Cell Culture Techniques

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Lec. 1: Introduction to cell lines & cell culture technique

What is Cell Culture?

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

The cells may be removed from the tissue directly and disaggregated by enzymatic or mechanical means before cultivation, or they may be derived from a cell line or cell strain that has been already established. It occurs *in vitro* (in glass) as opposed to *in vivo* (in life). The culture process allows single cells to act as independent units, much like a microorganism such as a bacterium or fungus. The cells are capable of dividing. They increase in size and, in a batch culture, can continue to grow until limited by some culture variable such as nutrient depletion.

Cultures normally contain cells of one type although mixed cultures are used in some studies. The cells in culture may be genetically identical (homogenous population) or may show some genetic variation (heterogeneous population). A homogenous population of cells derived from a single parental cell is called a clone. Therefore, all cells within a clonal population are genetically identical.

Freshly isolated cultures from mammalian tissues are known as **primary cultures** until **sub-cultured**. At this stage, cells are usually still closely represent the parent cell types as well as in the expression of tissue specific properties. After several sub-cultures onto fresh media, the cell line will either die out or ‘transform’ to become a continuous cell line. Such cell lines show many alterations from the primary cultures including change in morphology, chromosomal variation and increase in capacity to give rise to tumors in hosts with weak immune systems.

Animal cells can be grown either in an unattached suspension culture or attached to a solid surface. Suspension cultures have been successfully developed to quite large bioreactor volumes, with successful production of viruses and therapeutic proteins.

Types of Culture

Primary culture refers to the stage of culture after cells are isolated from the tissue and proliferated under the appropriate conditions until they occupy all of the available substrate (reach **confluence**). At this stage, the cells have to be **subcultured (passaged)** by transferring them to a new vessel with a fresh growth medium to provide more room for continued growth.

Cell Line

Cell culture is the process by which cells are grown under controlled conditions, generally outside their natural environment. After the cells of interest have been isolated from living tissue, they can subsequently be maintained under carefully controlled conditions. These conditions vary widely for each cell type, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel containing a substrate or medium that supplies cells with the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones and gases (O_2, CO_2), and regulates the physicochemical environment (pH, osmotic pressure, temperature).

A cell culture developed from a single cell and therefore consisting of cells with a uniform genetic make-up. After the first subculture, the primary culture becomes known as a cell line or subclone. Cell lines derived from primary cultures have a limited life span (they are finite), and as they are passaged, cells with the highest growth capacity predominate, resulting in a degree of genotypic and phenotypic uniformity in the population.

Most cells require a surface or an artificial substrate **(adherent or monolayer culture)**, whereas others can be grown free floating in culture medium **(suspension culture)**. The lifespan of most cells is genetically determined, but some cell culturing cells have been "transformed" into immortal cells which will reproduce indefinitely if the optimal conditions are provided.

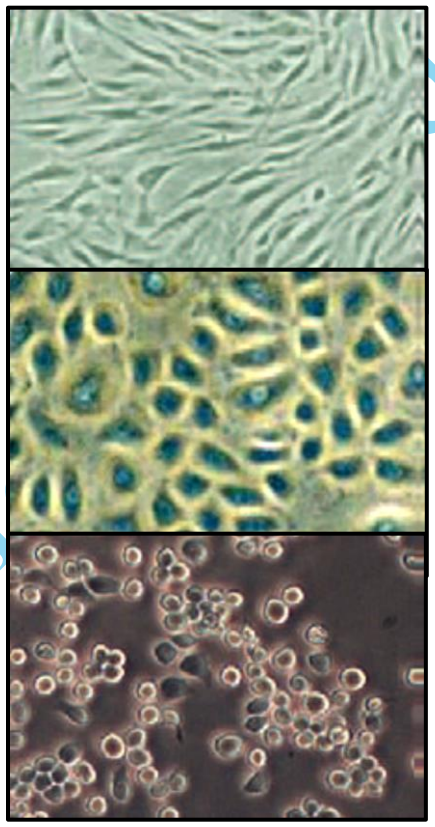
Morphology of Cells in Culture

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

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

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

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



Cell Strain

A cell strain is derived either from a primary culture or subpopulation of a cell line by the selection or cloning of cells having specific properties or characteristics which must be defined. A cell strain often acquires additional genetic changes subsequent to the initiation of the parent line.

Cell strains are cells that have been adapted to culture but, unlike cell lines, have a finite division potential. Non-immortalized cells stop dividing after 40 to 60 population doublings and, after this, they lose their ability to proliferate (a genetically determined event known as **senescence**).

Finite and Continuous Cell Line

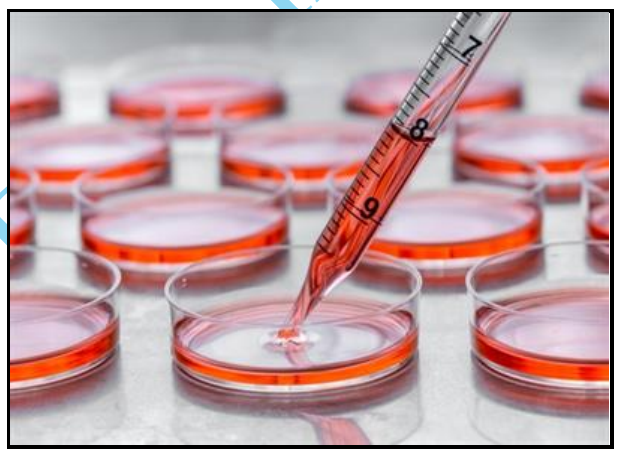
Normal cells usually divide only a limited number of times before losing their ability to proliferate, which is a genetically determined event known as **senescence (aging)**; these cell lines are known as **finite**. However, some cell lines become immortal through a process called transformation, which can occur spontaneously or can be chemically or virally induced. When a finite cell line undergoes transformation and acquires the ability to divide indefinitely, it becomes a **continuous cell line**.

Attempts have been made to culture almost every tissue, including neuronal cells, bone, cartilage and hair cells. Human fibroblasts are easier to culture than epithelial cells. Different epithelial cells show different responses to culture conditions. Despite advances in culturing techniques, human epithelial cells could not be maintained in culture for long time periods. The problem is the tendency of human cells to undergo senescence after a certain cell division.

Cell culture in two and three dimensions

Research in tissue engineering, stem cells and molecular biology primarily involves cultures of cells on flat plastic dishes. This technique is known as two-dimensional (2D) cell culture.

From the advance of polymer technology, today's standard plastic dish (commonly known as the Petri dish) had arose for the 2D cell culture. However, various researchers today also utilize culturing laboratory flasks and conicals.



Petri Dishes

Different sizes of cell culture flasks

In addition, cell culture in three dimensions has been described as "Biology's New Dimension". Currently, there is an increase in use of 3D cell cultures in research areas including drug discovery, cancer biology, regenerative medicine, nanomaterials assessment and basic life science research. A 3D cell culture is an artificially created environment in which biological cells are permitted to grow or interact with their surroundings in all three dimensions. Unlike 2D environments, a 3D cell culture allows cells *in vitro* to grow in all directions, similar to how they would *in vivo*. These three-dimensional cultures are usually grown in bioreactors, small capsules in which the cells can grow into spheroids, or 3D cell colonies. Approximately 300 spheroids are usually cultured per bioreactor.

Samples of Bioreactors

Sample of Bioreactor

Applications of Cell Culture (Why Grow Cells in Culture?)

The major application of human cell culture is in stem cell industry, where mesenchymal stem cells can be cultured and cryopreserved for future use. Tissue engineering potentially offers dramatic improvements in low cost medical care for hundreds of thousands of

patients annually, as well as Novel ideas in the field include recombinant DNA-based vaccines.

In addition, cell culture and cell lines have assumed an important role in studying physiological, pathophysiological and differentiation processes of specific cells. It allows the examination of stepwise alterations in the structure, biology and genetic makeup of the cell under controlled environments. This is especially valuable for complex tissues, such as the pancreas, which is composed of various cell types, where *in vivo* examination of individual cells is difficult, if not impossible. Cell culture is one of the major tools used in cellular and molecular biology, providing excellent model systems for studying:

1. The normal physiology and biochemistry of cells (e.g., metabolic studies, aging).

2. To test the effects of drugs and chemical and toxic compounds on specific cell types (normal or cancerous cells), as well as the mutagenesis and carcinogenesis.

3. It is used in drug screening and development.

4. To synthesize large scale of biological compounds and manufacturing valuable biological materials and compounds from large scale cell cultures. These biological materials encompass a broad range of cell products and include specific proteins or viruses that require animal cells for proliferation (e.g., vaccines, therapeutic proteins).

For example, therapeutic proteins can be synthesized in large quantities by growing genetically engineered cells in large-scale cultures. The number of such commercially valuable biologicals has increased rapidly over the last decade and has led to the present widespread interest in animal cell culture technology.

5. To study the sequential or parallel combination of various cell types to generate artificial tissues (e.g. artificial skin). Possibility of generating artificial tissues is an emerging and intensively studied area of biotechnology known as **“tissue engineering”**.

6. The milestone in the animal cell culture technology came in 1975 with the production of hybrid cells (known as hybridoma) from the fusion of two or more cells capable of continuous production of a single type of antibody. These antibodies have diagnostic and therapeutic value and are now produced commercially in kilogram quantities from large-scale cultures of hybridomas.

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.

The disadvantage is that, after a period of continuous growth, cell characteristics can change and may become quite different from those found in the starting population. Cells can also adapt to different culture environments (e.g. different nutrients, temperatures, salt concentrations etc.) by varying the activities of their enzymes. However, the growth rate of animal cells is relatively slow compared with bacteria. Whereas bacteria can double every 30 minutes or so, animal cells require anywhere from 18 to 24 hr to double. This makes the animal culture vulnerable to contamination, as a small number of bacteria would soon outgrow a larger population of animal cells.

Cryopreservation

If a surplus of cells are available from subculturing, they should be treated with the appropriate protective agent (e.g., DMSO or glycerol) and stored at temperatures below -130˚C (**cryopreservation**) until they are needed.

Cells Bank

The first step in generating a cell bank is the production of the Master Cell Bank **(MCB)** by expanding the cell substrate. The Working Cell Bank **(WCB)** that is used to start the manufacturing process is derived from a single or a few vials of cells of the MCB. Thus, using this cell bank system (MCB and WCB) providing a continuous supply of cells.

In both banks (MCB and WCB), different types of quality control tests are applied. The viability tests measure the proportion of viable cells following freezing or thawing cells in the banking process. The microbiological controls guarantee the absence of any adventitious agents like viruses, mycoplasma, bacteria, and fungi. Karyotyping assess the chromosomal stability of the cells in the continuous culture process. Finally, the DNA fingerprinting and isoenzyme analysis detect respectively the presence of intra-species and inter-species cross-contamination. Therefore, both techniques (DNA fingerprinting, and isoenzyme analysis) are an essential tool in the quality control of cell-banking. Today, the **American Type Culture Collection (ATCC)** carried out the identification of all their cell lines.

Cell line cross-contamination

The term of cross-contamination is used to indicate misidentification of one cell line by another, rather than contamination by a microbiological organism. While not as common as microbial contamination, extensive cross-contamination of many cell lines with HeLa and other fast growing cell lines is a clearly-established problem with serious consequences.

Obtaining cell lines from reputable cell banks, periodically checking the characteristics of the cell lines, and practicing good aseptic technique are practices that will help you avoid cross-contamination. DNA fingerprinting, karyotype analysis and isotype analysis can confirm the presence or absence of cross-contamination in your cell cultures.

Cell line cross-contamination can be a problem for scientists working with cultured cells. Studies suggest anywhere from 15-20% of the time, cells used in experiments have been misidentified or contaminated with another cell line. Problems with cell line cross-contamination have even been detected in lines from the NCI-60 panel (National Cancer Institute-USA), which are used routinely for drug-screening studies. Major cell line repositories, including the American Type Culture Collection (ATCC), the European Collection of Cell Cultures (ECACC) and the German Collection of Microorganisms and Cell Cultures (DSMZ), have received cell line submissions from researchers that were misidentified by them. Such contamination poses a problem for the quality of research produced using cell culture lines, and the major repositories are now authenticating all cell line submissions.

To address this problem of cell line cross-contamination, researchers are encouraged to authenticate their cell lines at an early passage to establish the identity of the cell line. Authentication should be repeated before freezing cell line stocks, every two months during active culturing and before any publication of research data generated using the cell lines. Many methods are used to identify cell lines, including isoenzyme analysis, human lymphocyte antigen (HLA) typing, chromosomal analysis, karyotyping, morphology and STR analysis (short tandem repeat).

Other technical issues

As cells generally continue to divide in culture, they generally grow to fill the available area or volume. This can generate several issues:

1. Nutrient depletion in the growth media.

2. Changes in the pH of the growth media.

3. Accumulation of apoptotic/necrotic (dead) cells.

4. Cell-to-cell contact can stimulate cell cycle arrest, causing cells to stop dividing which known as **contact inhibition**.

5. Cell-to-cell contact can stimulate cellular differentiation.

6. Genetic and epigenetic alterations, with a natural selection of the altered cells potentially leading to overgrowth of abnormal, culture-adapted cells with decreased differentiation and increased proliferative capacity.

7. The choice of culture medium might affect the physiological relevance of findings from cell culture experiments due to the differences in the nutrient composition and concentrations.

Aseptic Technique

Successful cell culture depends heavily on keeping the cells free from contamination by microorganisms as mentioned earlier. Non-sterile supplies, media and reagents, airborne particles laden with microorganisms, unclean incubators and dirty work surfaces are all sources of biological contamination. Aseptic technique designed to provide a barrier between the microorganisms in the environment and the sterile cell culture, depends upon a set of procedures to reduce the probability of contamination from these sources. The elements of aseptic technique are a sterile work area, good personal hygiene, sterile reagents and media, and sterile handling.

Manipulation of cultured cells

Among the common manipulations carried out on culture cells are media changes, passaging cells, and transfecting cells. These are generally performed using tissue culture methods that build on aseptic technique. Aseptic technique aims to avoid contamination with bacteria, yeast or other fungi, viruses or other cell lines.

Manipulations are typically carried out in a biosafety cabinet or laminar flow cabinet to exclude contaminating microorganisms. Antibiotics (e.g. penicillin and streptomycin) and antifungals (e.g. amphotericin B and Antibiotic-Antimycotic solution) can also be added to the growth media.

As cells undergo metabolic processes, acid is produced and the pH decreases. Often, a pH indicator is added to the medium to measure nutrient depletion.

Biosafety Cabinet

Culture Media

The culture medium is the most important component of the culture environment, because it provides the necessary nutrients, growth factors and hormones for cell growth, as well as regulating the pH and the osmotic pressure of the culture. Although initial cell culture experiments were performed using natural media obtained from tissue extracts and body fluids. The need for standardization, media quality and the increased demand led to the development of defined media. The three basic classes of media are **basal media**, **reduced-serum media**, and **serum-free media**, which differ in their requirement for supplementation with **serum**.

Serum is vitally important as a source of growth and adhesion factors, hormones, lipids and minerals for the culture of cells in basal media. In addition, serum also regulates cell membrane permeability and serves as a carrier for lipids, enzymes, micronutrients and trace elements into the cell. However, using serum in media has a number of disadvantages including high cost, problems with standardization, specificity, variability and unwanted effects such as stimulation or inhibition of growth and/or cellular function on certain cell cultures. If the serum is not obtained from reputable source, contamination can also pose a serious threat to successful cell culture experiments. To address this threat, all Invitrogen

and GIBCO products, including sera, are tested for contamination and guaranteed for their quality, safety, consistency and regulatory compliance.

Culture media

Media changes

In the case of adherent cultures, the media can be removed directly by aspiration, and then is replaced. Media changes in non-adherent cultures involve centrifuging the culture and resuspending the cells in fresh media.

Components of cell culture media

Component	Function
Carbon source (glucose/glutamine)	Source of energy
Amino acid	Building blocks of protein
Vitamins	Promote cell survival and growth
Balanced salt solution	An isotonic mixture of ions to maintain optimum osmotic pressure within the cells and provide

	essential metal ions to act as cofactors for enzymatic reactions, cell adhesion etc.
Phenol red dye	pH indicator. The color of phenol red changes from orange/red at pH 7–7.4 to yellow at acidic (lower) pH and purple at basic (higher) pH.
Bicarbonate /HEPES buffer	It is used to maintain a balanced pH in the media

Typical Growth conditions

Parameter	
Temperature	37 °C
CO_2	5%
Relative Humidity	95%

Tissue culture and engineering

Cell culture is a fundamental component of tissue culture and tissue engineering, as it establishes the basics of growing and maintaining cells *in vitro*. The major application of human cell culture is in stem cell industry, where mesenchymal stem cells can be cultured and cryopreserved for future use. Tissue engineering potentially offers dramatic improvements in low cost medical care for hundreds of thousands of patients annually.

Vaccines

Vaccines for polio, measles, mumps, rubella and chickenpox are currently made in cell cultures. Due to the H5N1 pandemic threat, research into using cell culture for influenza vaccines is being funded by the United States government. Novel ideas in the field include recombinant DNA-based vaccines, such as one made using human adenovirus (a common cold virus) as a vector, and novel adjuvants.