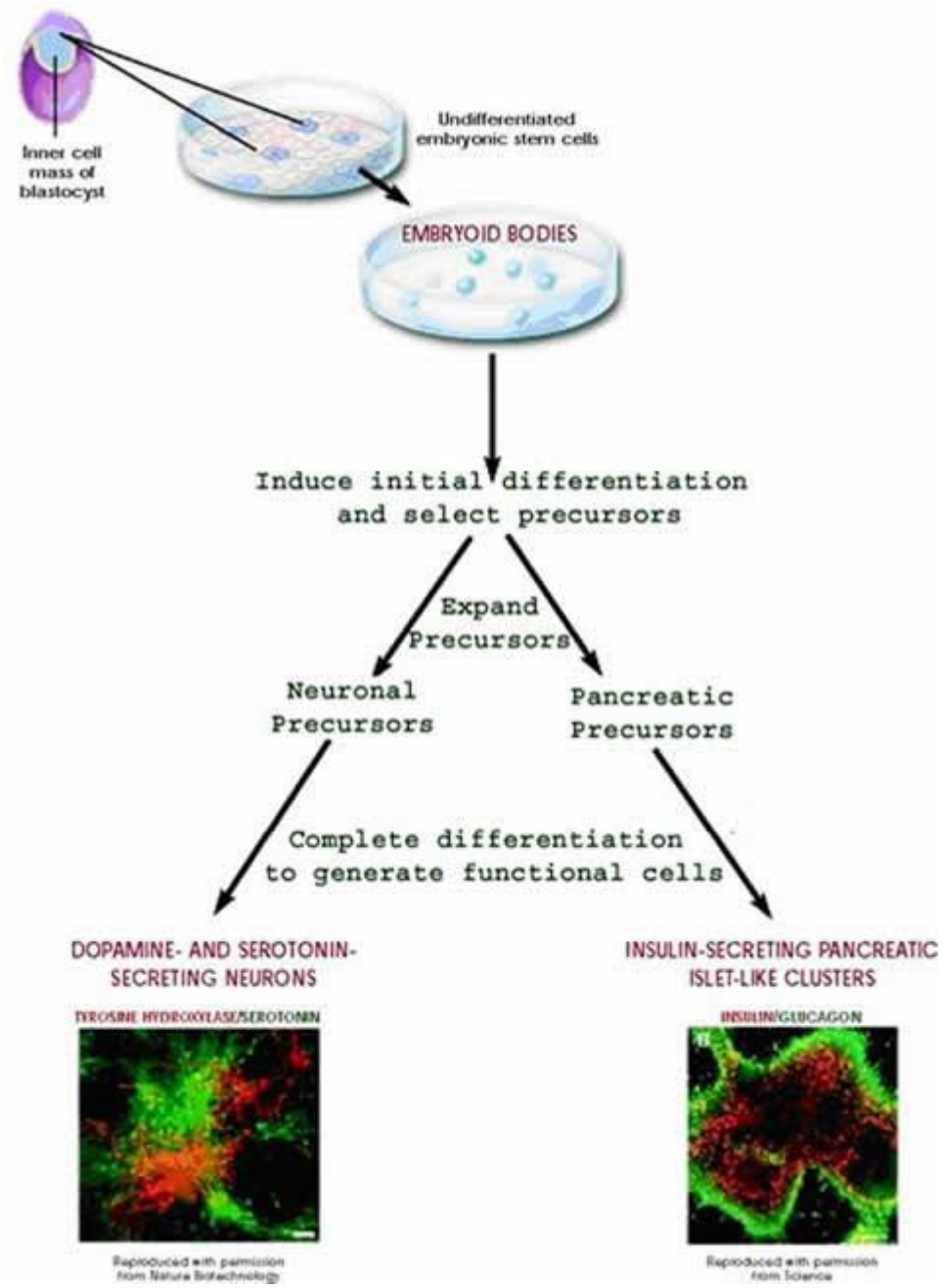
- Growing and subculturing the stem cells for many months. This ensures that the cells are capable of long-term growth and self-renewal. Scientists inspect the cultures through a microscope to see that the cells look healthy and remain undifferentiated.
- Using specific techniques to determine the presence of transcription factors that are typically produced by undifferentiated cells. Two of the most important transcription factors are Nanog and Oct4. Transcription factors help turn genes on and off at the right time, which is an important part of the processes of cell differentiation and embryonic development. In this case, both Oct 4 and Nanog are associated with maintaining the stem cells in an undifferentiated state, capable of self-renewal.
- Using specific techniques to determine the presence of particular cell surface markers that are typically produced by undifferentiated cells.
- Examining the chromosomes under a microscope. This is a method to assess whether the chromosomes are damaged or if the number of chromosomes has changed. It does not detect genetic mutations in the cells.
- Determining whether the cells can be re-grown, or subcultured, after freezing, thawing, and re-plating.

- Testing whether the human embryonic stem cells are pluripotent by 1) allowing the cells to differentiate spontaneously in cell culture; 2) manipulating the cells so they will differentiate to form cells characteristic of the three germ layers; or 3) injecting the cells into a mouse with a suppressed immune system to test for the formation of a benign tumor called a teratoma. Since the mouse's immune system is suppressed, the injected human stem cells are not rejected by the mouse immune system and scientists can observe growth and differentiation of the human stem cells. Teratomas typically contain a mixture of many differentiated or partly differentiated cell types—an indication that the embryonic stem cells are capable of differentiating into multiple cell types.

D. How are embryonic stem cells stimulated to differentiate?

As long as the embryonic stem cells in culture are grown under appropriate conditions, they can remain undifferentiated (unspecialized). But if cells are allowed to clump together to form embryoid bodies, they begin to differentiate spontaneously. They can form muscle cells, nerve cells, and many other cell types. Although spontaneous differentiation is a good indication that a culture of embryonic stem cells is healthy, the process is uncontrolled and therefore an inefficient strategy to produce cultures of specific cell types.

So, to generate cultures of specific types of differentiated cells—heart muscle cells, blood cells, or nerve cells, for example—scientists try to control the differentiation of embryonic stem cells. They change the chemical composition of the culture medium, alter the surface of the culture dish, or modify the cells by inserting specific genes. Through years of experimentation, scientists have established some basic protocols or "recipes" for the directed differentiation of embryonic stem cells into some specific cell types (Figure 1). (For additional examples of directed differentiation of embryonic stem cells, refer to the 2006 NIH stem cell report.)



If scientists can reliably direct the differentiation of embryonic stem cells into specific cell types, they may be able to use the resulting, differentiated cells to treat certain diseases in the future. Diseases that might be treated by transplanting cells generated from human embryonic stem cells include diabetes, traumatic spinal cord injury, Duchenne's muscular dystrophy, heart disease, and vision and hearing loss.

Embryonic stem cells (ESCs) are stem cells derived from the undifferentiated inner mass cells of a human embryo.

Embryonic stem cells are pluripotent, meaning they are able to grow (i.e. differentiate) into all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm.

In other words, they can develop into each of the more than 200 cell types of the adult body as long as they are specified to do so.

Embryonic stem cells are distinguished by two distinctive properties: their pluripotency, and their ability to replicate indefinitely.

ES cells are pluripotent, that is, they are able to differentiate into all derivatives of the three primary germ layers: ectoderm, endoderm, and mesoderm.

These include each of the more than 220 cell types in the adult body.

Pluripotency distinguishes embryonic stem cells from adult stem cells found in adults; while embryonic stem cells can generate all cell types in the body, adult stem cells are multipotent and can produce only a limited number of cell types.

Additionally, under defined conditions, embryonic stem cells are capable of propagating themselves indefinitely.

This allows embryonic stem cells to be employed as useful tools for both research and regenerative medicine, because they can produce limitless numbers of themselves for continued research or clinical use.

Because of their plasticity and potentially unlimited capacity for self-renewal, ES cell therapies have been proposed for regenerative medicine and tissue replacement after injury or disease.

Diseases that could potentially be treated by pluripotent stem cells include a number of blood and immune-system related genetic diseases, cancers, and disorders; juvenile diabetes;

Parkinson's; blindness and spinal cord injuries.

Besides the ethical concerns of stem cell therapy, there is a technical problem of graft-versus-host disease associated with allogeneic stem cell transplantation.

However, these problems associated with histocompatibility may be solved using autologous donor adult stem cells, therapeutic cloning, stem cell banks or more recently by reprogramming of somatic cells with defined factors (e.g. induced pluripotent stem cells).

Other potential uses of embryonic stem cells include investigation of early human development, study of genetic disease and as in vitro systems for toxicology testing.

Transgenic animals

Definition

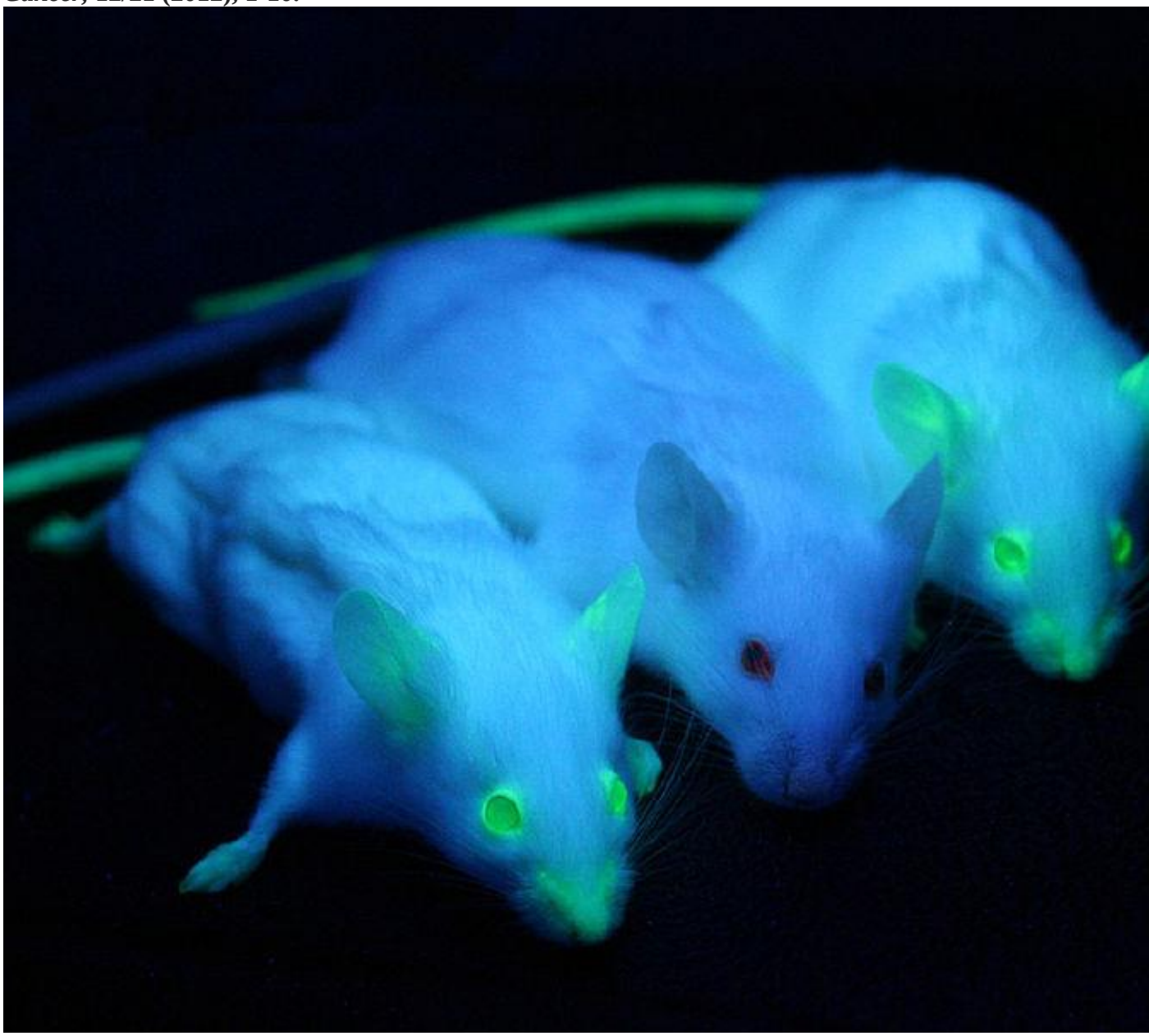
A transgenic animal is one whose genome has been altered by the transfer of a gene or genes

from another species or breed.

The photo shows two transgenic mice positioned either side of a plain mouse. The

transgenic mice have been genetically modified so that they carry a green fluorescent

protein which glows green under blue light. Credit: Ingrid Moen et alet al., *BMC Cancer,* 12/21 (2012), 1-10.



Connections Frank Ruddle | CRISPR | Monoclonal antibodies | Recombinant DNA

Importance

Transgenic animals are routinely used in the laboratory as models in biomedical research. Over 95 per cent of those used are genetically modified rodents, predominantly mice. They are important tools for researching human disease, being used to understand gene function in the context of disease susceptibility, progression and to determine responses to a therapeutic

intervention.

Mice have also been genetically modified to naturally produce human antibodies for use as therapeutics. Seven out of the eleven monoclonal antibody drugs approved by the FDA between 2006 and 2011 were derived from transgenic mice.

Transgenic farm animals are also being explored as a means to produce large quantities of complex human proteins for the treatment of human disease. Such therapeutic proteins are currently produced in mammalian cell-based reactors, but this production process is expensive. In 2008, for example, the building of a new cell-based manufacturing facility for one therapeutic protein was estimated to cost over US$500 million. A cheaper option would be to develop a means to produce recombinant proteins in the milk, blood or eggs of transgenic animals. Progress in this area, however, has been slow to-date. Only two biomedical products have so far received regulatory approval. The first is human antithrombin III, a therapeutic protein produced in the milk of transgenic goats, which is used to prevent clots in patients with hereditary antithrombin deficiency receiving surgery or undergoing childbirth. A relatively small herd of goats (about 80) can supply enough human antithrombin III for all of Europe. The second product is a recombinant human C12 esterase inhibitior produced in the milk of transgenic rabbits. This is used to treat hereditary angiodema, a rare genetic disorder which causes blood vessels in the blood to expand and cause skin swellings.

Discovery

The ability to produce transgenic animals is reliant on a number of components. One of the first things needed to generate transgenic animals is the ability to transfer embryos. The first successful transfer of embryos was achieved by Walter Heape in Angora rabbits in 1891. Another important component is the ability to manipulate the embryo. In vitro manipulation of embryos in mice was first reported in the 1940s using a culture system. What is also vital

is the ability to manipulate eggs. This was made possible through the efforts of Ralph Brinster, attached to the University of Pennsylvania, who in 1963 devised a reliable system to culture eggs, and that of Teh Ping Lin, based at the California School of Medicine, who in 1966 outlined a technique to micro-inject fertilised mouse eggs which enabled the accurate insertion of foreign DNA.

The first genetic modification of animals was reported in 1974 by the virologist Rudolph Jaenisch, then at the Salk Institute, and the mouse embryologist Beatrice Mintz at Fox Chase Cancer Center. They demonstrated the feasibility of modifying genes in mice by injecting the SV40 virus into early-stage mouse embryos. The resulting mice carried the modified gene in all their tissues. In 1976, Jaenisch reported that the Moloney Murine Leukemia Virus could also be passed on to offspring by infecting an embryo. Four years later, in 1980, Jon Gordon and George Scango together with Frank Ruddle, announced the birth of a mouse born with genetic material they had inserted into newly fertilised mouse eggs. By 1981 other scientists had reported the successful implantation of foreign DNA into mice, thereby altering the genetic makeup of the animals. This included Mintz with Tim Stewart and Erwin Wagner at the Fox Chase Cancer Center in Philadelphia; Brinster and Richard Palmiter at the University of Washington, Seattle; and Frank Costantini and Elizabeth Lacy at Oxford University.

Such work laid the basis for the creation of transgenic mice genetically modified to inherit particular forms of cancer. These mice were generated as a laboratory tool to better understand the onset and progression of cancer. The advantage of such mice is that they provide a model which closely mimics the human body. The mice not only provide a means to gain greater insight into cancer but also to test experimental drugs.

Application

Transgenic animals are animals (most commonly mice) that have had a foreign gene deliberately inserted into their genome. Such animals are most commonly created by the

microinjection of DNA into the pronuclei of a fertilised egg which is subsequently implanted into the oviduct of a pseudopregnant surrogate mother. This results in the recipient animal giving birth to genetically modified offspring. The progeny are then bred with other transgenic offspring to establish a transgenic line. Transgenic animals can also be created by inserting DNA into embryonic stem cells which are then micro-injected into an embryo which has developed for five or six days after fertilisation, or infecting an embryo with viruses that carry a DNA of interest. This final method is commonly used to manipulate a single gene, in most cases this involves removing or 'knocking out' a target gene. The end result is what is known as a ‘knockout’ animal.

Since the mid-1980s transgenic mice have become a key model for investigating disease. Mice are the model of choice not only because there is extensive analysis of its completed genome sequence, but its genome is similar to the human. Moreover, physiologic and behavioural tests performed on mice can be extrapolated directly to human disease. Robust and sophisticated techniques are also easily available for the generic manipulation of mouse cells and embryos. Another advantage of mice is the fact that they have a short reproduction cycle. Other transgenic species, such as pig, sheep and rats are also used, but their use in pharmaceutical research has so far been limited due to technical constraints. Recent technological advances, however, are laying the foundation for wider adoption of the transgenic rat.

Transgenic rodents play a number of critical roles in drug discovery and development. Importantly, they enable scientists to study the function of specific genes at the level of the whole organism which has enhanced the study of physiology and disease biology and facilitated the identification of new drug targets. Due to their similarity in physiology and gene function between humans and rodents, transgenic rodents can be developed to mimic human disease. Indeed, an array of transgenic mice models have been produced for this purpose. Mice are being used as models, for example, to study obesity, heart disease,

diabetes, arthritis, substance abuse, anxiety, ageing, Alzheimer's disease and Parkinson's disease. They are also used to study different forms of cancer. In addition, transgenic pigs are being investigated as a source of organs for transplants, which if proven clinically safe could overcome some of the severe donor organ shortages. The development of transgenic animals has recently been transformed by the emergence of the new gene editing tool CRISPR which greatly reduced the number of steps involved in the creation of transgenic animals, making the whole process much faster and less costly.

This section on transgenic mice was jointly written by Lara Marks and Dmitriy Myelnikov. For more information see D. Myelnikov, 'Transforming mice: technique and communication in the making of transgenic animals, 1974-1988', unpublished PhD, Cambridge University, 2015.

Transgenic animals: timeline of key events

Date	Event	People	Places
23 Jun 1925	Oliver Smithies was born in Halifax, United Kingdom	Smithes	University of Washington, University of North Carolina
1929	Jackson Memorial Laboratories established to develop inbred strains of mice to study the genetics of cancer and other diseases		Jackson Memorial Laboratoroies
19 Aug 1929	Frank Ruddle was born in West New York, New Jersey	Ruddle	Yale University
18 Sep 1951	Anthony J Clark was born in Blackpool, UK	Anthony Clark	Roslin Institute

Date	Event	People	Places
1974	First publication on inserting foreign DNA into mice	Jaenisch, Mintz	Salk Institute, Fox Chase Institute for Cancer Research
September 1980	Scientists reported the first successful development of transgenic mice	Barbosa, Gordon, Plotkin, Ruddle, Scangos	Yale University
November 1980	Technique published using fine glass micropipettes to inject DNA directly into the nuclei of cultured mammalian cells. High efficiency of the method enables investigators to generate transgenic mice containing random insertions of exogenous DNA.	Capecchi	University of Utah
5 Nov 1981	First successful transmission of foreign DNA into laboratory mice	Constantini, Lacy	Oxford University, Yale University
December 1982	Giant mice made with the injection of rat growth hormone	Brinster, Palmiter	University of Pennsylvania, University of Washington Seattle
1983	Course started in the molecular embyology of mice	Costantini, Hogan,	Cold Spring Harbour

Date	Event	People	Places
		Lacy	Laboratory, NIMR, Sloan Kettering Cancer Research Center, Columbia University
1985	First transgenic mice created with with genes coding for both the heavy and light chain domains in an antibody.	Kohler, Rusconi	Max-Planck Institute
6 Nov 1987	Publication of gene targeting technique for targetting mutations in any gene	Thomas, Capecchi	University of Utah
1988	Patent application filed for a method to create transgenic mice for the production of human antibodies	Bruggeman, Caskey, Neuberger, Surani, Teale, Waldmann, Williams	Laboratory of Molecular Biology, Babraham Institute, Cambridge University
12 Apr 1988	OncoMouse patent granted	Leder, Stewart	Harvard University
12 Jun 1992	First transgenic mouse model created for studying link between DNA methylation and disease	Li, Bestor, Jaenisch	Whitehead Institute for Biomedical

Date	Event	People	Places
			Research
1994	First transgenic mice strains reported for producing human monoclonal antibodies	Bruggemann, S.Green, Lonsberg, Neuberger	Cell Genesys, GenPharm, Laboratory of Molecular Biology
5 Jul 1996	Dolly the sheep, the first cloned mammal, was born	Wilmut, Campbell	Roslin Institute
9 Jul 1997	Birth of first sheep cloned with human genes	Schnieke, Kind, Ritchie, Mycock, Scott, Wilmutt, Colman, Campbell	PPL Therapeutics, Roslin Institute
14 Feb 2003	Dolly the sheep, the first cloned mammal, died	Wilmut	Roslin Institute
12 Aug 2004	Anthony J Clark died	Anthony Clark	Roslin Institute
September 2006	First fully human monoclonal antibody drug approved		Agensys, Amgen
2007	Nobel Prize for Physiology for Medicine awarded for	Capecchi	University of

Date	Event	People	Places
	discoveries enabling germline gene modification in mice using embryonic stem cells	, Evans, Smithies	North Carolina, University of Utah
10 Mar 2013	Frank Ruddle died in New Haven, Connecticut	Ruddle	Yale University
26 Oct 2013	Michael Neuberger died	Neuberger	Laboratory of Molecular Biology
23 Sep 2015	Beijing Genomics Institute announced the sale of the first micropigs created with the help of the TALENs gene-editing technique		Beijing Genomics Institute
5 Oct 2015	CRISPR/Cas9 modified 60 genes in pig embryos in first step to create organs suitable for human transplants	Church	Harvard University
10 Jan 2017	Oliver Smithies died	Smithies	University of Washington, University of North Carolina
20 Apr 2017	Diabetes research using transgenic mice shows the protein P2X7R plays important role in inflammation and immune system offering new avenue for treating kidney disease	Menzies	University of Edinburgh, University College London, Imperial College
23 Jan 2019	CRISPR-Cas9 used to control genetic inheritance in	Grunwald, Gntz,	University of California San

Date	Event	People	Places
	mice	Poplawski, Xu, Bier, Cooper	Diego

Xenotransplantation is any procedure that involves the transplantation, implantation or infusion into a human recipient of either (a) live cells, tissues, or organs from a nonhuman animal source, or (b) human body fluids, cells, tissues or organs that have had *ex vivo* contact with live nonhuman animal cells, tissues or organs. The development of xenotransplantation is, in part, driven by the fact that the demand for human organs for clinical transplantation far exceeds the supply.

Currently ten patients die each day in the United States while on the waiting list to receive lifesaving vital organ transplants. Moreover, recent evidence has suggested that transplantation of cells and tissues may be therapeutic for certain diseases such as neurodegenerative disorders and diabetes, where, again human materials are not usually available.

Although the potential benefits are considerable, the use of xenotransplantation raises concerns regarding the potential infection of recipients with both recognized and unrecognized infectious agents and the possible subsequent transmission to their close contacts and into the general human population. Of public health concern is the potential for cross-species infection by retroviruses, which may be latent and lead to disease years after infection. Moreover, new infectious agents may not be readily identifiable with current techniques.

1. Which of the following is not a source of energy in active muscle cells?	Creatine phosphate	ATP	Lactic acid	Glucose	Lactic acid
2. Higher dissolved oxygen concentration in the culture media are toxic and lead to	DNA degradation	lipid peroxidation	metabolism of nutrients in culture media at a rate greater than that required for consumptio	all of the above	all of the above
3. Which of the following is the technique used for the embryo culture?	Organ cultures on plasma clots	Organ cultures on agar	Whole embryo cultures	All of these	All of these
The major problem associated with the isolation of free cells and cell aggregates from organs is that of	releasing the cells from their supporting matrix	inhibiting the cells from their supporting matrix	disintegrating the cells from their supporting matrix	none of the above	releasing the cells from their supporting matrix
Which of the following cells would be considered differentiated?	Blastomere	Spemann organizer	Myotome of the somite	Muscle cell	Muscle cell
Where are the stem cells that renew the epithelium of the gut found?	in the bone marrow	in the inner cell mass	in the villi, underlying the dead outer layer of keratinocytes	near the bottom of the crypt	near the bottom of the crypt