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الفصل الدراسي الثاني

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The Historical Background

Tissue culture was first devised at the beginning of the twentieth century as a method for studying the behavior of animal cells free of systemic variations that might arise in vivo both during normal homeostasis and under the stress of an experiment. As the name implies, the technique was elaborated first with undisaggregated fragments of tissue, and growth was restricted to the migration of cells from the tissue fragment, with occasional mitoses in the outgrowth.

As culture of cells from such primary explants of tissue dominated the field for more than 50 years [Fischer, 1925; Parker, 1961], it is not surprising that the name ‘‘tissue culture’’ has remained in use as a generic term despite the fact that most of the explosive expansion in this area in the second half of the twentieth century was made possible by the use of dispersed cell cultures.

Disaggregation of explanted cells and subsequent plating out of the dispersed cells was first demonstrated by Rous [Rous and Jones, 1916], although passage was more often by surgical subdivision of the culture [Fischer, Carrel, and others] to generate what were then termed cell strains.

L929 was the first cloned cell strain, isolated by capillary cloning from mouse L-cells [Sanford et al., 1948]. It was not until the 1950s that trypsin became more generally used for subculture, following procedures described by Dulbecco to obtain passaged monolayer cultures for viral plaque assays [Dulbecco, 1952], and the generation of a single cell suspension by trypsinization, which facilitated the further development of single cell cloning.

Gey established the first continuous human cell line, HeLa [Gey et al., 1952]; this was subsequently cloned by Puck [Puck and Marcus, 1955] when the concept of an X-irradiated feeder layer was introduced into cloning.

Tissue culture became more widely used at this time because of the introduction of antibiotics, which facilitated long-term cell line propagation although many people were already warning against continuous use and the associated risk of harboring cryptic, or antibiotic-resistant, contaminations[Parker, 1961]. The 1950s were also the years of the development of defined media [Morgan et al., 1950; Parker et al., 1954; Eagle, 1955, 1959; Waymouth, 1959],which led ultimately to the development of serum-free media [Ham, 1963, 1965].

Tissue culture is used as a generic term to include organ culture and cell culture.

Organ culture will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue in vivo.

Cell culture refers to a culture derived from dispersed cells taken from original tissue, from a primary culture, or from a cell line or cell strain by enzymatic, mechanical, or chemical disaggregation.

Histotypic culture implies that cells have been reaggregated or grown to re-create a three-dimensional structure with tissue like cell density, e.g., by cultivation at high density in a filter well, perfusion and overgrowth of a monolayer in a flask or dish, reaggregation in suspension over agar or in real or simulated zero gravity, or infiltration of a three-dimensional matrix such as collagen gel.

Organotypic implies the same procedures but recombining cells of different lineages, e.g., epidermal keratinocytes in combined culture with dermal fibroblasts, in an attempt to generate a tissue equivalent.

First experiment

Harrison [1907] chose the frog as his source of tissue, presumably because it was a cold-blooded animal, and consequently, incubation was not required. Furthermore, because tissue regeneration is more common in lower vertebrates, he perhaps felt that growth was more likely to occur than with mammalian tissue. Although his technique initiated a new wave of interest in the cultivation of tissue in vitro, few later workers were to follow his

example in the selection of species. The stimulus from medical science carried future interest into warm-blooded animals, in which both normal development and pathological development are closer to that found in humans.

Applications

-INTRACELLULAR ACTIVITY: DNA transcription, protein synthesis, energy metabolism, drug metabolism, cell cycle, differentiation, apoptosis.

-INTRACELLULAR FLUX: RNA processing, hormone receptors, metabolite flux, calcium mobilization, signal transduction, membrane trafficking.

-PHARMACOLOGY: Drug action, ligand receptor interactions, drug metabolism, drug resistance

-CELL-CELL INTERACTION: Morphogenesis, paracrine control, cell proliferation kinetics, metabolic cooperation, cell adhesion and motility, matrix interaction, invasion.

-GENOMICS: Genetic analysis, transfection, infection, transformation, immortalization, senescence.

-CELL PRODUCTS: Proteomics, secretion, biotechnology, biorector design, product harvesting, downstream processing.

-TISSUE ENGINEERING: Tissue constructs, matrices and scaffolds, stem cell sources, propagation, differentiation.

-IMMUNOLOGY: Cell surface epitopes, hybridomas, cytokines and signaling, inflammation

-TOXICOLOGY: Infection, cytotoxicity, mutagenesis, carcinogenesis, irritation, inflammation.

ADVANTAGES OF TISSUE CULTURE

Control of the Environment. The two major advantages of tissue culture are control of the physiochemical environment (pH, temperature, osmotic pressure, and O2 and CO2 tension), which may be controlled very precisely, and the physiological conditions, which may be kept relatively constant, but cannot always be defined. Most cell lines still require supplementation of the medium with serum or other poorly defined constituents. These supplements are prone to batch variation and contain undefined elements such as hormones and other regulatory substances. The identification of some of the essential components of serum, together with a better understanding of factors regulating cell proliferation, has made the replacement of serum with defined constituents feasible.

Characterization and Homogeneity of Sample Tissue samples are invariably heterogeneous. Replicates—even from one tissue—vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous (or at least uniform) constitution, as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture, replicate samples are identical to each other, and the characteristics of the line may be perpetuated over several generations, or even indefinitely if the cell line is stored in liquid nitrogen. Because experimental replicates are virtually identical, the need for statistical analysis of variance is reduced.

Economy, Scale, and Mechanization Cultures may be exposed directly to a reagent at a lower, and defined, concentration and with direct access to the cell. Consequently, less reagent is required than for injection in vivo, Cultures may be exposed directly to a reagent at a lower, and defined, concentration and with direct access to the cell. Consequently, less reagent is required than for injection in vivo.

LIMITATIONS

Expertise: Culture techniques must be carried out under strict aseptic conditions, because animal cells grow much less rapidly than many of the common contaminants, such as bacteria, molds, and yeasts. Furthermore, unlike microorganisms, cells from multicellular animals do not normally exist in isolation and, consequently, are not able to sustain an independent existence without the provision of a complex environment simulating blood plasma or interstitial fluid.

Quantity: A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. The cost of producing cells in culture is about 10 times that of using animal tissue. Consequently, if large amounts of tissue (>10 g) are required, the reasons for providing them by culture must be very compelling.

Dedifferentiation and Selection: When the first major advances in cell line propagation were achieved in the 1950s, many workers observed the loss of the phenotypic characteristics typical of the tissue from which the cells had been isolated. This effect was blamed on dedifferentiation, a process assumed to be the reversal of differentiation, but later shown to be largely due to the overgrowth of undifferentiated cells of the same or a different lineage. The development of serum-free selective media has now made the isolation of specific lineages quite possible, and it can be seen that, under the right conditions, many of the differentiated properties of these cells may be restored.

Origin of Cells: If differentiated properties are lost, for whatever reason, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived. Stable markers are required for characterization of the cells. In addition, the culture conditions may need to be modified so that these markers are expressed.

Instability: Instability is a major problem with many continuous cell lines, resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures of untransformed cells, heterogeneity in growth rate and the capacity to differentiate within the population can produce variability from one passage to the next

TYPES OF TISSUE CULTURE

There are three main methods of initiating a culture:

(1) **Organ culture** implies that the architecture characteristic of the tissue in vivo is retained, at least in part, in the culture Toward this end, the tissue is cultured at the liquid–gas interface (on a raft, grid, or gel), which favors the retention of a spherical or three-dimensional shape.

(2) **In primary explant culture**, a fragment of tissue is placed at a glass (or plastic)–liquid interface, where, after attachment, migration is promoted in the plane of the solid substrate.

(3) **Cell culture** implies that the tissue, or outgrowth from the primary explant, is dispersed (mechanically or enzymatically) into a cell suspension, which may then be cultured as an adherent monolayer on a solid substrate or as a suspension in the culture medium Because of the retention of cell interactions found in the tissue from which the culture was derived, organ cultures tend to retain the differentiated properties of that tissue. They do not grow rapidly (cell proliferation is limited to the periphery of the explant and is restricted mainly to embryonic tissue).

The formation of a cell line from a primary culture implies: (1) an increase in the total number of cells over several generations and (2) the ultimate predominance of cells or cell lineages with the capacity for high growth, resulting in (3) a degree of uniformity in the cell population. The line may be characterized, and the characteristics will apply for most of its finite life span.

The derivation of **continuous** (or ‘‘established,’’ as they were once known) cell lines usually implies a phenotypic change, or transformation. When cells are selected from a culture, by cloning or by some other method, the subline is known as a **cell strain**.

A detailed characterization is then implied. Cell lines or cell strains may be propagated as an adherent monolayer or in suspension. Monolayer culture signifies that, given the opportunity, the cells will attach to the substrate and that normally the cells will be propagated in this mode. Anchorage dependence means that attachment to (and usually, some degree of spreading onto) the substrate is a prerequisite for cell proliferation.

Monolayer culture is the mode of culture common to most normal cells, with the exception of hematopoietic cells.

Suspension cultures are derived from cells that can survive and proliferate without attachment (anchorage independent); this ability is restricted to hematopoietic cells, transformed cell lines, and cells from malignant tumors. It can be shown, however, that a small proportion of cells that are capable of proliferation in suspension exists in many normal tissues. The identity of these cells remains unclear, but a relationship to the stem cell or uncommitted precursor cell compartment has been postulated.

Cultured cell lines are more representative of precursor cell compartments in vivo than of fully differentiated cells, as, normally, most differentiated cells do not divide. Because they may be propagated as a uniform cell suspension or monolayer, cell cultures have many advantages, in quantitation, characterization, and replicate sampling, but lack the potential for cell–cell interaction and cell–matrix interaction afforded by organ cultures.

Primary Culture

A primary culture is that stage of the culture after isolation of the cells but before the first subculture. There are four stages to consider: (1) acquisition of the sample, (2) isolation of the tissue, (3) dissection and/or disaggregation, and (4) culture after seeding into the culture vessel. After isolation, a primary cell culture may be obtained either by allowing cells to migrate out from fragments of tissue adhering to a suitable substrate or by disaggregating the tissue mechanically or enzymatically to produce a suspension of cells, some of which will ultimately attach to the substrate.

The enzymes used most frequently for tissue disaggregation are crude preparations of trypsin, collagenase, elastase, pronase, dispase, DNase, and hyaluronidase.

Trypsin and pronase give the most complete disaggregation, but may damagethe cells. Collagenase and dispase, on the other hand, give incomplete disaggregation, but are less harmful. Hyaluronidase can be used in conjunction with collagenase to digest the intracellular matrix, and DNase is used to disperse DNA released from lysed cells.

Although each tissue may require a different set of conditions, certain requirements are shared by most of them:

(1) Fat and necrotic tissue are best removed during dissection.

(2) The tissue should be chopped finely with sharp instruments to cause minimum damage.
(3) Enzymes used for disaggregation should be removed subsequently by gentle centrifugation.

(4) The concentration of cells in the primary culture should be much higher than that normally used for subculture, because the proportion of cells from the tissue that survives in primary culture may be quite low.

(5) A rich medium, such as Ham's F12, is preferable to a simple medium, such as Eagle's MEM, and, if serum is required, fetal bovine often gives better survival than does calf or horse. Isolation of specific cell types will probably require selective media.

(6) Embryonic tissue disaggregates more readily, yields more viable cells, and proliferates more rapidly in primary culture than does adult tissue.

ISOLATION OF THE TISSUE

Before attempting to work with human or animal tissue, make sure that your work fits within medical ethical rules or current legislation on experimentation with animals. For example, in the United Kingdom, the use of embryos or fetuses beyond 50% gestation or incubation is regulated under the Animal Experiments. Work with human biopsies or fetal material usually requires the consent of the local ethical committee and the patient and/or his or her relatives.

-Mouse Embryo

Mouse embryos are a convenient source of cells for undifferentiated fibroblastic cultures. They are often used as feeder layers. The optimal age for preparing cultures from a whole disaggregated embryo is around 13 days, when the embryo is relatively large, but still contains a high proportion of undifferentiated mesenchyme, which is the main source of the culture. However, isolation and handling embryos beyond 50% full-term may require a license (e.g., in the United Kingdom) so 9- or 10-day embryos may be preferable.

Although the amount of tissue recovered from these embryos will be substantially less, a higher proportion of the cells will grow.

Most individual organs, with the exception of the brain and the heart, begin to form at about the 9th day of gestation, but are difficult to isolate until about the 11th day. Dissection of individual organs is easier at 13–14 days, and most of the organs are completely formed by the 18th day.

-Chick Embryo

Chick embryos are easier to dissect, as they are larger than mouse embryos at the equivalent stage of development. Like mouse embryos, chick embryos are used to provide predominantly mesenchymal cell primary cultures for cell proliferation analysis, to provide feeder layers, and as a substrate for viral propagation. Because of their larger size, it is easier to dissect out individual organs to generate specific cell types, such as hepatocytes, cardiac muscle, and lung epithelium. As with mouse embryos, the use of chick embryos may be subject to animal legislation.

-Human Biopsy Material

Handling human biopsy material presents certain problems that are not encountered with animal tissue. It usually is necessary to obtain consent (1) from the hospital ethical committee, (2) from the attending physician or surgeon, and (3) from the donor or patient or the patient's relatives (see Section 7.9.2). Furthermore, biopsy sampling is usually performed for diagnostic purposes, and hence the need of the pathologist must be met first. This factor is less of a problem if extensive surgical resection or nonpathological tissue (e.g., placenta or umbilical cord) is involved.

Methods for formation of primary culture

1.Primary Explant : The primary explant technique was the original method developed by Harrison [1907], Carrel [1912], and others for initiating a tissue culture. As originally performed, a fragment of tissue was embedded in blood plasma or lymph, mixed with heterologous serum and embryo extract, and placed on a cover slip that was inverted over a concavity slide. The clotted plasma held the tissue in place, and the explant could be

examined with a conventional microscope. The heterologous serum induced clotting of the plasma, and the embryo extract and serum, together with the plasma, supplied nutrients and growth factors and stimulated cell migration from the explant.

2. Enzymatic Disaggregation: Cell–cell adhesion in tissues is mediated by a variety of homotypic interacting glycopeptides (celladhesionmolecules, or CAMs), some of which are calcium dependent (cadherins) and hence are sensitive to chelating agents such as EDTA or EGTA. Integrins, which bind to the arginine-glycine-aspartic acid (RGD) motif in extracellular matrix, also have Ca2+-binding domains and are affected by Ca2+depletion. Intercellular matrix and basement membranes contain other glycoproteins, such as fibronectin and laminin, which are protease sensitive, and proteoglycans, which are less so but can sometimes be degraded by glycanases, such as hyaluronidase or heparinase. The easiest approach is to proceed from a simple disaggregation solution to a more complex solution with trypsin alone or trypsin/EDTA as a starting point, adding other proteases to improve disaggregation, and deleting trypsin if necessary to increase viability.

Embryonic tissue disperses more readily and gives a higher yield of proliferating cells than newborn or adult tissue. The increasing difficulty in obtaining viable proliferating cells with increasing age is due to several factors, including the onset of differentiation, an increase in fibrous connective tissue and extracellular matrix, and a reduction of the undifferentiated proliferating cell pool. When procedures of greater severity are required to disaggregate the tissue (e.g., longer trypsinization or increased agitation), the more fragile components of the tissue may be destroyed. In fibrous tumors, for example, it is very difficult to obtain complete dissociation with trypsin while still retaining viable carcinoma cells.

The choice of which trypsin grade to use has always been difficult, as there are two opposing trends: (1) The purer the trypsin, the less toxic it becomes, and the more predictable its action; (2) the cruder the trypsin, the more effective it may be, because of other proteases. In practice, a preliminary test experiment may be necessary to determine the optimum grade for viable cell yield, as the balance between sensitivity to toxic effects and disaggregation ability may be difficult to predict.

Crude trypsin is by far the most common enzyme used in tissue disaggregation as it is tolerated quite well by many cells and is effective for many tissues. Residual activity left after washing is neutralized by the serum of the culture medium, or by a trypsin inhibitor (e.g., soya bean trypsin inhibitor) when serum-free medium is used.

3. Mechanical Disaggregation: The outgrowth of cells from primary explants is a relatively slow process and can be highly selective. Enzymatic digestion is rather more labor intensive, although, potentially, it gives a culture that is more representative of the tissue. As there is a risk of proteolytic damage to cells during enzymatic digestion, many people have chosen to use the alternative of mechanvvy676jm,ji 76ujkm]’’’’’’’’’’’’’’’’’’’’’’’’’’’’ical disaggregation, e.g., collecting the cells that spill out when the tissue is carefully sliced, pressing the dissected tissue through a series of sieves for which the mesh is gradually reduced in size, or, alternatively forcing the tissue fragments through a syringe (with or without a wide-gauge needle). This procedure gives a cell suspension more quickly than does enzymatic digestion but may cause mechanical damage.

Separation of Viable and Nonviable Cells

When an adherent primary culture is prepared from dissociated cells, nonviable cells are removed at the first change of medium. With primary cultures maintained in suspension, nonviable cells are gradually diluted out when cell proliferation starts. If necessary, however, nonviable cells may be removed from the primary disaggregate by centrifuging the cells on a mixture of Ficoll and sodium metrizoate (e.g., Hypaque or Triosil) [Vries et al., 1973]. This technique is similar to the preparation of lymphocytes from peripheral blood (see Protocol 27.1). The viable cells collect at the interface between the medium and the Ficoll/metrizoate, and the dead cells form a pellet at the bottom of the tube.

Primary Culture in Summary

The disaggregation of tissue and preparation of the primary culture make up the first, and perhaps most vital, stage in the culture of cells with specific functions. If the required cells are lost at this stage, then the loss is irrevocable. Many different cell types may be

cultured by choosing the correct techniques (see Section 10.2.1 and Chapter 23). In general, trypsin is more severe than collagenase, but is some times more effective in creating a single-cell suspension. Collagenase does not dissociate epithelial cells readily, but this characteristic can be an advantage for separating the epithelial cells from stromal cells. Mechanical disaggregation is much quicker than the procedure using collagenase, but damages more cells.. If none of those methods is successful, try using additional enzymes, such as pronase, dispase, Accutase, and DNase, and consult the literature for examples of previous work with the tissue in which you are interested.

THE CULTURE ENVIRONMENT

The validity of the cultured cell as a model of physiological function in vivo has frequently been criticized. Often, the cell does not express the correct in vivo phenotype because the cell's microenvironment has changed. Cell–cell and cell–matrix interactions are reduced because the cells lack the heterogeneity and three-dimensional architecture found in vivo, and many hormonal and nutritional stimuli are absent. This creates an environment that favors the spreading, migration, and proliferation of unspecialized progenitor cells, rather than the expression of differentiated functions.

The influence of the environment on the culture is expressed via five routes:

(1) the nature of the substrate on or in which the cells grow—solid, as on plastic or other rigid matrix, semisolid, as in a gel such as collagen or agar, or liquid, as in a suspension culture

(2) the degree of contact with other cells

(3) the physicochemical and physiological constitution of the medium

(4) the constitution of the gas phase

(5) the incubation temperature. The provision of the appropriate environment, including substrate adhesion, nutrient and hormone or growth factor concentration, and cell interaction, is fundamental to the expression of specialized functions.

CELL ADHESION

Most cells from solid tissues grow as adherent monolayers, and, unless they have transformed and become anchorage independent after tissue disaggregation or subculture they will need to attach and spread out on the substrate before they will start to proliferate. Originally, it was found that cells would attach to, and spread on, glass that had a slight net negative charge. Subsequently, it was shown that cells would attach to some plastics, such as polystyrene, if the plastic was appropriately treated with an electric ion discharge or high-energy ionizing radiation.

We now know that cell adhesion is mediated by specific cell surface receptors for molecules in the extracellular matrix (see also Sections 8.4, 17.7.3), so it seems likely that spreading may be preceded by the secretion of extracellular matrix proteins and proteoglycans by the cells.

The matrix adheres to the charged substrate, and the cells then bind to the matrix via specific receptors. Hence, glass or plastic that has been conditioned by previous cell growth can often provide a better surface for attachment, and substrates pretreated with matrix constituents, such as fibronectin or collagen, or derivatives, such as gelatin, will help the more fastidious cells to attach and proliferate.

With fibroblast-like cells, the main requirement is for substrate attachment and spreading and the cells migrate individually at low densities. Epithelial cells may also require cell–cell adhesion for optimum survival and growth and, consequently, they tend to grow in patches.

Cell Adhesion Molecules

Three major classes of transmembrane proteins have been shown to be involved in cell–cell and cell–substrate adhesion. Cell–cell adhesion molecules, CAMs (Ca2+ independent), and cadherins (Ca2+ dependent) are involved primarily in interactions between homologous cells. These proteins are self-interactive; that is, homologous molecules in opposing cells interact with each other. Cell–substrate interactions are mediated primarily by integrins, receptors for matrix molecules such as fibronectin, entactin, laminin, and collagen, which bind to them via a specific motif usually containing the arginine–glycine–aspartic acid (RGD).

The third group of cell adhesion molecules is the transmembrane proteoglycans, also interacting with matrix constituents such as other proteoglycans or collagen, but not via the RGD motif.

Intercellular Junctions

Although some cell adhesion molecules are diffusely arranged in the plasma membrane, others are organized into intercellular junctions. The role of the junctions varies between mechanical, such as the desmosomes and adherens junctions, which hold epithelial cells together, tight junctions, which seal the space between cells, e.g. between secretory cells in an acinus or duct or between endothelial cells in a blood vessel, and gap junctions, which allow ions, nutrients, and small signaling molecules such as cyclic adenosine monophosphate (cAMP) to pass between cells in contact.

Disaggregation of the tissue, or an attached monolayer culture, with protease will digest some of the extracellular matrix and may even degrade some of the extracellular domains of transmembrane proteins, allowing cells to become dissociated from each other. Endothelial cells may also express tight junctions in culture, especially if left at confluence for prolonged periods on a preformed matrix, and can be difficult to dissociate. In each case, the cells must resynthesize matrix proteins before they attach or must be provided with a matrix-coated substrate.

Extracellular Matrix

Intercellular spaces in tissues are filled with extracellular matrix (ECM), the constitution of which is determined by the cell type, e.g., fibrocytes secrete type I collagen and fibronectin into the matrix, whereas epithelial cells produce laminin. Where adjacent cell types are different, e.g., at the boundary of the dermis (fibrocytes) and epidermis (epithelial keratinocytes), both cell types will contribute to the composition of the ECM, often producing a basal lamina. The complexity of the ECM is a significant component in the phenotypic expression of the cells attached to it, so a dynamic equilibrium exists in which the cells attached to the ECM control its composition and, in turn, the composition of the ECM regulates the cell phenotype. Hence a proliferating, migratory fibroblast will require a different ECM from a differentiating epithelial cell or neuron. Mostly, cultured

cell lines are allowed to generate their own ECM, but primary culture and propagation of some specialized cells, and the induction of their differentiation, may require exogenous provision of ECM.

for the expression of some specialized functions at least two components of interaction with the substrate may be recognized: (1) adhesion, to allow the attachment and spreading that are necessary for cell proliferation and (2) specific interactions, reminiscent of the interaction of an epithelial cell with basement membrane, with other ECM constituents, or with adjacent tissue cells, and required.

CELL PROLIFERATION

Cell Cycle

The cell cycle is made up of four phases (Fig. 3.4). In the M phase (M=mitosis), the chromatin condenses into chromosomes, and the two individual chromatids, which make up the chromosome, segregate to each daughter cell. In the G1 (Gap 1) phase, the cell either progresses toward DNA synthesis or another division cycle or exits the cell cycle reversibly (G0) or irreversibly to commit to differentiation.

Control of Cell Proliferation

Entry into the cell cycle is regulated by signals from the environment. Low cell density leaves cells with free edges and renders them capable of spreading, which permits their entry into the cycle in the presence of mitogenic growth factors, such as epidermal growth factor (EGF), fibroblast growth factors (FGFs), or platelet-derived growth factor (PDGF) interacting with cell surface receptors. High cell density inhibits the proliferation of normal cells (though not transformed cells). Inhibition of proliferation is initiated by cell contact and is accentuated by crowding and the resultant change in the shape of the cell and reduced spreading.

Intracellular control is mediated by positive-acting factors, such as the cyclins which are upregulated by signal transduction cascades activated by phosphorylation of the intracellular domain of the receptor when it is bound to growth factor. Negative-acting factors such as p53 product block cell cycle progression at restriction points or checkpoints.

DIFFERENTIATION

Maintenance of Differentiation

It has been recognized for many years that specific functions are retained longer when the three-dimensional structure of

the tissue is retained, as in organ culture (see Section 25.2). Unfortunately, organ cultures cannot be propagated, must be prepared de novo for each experiment, and are more difficult to quantify than cell cultures. Re-creating three dimensional structures by perfusing monolayer cultures and culturing cells on or in special matrices, such as collagen gel, cellulose, or gelatin sponge, or other matrices may be a better option.

CELL SIGNALING

Cell proliferation, migration, differentiation, and apoptosis in vivo are regulated by cell–cell interaction, cell–matrix interaction, and nutritional and hormonal signals. Some signaling is contact-mediated via cell adhesion molecules, but signaling can also result from soluble, diffusible factors. Signals that reach the cell from another tissue via the systemic vasculature are called endocrine, and those that diffuse from adjacent cells without entering the bloodstream are called paracrine.

It is useful to recognize that some soluble signals arise in, and interact with, the same type of cell call this homotypic paracrine, or homocrine, signaling. Signals that arise in a cell type different from the responding cells are heterotypic paracrine and will be referred to simply asparacrine in any subsequent discussion. A cell can also generate its own signaling factors that bind to its own receptors, and this is called autocrine signaling.

INITIATION OF THE CULTURE

Briefly, a culture is derived either by the outgrowth of migrating cells from a fragment of tissue or by enzymatic or mechanical dispersal of the tissue. Regardless of the method employed, primary culture is the first in a series of selective processes that may ultimately give rise to a relatively uniform cell line. In primary explantation (see Section 12.3.1), selection occurs by virtue of the cells' capacity to migrate from the explant, whereas with dispersed cells, only those cells that both survive the disaggregation techniqueandadheretothesubstrateorsurviveinsuspension will form the basis of a primary culture. If the primary culture is maintained for more than a few hours, a further selection step will occur. Cells that are capable of proliferation will increase, some cell types will survive but not increase, and yet others will be unable to survive under the particular conditions of the culture. Hence, the relative proportion of each cell type will change and will continue to do so until, in the case of monolayer cultures, all the available culture substrate is occupied. It should be realized that primary cultures, although suitable for some studies such as cytogenetic analysis, may be unsuitable for other studies because of their instability. Both cell population changes and adaptive modifications within the cells are occurring continuously throughout the culture, making it difficult to select a period when the culture may be regarded as homogeneous or stable.

EVOLUTION OF CELLLINES

After the first subculture, or passage the primary culture becomes known as a cell line and may be propagated and subcultured several times. With each successive subculture, the component of the population with the ability to proliferate most rapidly will gradually predominate, and nonproliferating or slowly proliferating cells will be diluted out. This is most strikingly apparent after the first subculture, in which differences in proliferative capacity are compounded with varying abilities to withstand the trauma of trypsinization and transfer.

Although some selection and phenotypic drift will continue, by the third passage the culture becomes more stable and is typified by a rather hardy, rapidly proliferating cell. In the presence of serum and without specific selection conditions, mesenchymal cells derived from connective tissue fibroblasts or vascular elements frequently overgrow the culture.

Although this has given rise to some very useful cell lines (e.g., WI-38 human embryonic lung fibroblasts, BHK21 baby hamster kidney fibroblasts, COS cells [and perhaps the most famous of all, the L-cell, a mouse subcutaneous fibroblast treated with methylcholanthrene, this overgrowth represents one of the major challenges of tissue cultures in ceitsinception—namely, how to prevent the over growth of the more fragile or slower-growing specialized cells such as hepatic parenchyma or epidermal keratinocytes.

Senescence

Normal cells can divide a limited number of times; hence, cell lines derived from normal tissue will die out after a fixed number of population doublings. This is a genetically determined event involving several different genes and is known as senescence. It is

Subculture and Cell Lines

SUBCULTURE AND PROPAGATION

The first subculture represents an important transition for a culture. The need to subculture implies that the primary culture has increased to occupy all of the available substrate.

Hence, cell proliferation has become an important feature. Although the primary culture may have a variable growth fraction , depending on the type of cells present in the culture, after the first subculture, the growth fraction is usually high (80% or more). From a very heterogeneous primary culture, containing many of the cell types present in the original tissue, a more homogeneous cell line emerges. In addition to its biological significance, this process has considerable practical importance, as the culture can now be propagated, characterized, and stored, and the potential increase in cell number and the uniformity of the cells open up a much wider range of experimental possibilities.

The first subculture gives rise to a secondary culture, the secondary to a tertiary, and so on, and the passage number is the number of times that the culture has been subcultured,

Once a primary culture is subcultured (or passaged), it becomes known as a **cell line**. This term implies the presence of several cell lineages of either similar or distinct phenotypes. If one cell lineage is selected, to have certain specific properties that have been identified in the bulk of the cells in the culture, this cell line becomes known as a **cell strain.**

CULTURE AGE

Cell lines with limited culture life spans are known as finite cell lines and behave in a fairly reproducible fashion. They grow through a limited number of cell generations, usually between 20 and 80 cell population doublings, before extinction. The actual number of doublings depends on species and cell lineage differences, clonal variation, and culture conditions, but it is consistent for one cell line grown under the same conditions. It is therefore important that reference to a cell line should express the approximate generation number or number of doublings since explantation; which will ‘‘approximate’’ because the number of generations that have elapsed in the primary culture is difficult to assess.

Continuous cell lines have escaped from senescence control, so the generation number becomes less important and the number of passages since last thawed from storage becomes more important (see Section 13.7.2). In addition, because of the increased cell proliferation rate and saturation density, split ratios become much greater (1:20–1:100) and cell concentration at subculture becomes much more critical.

CELL LINE DESIGNATIONS

New cell lines should be given a code or designation [e.g., normal human brain (NHB)]; a cell strain or cell line number (if several cell lines were derived from the same source; e.g., NHB1, NHB2, etc.); and, if cloned, a clone number (e.g., NHB2-1, NHB2-2, etc.).

CHOOSING A CELL LINE

A part from specific functional requirements, there are a number of general parameters to consider in selecting a cell line:

(1) **Finite vs. Continuous**. Is there a continuous cell line that expresses the right functions? A continuous cell line generally is easier to maintain, grows faster, clones more easily, produces a higher cell yield per flask, and is more readily adapted to serum-free medium .

(2) **Normal or Transformed**. Is it important whether the line is malignantly transformed or not? If it is, then it might be possible to obtain an immortal line that is not tumorigenic, e.g., 3T3 cells or BKK21-C13.

(3**) Species**. Is species important? Nonhuman cell lines have fewer biohazard restrictions and have the advantage that the original tissue may be more accessible.

(4) **Growth Characteristics**. What do you require in terms of growth rate, yield, plating efficiency, and ease of harvesting? You will need to consider the following parameters:

a) Population-doubling time.

b) Saturation density.

c) Plating efficiency.

d) Growth fraction.

e) Ability to grow in suspension.

(5) **Availability**. If you have to use a finite cell line, are there sufficient stocks available, or will you have to generate your own line(s)? If you choose a continuous cell line, are authenticated stocks available?

(6) **Validation**. How well characterized is the line, if it exists already, or, if not, can you do the necessary. It is vital to eliminate the possibility of cross-contamination before embarking on a program of work with a cell line, as so many cross contaminations have been reported.

(7) **Phenotypic Expression**. Can the line be made to express the right characteristics?

(8) **Control Cell Line**. If you are using a mutant, transfected, transformed, or abnormal cell line, is there a normal equivalent available, should it be required?

(9) **Stability**. How stable is the cell line? Has it been cloned? If not, can you clone it.

ROUTINE MAINTENANCE

Once a culture is initiated, whether it is a primary culture or a subculture of a cell line, it will need a periodic medium change, or ‘‘feeding,’’ followed eventually by subculture if the cells are proliferating. In nonproliferating cultures, the medium will still need to be changed periodically, as the cells will still metabolize and some constituents of the medium will become exhausted or will degrade spontaneously. Intervals between medium changes and between subcultures vary from one cell line to another, depending on the rate of growth and metabolism; rapidly growing transformed cell lines, such as HeLa, are usually subcultured once per week, and the medium should be changed four days later. More slowly growing, particularly nontransformed, cell lines may need to be subcultured only every two, three, or even four weeks, and the medium should be changed weekly between subcultures.

Significance of Cell Morphology

Whatever procedure is undertaken, it is vital that the culture be examined carefully to confirm the absence of contamination. The cells should also be checked for any signs of deterioration, such as granularity around the nucleus, cytoplasmic vacuolation, and rounding up of the cells with detachment from the substrate. Such signs may imply that the culture requires a medium change, or may indicate a more serious problem, e.g., inadequate or toxic medium or serum, microbial contamination, or senescence of the cell line. Medium deficiencies can also initiate apoptosis.

Replacement of Medium

Four factors indicate the need for the replacement of culture medium:

(1) **A Drop in pH**. The rate of fall and absolute level should be considered. Most cells stop growing as the pH falls from pH 7.0 to pH 6.5 and start to lose viability between pH 6.5 and pH 6.0, so if the medium goes from red through orange to yellow, the medium should be changed. Try to estimate the rate of fall; a culture at pH 7.0 that falls 0.1 pH units in one

day will not come to harm if left a day or two longer before feeding, but a culture that falls 0.4 pH units in one day will need to be fed within 24–48 h and cannot be left over a weekend without feeding.

(2) **Cell Concentration**. Cultures at a high cell concentration exhaust the medium faster than those at a low concentration. This factor is usually evident in the rate of change of pH, but not always.

(3) **Cell Type**. Normal cells (e.g. diploid fibroblasts) usually stop dividing at a high cell density, because of cell crowding, growth factor depletion, and other reasons. The cells block in the G1 phase of the cell cycle and deteriorate very little, even if left for two to three weeks or longer. Transformed cells, continuous cell lines, and some embryonic cells, however, deteriorate rapidly at high cell densities unless the medium is changed daily or they are subcultured.

(4) **Morphological Deterioration**. This factor must be anticipated by regular examination and familiarity with the cell line. If deterioration is allowed to progress too far, it will be irreversible, as the cells will tend to enter apoptosis.

SUBCULTURE

When a cell line is subcultured the regrowth of the cells to a point ready for the next subculture usually follows a standard pattern. A lag period after seeding is followed by a period of exponential growth, called the log phase. When the cell density (cells/cm2 substrate) reaches a level such that all of the available substrate is occupied, or when the cell concentration (cells/mL medium) exceeds the capacity of the medium, growth ceases or is greatly reduced. Then either the medium must be changed more frequently or the culture must be divided.

For an adherent cell line, dividing a culture, or subculture as it is called, usually involves removal of the medium and dissociation of the cells in the monolayer with trypsin, although some loosely adherent cells (e.g., HeLa-S3) may be subcultured by shaking the bottle, collecting the cells in the medium, and diluting as appropriate in fresh medium in new bottles. Exceptionally, some cell monolayers cannot be dissociated in trypsin and require the action of alternative proteases, such as pronase, dispase, and collagenase. Of

these proteases, pronase is the most effective but can be toxic to some cells. Dispase and collagenase are generally less toxic than trypsin but may not give complete dissociation of epithelial cells.

Other proteases, such as Accutase, Accumax (invertebrate proteases), and Trypzean or TrypLE (recombinant trypsins), are available, and their efficacy should be tested where either there is a problem with standard disaggregation protocols or there is a need to avoid mammalian (e.g., porcine trypsin) or bacterial (e.g., Pronase) proteases. The severity of the treatment required depends on the cell type, as does the sensitivity of the cells to proteolysis, and a protocol should be selected with the least severity that is compatible with the generation of a single-cell suspension of high viability. The attachment of cells to each other and to the culture substrate is mediated by cell surface glycoproteins and Ca2+ (see Section 3.2). Other proteins, and proteoglycans, derived from the cells and from the serum, become associated with the cell surface and the surface of the substrate and facilitate cell adhesion. Subculture usually requires chelation of Ca2+ and degradation of extracellular matrix and, potentially, the extracellular domains of some cell adhesion molecules.

Criteria for Subculture

The need to subculture a monolayer is determined by the following criteria:

(1) **Density of Culture**. Normal cells should be subcultured as soon as they reach confluence. If left more than 24 h, they will withdraw from the cycle and take longer to recover when reseeded. Transformed cells should also be subcultured on reaching confluence; although they will continue to proliferate beyond confluence, they will start to deteriorate after about two doublings, and reseeding efficiency will decline. (2) Exhaustion of Medium. Exhaustion of the medium (see Section 13.6.2) usually indicates that the medium requires replacement, but if a fall in pH occurs so rapidly that the medium must be changed more frequently, then subculture may be required. Usually, a drop in pH is accompanied by an increase in cell density, which is the prime indicator of the need to subculture. Note that a sudden drop in pH can also result from contamination, so be sure to check.

(3) **Time since Last Subculture**. Routine subculture is best performed according to a strict schedule, so that reproducible behavior is achieved and monitored. If cells have not reached a high-enough density (i.e., they are not confluent) by the appropriate time, then increase the seeding density, or if they reach confluence too soon, then reduce the seeding density. Once this routine is established, the recurrent growth should be consistent in duration and cell yield from a given seeding density. Deviations from this pattern then signify a departure from normal conditions or indicate deterioration of the cells. Ideally, a cell concentration should be found that allows for the cells to be subcultured after 7 days, with the medium being changed after 3–4 days.

(4) **Requirements for Other Procedures**. When cells are required for purposes other than routine propagation, they also have to be subcultured, in order to increase the stock or to change the type of culture vessel or medium. Ideally, this procedure should be done at the regular subculture time, when it will be known that the culture is performing routinely, what the reseeding conditions should be, and what outcome can be expecte.

CROSS-CONTAMINATION

During the development of tissue culture, a number of cell strains have evolved with very short doubling times and high plating efficiencies. Although these properties make such cell lines valuable experimental material, they also make them potentially hazardous for cross-infecting other cell lines. The extensive cross contamination of many cell lines with HeLa and other rapidly growing cell lines is now clearly established, but many operators are still unaware of the seriousness of the risk. The responsibility lies with supervisors to impress upon new personnel the severity of the risks, with journal editors and referees to reject manuscripts, and with grant review bodies to reject grant proposals without evidence of proper authentication of cell lines. Without he acceptance of these obligations, the situation can only get worse.

The following practices help avoid cross-contamination:

(1) Obtain cell lines from a reputable cell bank that has performed the appropriate validation of the cell line.

(2) Do not have culture flasks of more than one cell line, or media bottles used with them, open simultaneously.

(3) Handle rapidly growing lines, such as HeLa, on their own and after other cultures.

(4) Never use the same pipette for different cell lines.

(5) Never use the same bottle of medium, trypsin, etc., for different cell lines.

(6) Do not put a pipette back into a bottle of medium, trypsin, etc., after it has been in a culture flask containing cells.

(7) Add medium and any other reagents to the flask first, and then add the cells last.

(8) Do not use unplugged pipettes, or pipettors without plugged tips, for routine maintenance.

(9) Check the characteristics of the culture regularly, and suspect any sudden change in morphology, growth rate, or other phenotypic properties. Cross-contamination or its absence may be confirmed by DNA fingerprinting, karyotype and others.

, in part, by the inability of terminal sequences of the DNA in the telomeres to replicate at each cell division. The result is a progressive shortening of the telomeres until, finally, the cell is unable to divide further. Exceptions to this rule are germ cells, stem cells, and transformed cells, which often express the enzyme telomerase, which is capable of replicating the terminal sequences of DNA in the telomere and extending the life span of the cells, infinitely in the case of germ cells and some tumor cells

Production from cell culture

Why use animal cell culture for production?

Many healthcare products can be derived from fractionation of blood or extraction from human tissue. However, it is difficult to ensure that such products are free from contamination, particularly by viruses. This risk can be reduced considerably by using

well-characterized animal cell lines that are shown to be free of contaminating viruses. As an example, factor VIII used for the treatment of hemophilia was purified from pooled human plasma in the early 1980s. This process was discontinued when samples were found to be contaminated with human immunodeficiency virus (HIV). The safety of the present recombinant product is now ensured by careful screening of the mammalian cell line used in the large-scale bioprocess.

Genetically engineered mammalian cells can overexpress and secrete glycoprotein products into the culture medium. Protein extraction from the culture medium is relatively efficient particularly if a low-protein culture medium is used. In comparison, secretion of protein from genetically engineered bacteria can be more problematic. The synthesized protein usually accumulates as insoluble aggregates within bacterial cells and so requires extraction from the cell lysate. This is more difficult because of the high content of other proteins (including some endotoxins) that must be completely removed from the final product. Thus, the recovery process from the cell lysate is more complex and the final yield of purified product may be lower.

How to produce biologicals from cell culture

The first obvious questions to ask before setting up a cell culture process for production are:

■ What product do I want?

■ How pure do I want it?

■ How much do I want?

The type of process, strategy and design will depend upon the answers to these three critical questions.

1-What product?

The type of product required is the key to deciding which type of cell to use for production. For simple proteins such as insulin or growth hormone that have low molecular weights and no attached carbohydrate groups, a genetically engineered Escherichia coli culture producing the recombinant protein may be preferred. Bacteria grow faster and

under more robust conditions than animal cells. However, there are limitations of using bacteria for the expression and production of more complex mammalian proteins. The most serious consideration is that there are a number of posttranslational processes that are necessary to modify proteins before they become biologically active:

- proteolytic cleavage;
- subunit association;
- chemical derivatization;
- glycosylation;
- phosphorylation;
- fatty acylation.

In particular, glycosylation has received a lot of attention. Glycosylation is the intracellular process that involves the addition of a carbohydrate group on to the synthesized protein. The process occurs in the Golgi apparatus prior to secretion through the cell membrane. The carbohydrate component of glycoproteins is often essential for biological activity.

Proteins that are under-glycosylated are often cleared from the bloodstream too quickly Prokaryotes (such as E. coli) are not capable of glycosylation. Yeast cells may be genetically engineered to produce a protein from a transfected mammalian gene. Yeast cells can glycosylate proteins but the carbohydrate added may be different from that in the authentic mammalian glycoprotein and this could influence the therapeutic activity of the molecule. The carbohydrate content of naturally occurring glycoproteins can vary from 3% (e.g. immunoglobulin) to 40% (e.g. erythropoietin) of the total weight and can be important for many biological properties.

Glycoprotein extracted from a culture may be in various glycoforms. These are molecules having an identical protein structure but with differing carbohydrate additives. This type of variation may be unacceptable for some applications, such as for therapeutic use, where a single glycoform may be required. The extent of glycosylation of proteins produced in culture depends on the conditions. The optimal conditions for full

glycosylation are often unknown but it has been shown that reduced glycosylation may occur at the end of the growth phase and during the stationary phase of culture.

In most cases, a genetically modified cell line will be required to obtain a specific glycoprotein at a reasonable yield. The most commonly used host cell lines for such production are Chinese hamster ovary (CHO) and baby hamster kidney (BHK) because their genetics and growth characteristics have been well characterized. However, in some cases a mammalian cell line may be found that will produce the required glycoprotein product without any genetic manipulation. An example of this is interferon (alpha or beta) production from human fibroblasts. An induction process has been developed which allows high productivity from these cells.

The production of animal viruses from cell culture requires some consideration of an acceptable host cell. In this process it is important to know which cell line is susceptible to the particular strain of virus required. Also, the mode of culture may be important. For example, the extent of viral infection is often greater in anchorage-dependent cells attached to a substratum.

2. How pure?

If a product is required for human injection—as a therapeutic agent or as a prophylactic viral vaccine—then high standards of purification are needed with an absolute requirement to ensure the absence of known risk factors. If a product is required as a diagnostic agent or for laboratory use, then the conditions of purification may be less stringent. Electrophoresis is a powerful method for discriminating between proteins. Thus the visualization of a single stained protein band by gel electrophoresis is a good indicator of the purity of a protein. The purity requirements for the final product may influence not only the purification process (downstream processing) chosen but also the conditions of culture. For example, purification is easier if the cell product is secreted into a low-protein or protein-free culture media. Also, it may be necessary to avoid antifoam or shear protective agents as these can foul membrane-based purification processes. The timing of product harvest may be important because of the risk of product exposure to proteases or glycosidases,t hat may arise from cell lysis. These enzymes will cause breakdown of the protein backbone or carbohydrate group of a glycoprotein product

3-How much?

Deciding the quantity of cell product required over a set period of time is vital to planning a production process. This is equally important for a laboratory preparation as well as a large-scale commercial enterpris. The importance of this question to developing a process strategy can be best illustrated by an example of the production of a hypothetical glycoprotein (Biolikin). The biotechnology company, Candogene, planned to make 10 kg of Biolikin from a cell with a product yield of 1 pg/cell-day. Could this requirement for 1016 cell-days of production be met by their stirred tank reactor at 106 cells/ml? The strategic planners at Candogene determined that this would require a day's run on a 10 million (107)-liter fermenter and abandoned the project as beyond their scope. However, an alternative company decided to take up this project by a three-stage plan.

1. They improved the specific productivity of the cell line (×100) by gene amplification. This reduced the required culture capacity to 105 liters.

2. They decided to use a perfusion culture that increased the final cell concentration to 4×107/ml. This reduced the required culture capacity to 2500 liters.

3. They decided that there was no need to make the product in 1 day but could continue the process over 100 days. This reduced the required culture capacity to 25 liters, which was well within the fermenter size available in their small laboratory.

This example illustrates that missed opportunities can arise from poor or no planning and by not asking the right questions from the onset of the work.

How to purify the final product

The process of purification—often called 'downstream processing'—is dependent on the product and the degree of purification required. However, some general principles can be provided here.

Primary separation involves the removal of cells and debris from the medium. In the case of immobilized cell cultures, this can occur by sedimentation of the cell-loaded particles. For suspension cultures, cells are normally removed by low-speed centrifugation or by filtration. It is important in this primary separation to minimize cell lysis and ensure

the removal of all cell debris as this can give rise to degradative enzymes which are capable of causing product breakdown. Chilling the medium (4°C) can also minimize the activity of these enzymes.

Product concentrations arising from a cell culture are generally low -10–100 mg/1. So, the next stage of purification often involves increasing the concentration by a de-watering process. This can be accomplished by precipitation with a suitable agent such as ethanol, polyethylene glycol or ammonium sulfate. These precipitating agents can be specific for certain protein types and it is important to ensure that the conditions and concentration are appropriate for precipitating the protein required.

An alternative method of concentration is by ultrafiltration using a hollow-fiber cartridge, which can concentrate by 10–100-fold. The hollow-fiber cartridge should have a high-molecular-weight cut-off, so that the product, but not the cells, passes through the fiber. For example, with a 10-kDa cut-off, the concentrated 'retentate' will include all substances above that molecular weight while all lower-molecular-weight substances will be drained off as 'permeate'.

To maximize the yield of the final product extracted from culture media, it is important to have as few purification steps as possible. This can be achieved particularly if highly selective fractionation procedures are available. Bioselective adsorption methods are particularly valuable because of the selectivity for a single compound or group of compounds. For example, Protein A and Protein G have specificity for binding immunoglobulins. Also, monoclonal antibodies are useful because of their affinity for particular compounds.

The adsorption reagents can be prepared by immobilization of an antibody to a solid matrix such as Sepharose. This is suitable for column chromatography because of the open-pore structure which allows a reasonably high liquid flow rate. Reactive forms such as cyanogen bromide-activated Sepharose are available commercially and can be bound covalently to a protein ligand (such as an antibody). Figure 11.1 shows the principle of immunoaffinity column purification. A protein extract is loaded on to the column at neutral pH, allowing a single protein (the antigen) to bind to the antibody sites. After washing the

column with further neutral pH buffer, the antigen is eluted in a pure state with a low pH buffer.

Lecture Six **Dr. Rasha Al- Sahlanee**

Cloning and selection

A clone is a population of cells that are descended from a single parental cell. Clones may be derived from continuous cell lines or from primary cultures, but in either case the purpose of cloning is the same: to minimize as far as possible the degree of genetic and phenotypic variation within a cell population. This is done by isolating a single cell under suitable conditions and then allowing it to multiply to produce a sufficient number of cells for the required purpose.

Cell cloning

The traditional microbiological approach to the problem of culture heterogeneity is to isolate pure cell strains by cloning, but, although this technique is relatively easy for continuous cell lines, its success in most primary cultures is limited by poor cloning efficiencies. However, the cloning of primary cultures can be successful, e.g., Sertoli cells and glomerular cells from kidney. In general, the cloning of primary cells is less successful than the cloning of established cell lines, as they tend to have a low colony-forming efficiency (CFE) and 'normal' cells can only undergo a limited number of population, which may prevent the generation of a sufficient number of cells for future use. Nevertheless, it is sometimes possible to isolate specific cell types from a mixed primary population, and to develop clones large enough for subsequent studies and free of unwanted cell types (often fibroblasts) that might otherwise overgrow the culture.

Colony-forming efficiency (CFE): Number of colonies obtained/Number of individual cells plated x100%

The CFE may vary from less than 1% for some primary cells, to practically 100% for some established cell lines.

Uses of cloning

Cloning cells from continuous lines has a number of applications:

1- Many continuous lines are genetically unstable and their properties may alter during passage. Cloning can be used to isolate cultures with properties more closely resembling those of the original population (but these may have to be recloned at intervals if instability is pronounced). Conversely, cloning procedures may be used to isolate variants. Examples of the latter may include karyological and biochemical variants and cells that exhibit different levels of product secretion or different susceptibilities to viruses. Treatment with mutagens can be used in order to increase the proportion of variant cells.

2- Hybridoma cells are cloned to generate cultures that secrete monoclonal antibodies.

3- Variation within a continuous cell line may be studied by examining the properties of panels of clones established at different passage levels.

4- Cells transfected with DNA do not necessarily form populations with homogenous genetic constitutions, and cloning can enable cells to be selected and cultures developed with the required characteristics.

5- In pharmaceutical production, it may be desirable from a regulatory standpoint that a cell line used to derive a product can be defined as originating from a single cell.

6- Cloning is also used as a survival assay for optimizing growth conditions and for determining chemosensitivity and radiosensitivity .

Cloning of attached cells may be carried out in Petri dishes, multiwell plates, or flasks, and it is relatively easy to discern individual colonies. Micromanipulation is the only conclusive method for determining genuine clonality (i.e., that a colony was derived from one cell), but when symmetrical colonies are derived from a single-cell suspension, particularly if colony formation is monitored at the early stages, then it is probable that the colonies are clones.

Cloning can also be carried out in suspension by seeding cells into a gel, such as agar or agarose, or a viscous solution, such as Methocel, with an agar or agarose underlay. The stability of the gel, or viscosity of the Methocel, ensures that daughter cells do not break

away from the colony as it forms. Even in monolayer cloning, some cell lines, such as HeLa-S3 and CHO, are poorly attached, and cells can detach from colonies as they form and generate daughter colonies, which will give an erroneous plating efficiency.

Continuous cell lines generally have a high plating efficiency in monolayer and in suspension because of their transformed status, whereas normal cells, which may have a moderately high cloning efficiency in monolayer, have a very low cloning efficiency in suspension, because of their need to attach and spread out to enter the cell proliferation cycle.

Dilution cloning

Dilution cloning is the technique that is used most widely, based on the observation that cells diluted below a certain density form discrete colonies. Seed the cells at low density and incubate until colonies form. Stain the cells (for plating efficiency and survival assays As the density of cells during cloning is very low, the need to feed the dishes after one week is debatable. Feeding mainly counteracts the loss of nutrients (such as glutamine), which are unstable, and replaces growth factors that have degraded. However, it also increases the risk of contamination, so it is reasonable to leave dishes for two weeks without feeding. If it is necessary to leave the dishes for a third week, then the medium should be replaced, or at least half of it.

Stimulation of plating efficiency

When cells are plated at low densities, the rate of survival falls in all but a few cell lines. This does not usually present a severe problem with continuous cell lines, for which the plating efficiency rarely drops below 10%, but with primary cultures and finite cell lines, the plating efficiency may be quite low—0.5–5%, or even zero. Numerous attempts have been made to improve plating efficiencies, based on the assumption either that cells require a greater range of nutrients at low densities, because of loss by leakage, or that cell-derived diffusible signals or conditioning factors are present in high-density cultures and are absent or too dilute at low densities. The intracellular metabolic pool of a leaky cell in a dense population will soon reach equilibrium with the surrounding medium, but that of an isolated cell never will. This principle was the basis of the capillary technique of Sanford,

by which the L929 clone of L-cells was first produced. The confines of the capillary tube allowed the cell to create a locally

enriched environment that mimicked a higher cell concentration. In microdrop techniques developed later, the cells were seeded as a microdrop under liquid paraffin, again maintaining a relatively high cell concentration, keeping one colony separate from another, and facilitating subsequent isolation. As media improved, however, plating efficiencies increased, and Puck and Marcus 1955 were able to show that cloning cells by simple dilution in association with a feeder layer of irradiated mouse embryo fibroblasts gave acceptable cloning efficiencies, although subsequent isolation required trypsinization from within a collar placed over each colony .

Conditions that improve clonal growth

(1) Medium: Choose a rich medium, such as Ham's F12,or a medium that has been optimized for the cell type in use (e.g., MCDB 110 for human fibroblasts, Ham's F12 or MCDB 302 for CHO.

(2) Serum. When serum is required, fetal bovine is generally better than calf or horse. Select a batch for cloning experiments that gives a high plating efficiency during tests .

(3) Hormones. Insulin, 1×10^{-10} IU/mL, has been found to increase the plating efficiency of several cell types Dexamethasone, 2.5×10^{-5} M, 10 μg/mL, a soluble synthetic hydrocortisone analog, improves the plating efficiency of chick myoblasts and human normal glia, glioma, fibroblasts, and melanoma and gives increased clonal growth (colony size) if removed five days after plating . Lower concentrations (e.g., 1×10^{-7} M) have been found to be preferable for epithelial cells .

(4) Intermediary Metabolites. Oxo-acids (previously known as keto-acids)—e.g., pyruvate or α-oxoglutarate (α-ketoglutarate) and nucleosides [α-MEM] have been used to supplement media and are already included in the formulation of a rich medium, such as Ham's F12. Pyruvate is also added to Dulbecco's modification of Eagle's MEM .

(5) Carbon Dioxide. CO_2 is essential for obtaining maximum cloning efficiency for most cells. Although 5% CO_2 is usually used, 2% is sufficient for many cells and may even be

slightly better for human glia and fibroblasts. HEPES (20 mM) may be used with 2% CO2, protecting the cells against pH fluctuations during feeding and in the event of failure of the CO2 supply.

(6) Treatment of Substrate. Polylysine improves the plating efficiency of human fibroblasts in low serum concentrations

(a) Add 1 mg/mL of poly-D-lysine in UPW to the plates (~5 mL/25 cm2).

(b) Remove and wash the plates with 5 mL of D-PBSA per 25 cm2. The plates may be used immediately or stored for several weeks before use. Fibronectin also improves the plating of many cells. The plates may be pretreated with 5 μg/mL of fibronectin incorporated in the medium.

(7) Trypsin. Purified (twice recrystallized) trypsin used at 0.05 μg/mL may be preferable to crude trypsin .

Suspension cloning

Some cells, particularly hematopoietic stem cells and virally transformed fibroblasts, clone readily in suspension. To hold the colony together and prevent mixing, the cells are suspended in agar or Methocel and plated on an agar underlay or into dishes that need not be treated for tissue culture.

Agarose, which has a reduced component of sulphated polysaccharides, can be substituted for agar. Some types of agarose have a lower gelling temperature and can be manipulated more easily at 37◦C. They are gelled at 4◦C and then are returned to 37◦C. Because of the complexity of handling melted agar with cells, and the impurities that may be present in agar, some laboratories prefer to use Methocel, which is a viscous solution and not a gel . It has a higher viscosity when warm. Because it is a sol and not a gel, cells will sediment through it slowly. It is, therefore, essential to use an underlay with Methocel. Colonies form at the interface between the Methocel and the agar (or agarose) underlay, placing themselves in the same focal plane and making analysis and photography easier.

And for the isolation of suspension clones : The isolation of colonies growing in suspension is simple but

requires a dissection microscope. It’s done by Draw the colony into a pipettor or Pasteur pipette, and transfer the colony to a flask or the well of a multiwell plate.

Isolation of clones

When cloning is used for the selection of specific cell strains, the colonies that form need to be isolated for further propagation. If monolayer cells are cloned directly into multiwall plates, then colonies may be isolated by trypsinizing individual wells. It is, however, necessary to confirm the clonal origin of the colony during its formation by regular microscopic observation. If cloning is performed in Petri dishes, there is no physical separation between colonies. This separation must be created by removing the medium and placing a stainless steel or ceramic ring around the colony to be isolated , The colony is trypsinized from within a porcelain, glass or stainless steel ring and transferred to one of the wells of a 24- or 12-well plate, or directly to a 25-cm2 flask.

Other Isolation Techniques for Monolayer Clones:

(1) Distribute small coverslips or broken fragments of coverslips on the bottom of a Petri dish. When plated out at the correct density, some colonies are found to be singly distributed on a piece of glass and may be transferred to a fresh dish or multiwell plate.

(2) Use the capillary technique of Sanford . A dilute cell suspension is drawn into a sterile glass capillary tube (e.g., a 50-µL Drummond Microcap), allowing colonies to form inside the tube. The tube is then carefully broken on either side of a colony and transferred to a fresh plate.

(3) Cells may be cloned in the OptiCell chamber, which is made up of two opposing growth surfaces of thin flexible plastic that may be cut with a scalpel or scissors.

Provided the outer surfaces are kept sterile, this can be used to cut out segments with colonies, which are then trypsinized into a multiwell plate or flask.

The isolation of colonies growing in suspension is simple but requires a dissection microscope. It done by drawing the colony into a pipette or Pasteur pipette, and transfer the colony to a flask or the well of a multiwell plate.

Lecture 7 and 8 **Dr. Rasha Al- Sahlanee**
Cloning and selection **March, 2019**

Replica plating :

Bacterial colonies can be replated by pressing a moist pad gently down onto colonies growing on a nutrient agar plate and transferring the pad to a second, fresh agar plate. Various attempts have been made to adapt this technique to cell culture, usually by placing a mesh screen or filter over monolayer clones and transferring it to a fresh dish after a few days . For clones that have been developed in microtitration plates, there are a number of transfer devices available—e.g., the Corning Transtar , which can be used with suspension cultures directly after agitating the culture or with monolayer cultures after trypsinization and resuspension.

Transfer Device. Transtar (Corning)

Selective inhibitors :

Manipulating the conditions of a culture by using a selective medium is a standard method for selecting microorganisms. Its application to animal cells in culture is limited due to the effect of serum, which tends to mask the selective properties of different

media. Most selective media that have been shown to be generally successful have been serum-free formulations A number of metabolic inhibitors, however, have had recurrent success. Gilbert and Migeon replaced the L-valine in the culture medium with D-valine and demonstrated that cells possessing D-amino acid oxidase would grow preferentially. Which used to select different cell types like Kidney tubular epithelia , bovine mammary epithelia , endothelial cells from rat brain , and Schwann cell but this technique is not effective against human fibroblasts.

Much of the effort in developing selective conditions has been aimed at suppressing fibroblastic overgrowth. Using cis-OH-proline it can prove toxic to other cells. Fibroblasts also tend to be more sensitive to geneticin (G418)

at 100 µg/mL. phenobarbitone inhibited fibroblastic overgrowth in cultures of hepatocytes.

One of the more successful approaches was the development of a monoclonal antibody to the stromal cells

of a human breast carcinoma. Used with complement, this antibody proved to be cytotoxic to fibroblasts from several tumors and helped to purify a number of malignant cell lines. Selective media are also commonly used to isolate hybrid clones from somatic hybridization experiments. Transfected cells are also selected by resistance to a number of drugs, such as neomycin, its analog geneticin (G418), hygromycin, and methotrexate, by including a resistance conferring gene in the construct used for transfection

Isolation of genetic variations :

the development of mutant cell lines that amplify the dihydrofolate reductase (DHFR) gene. The principle of this method was to exposed cells to gradually increasing concentrations of folic acid antagonists, such as methotrexate (MTX), over a prolonged period of time will develop resistance to the toxic effects of the drug .Resistance resulting from amplification of the DHFR gene generally develops the most rapidly, although other

mechanisms—e.g., alteration in antifolate transport and/or mutations affecting enzyme structure or affinity—may confer part or all of the resistant phenotype.

Characterize resistant cells for levels of resistance to the drug in a clonal growth assay for increase in activity or amount of DHFR by biochemical or gel electrophoresis techniques and/or for increase in mRNA and copy number of the reductase gene by Northern, Southern, or dot blots with DHFR-specific probes to determine the mechanism(s) of resistance.

composition of the medium (e.g., folic acid content) can be expected to influence the rate and type of MTX resistance development. Media containing thymidine, hypoxanthine, and glycine prevent the development of antifolate resistance and should be avoided. Cells can be treated with chemical mutagens before selection ; this treatment may also alter the rate and type of mutant selection. Selection can also be done with cells plated in the drug at low density in 10-cm tissue culture dishes (with the isolation of individual colonies with cloning rings, using single cells in 96-well cluster dishes, or in soft agar, to enable the isolation of one or multiple clonal populations at each or any step during resistance development.

Cells can be made to be resistant to a number of other agents, such as antibiotics, other antimetabolites, toxic metals, and so on, by similar techniques; differences in the mechanism of action or degree of toxicity of the agents.

Interaction with substrate:

1- Nature of substrate

Although several sources of ECM are now available , the emphasis so far has been on promoting cell survival or differentiation, and little has been made of the potential for selectivity in ‘‘designer’’ matrices, although collagen has been reported to favour epithelial proliferation and Matrigel also favors epithelial survival and differentiation . Because the constituents are now better understood, mixing various collagens with proteoglycans, laminin, and other matrix proteins could be used to create more selective

substrates. The selective effect of substrates on growth may depend on differential rates both of attachment and of growth, or the net result of both. Collagen and fibronectin coating has been used to enhance epithelial cell attachment and growth and to support endothelial cell growth and function . Primaria plastics have a charge on the plastic surface different from that of conventional tissue culture plastics and are designed to enhance epithelial growth relative to fibroblasts.

2- Selective feeder layers

As well as conditioning the substrate , feeder layers can also be used for the selective growth of epidermal cells and for repressing stromal overgrowth in cultures of breast and colon carcinoma . The role of the feeder layer is probably quite complex; it provides not only extracellular matrix for adhesion of the epithelium, but also positively acting growth factors and negative regulators that inactivate TGF-β . Human glioma will grow on confluent feeder layers of normal glia, whereas cells derived from normal brain will not. Treating feeder cells in the mid-exponential phase with mitomycin C, and reseed the cells to give a confluent monolayer. Seeding tumor cells, dissociated from the biopsy by collagenase digestion, or from a primary culture with trypsin, onto the confluent monolayer . Colonies from epithelial tumors may form in 3 weeks to 3 months. Fibrosarcoma and gliomas do not always form colonies, but may infiltrate the feeder layer and gradually overgrow.

3- Selection by semisolid media

The transformation of many fibroblast cultures reduces the anchorage dependence of cell proliferation . By culturing the cells in agar after viral transformation, it is possible to isolate colonies of transformed cells and exclude most of the normal cells. Normal cells will not form colonies in suspension with the high efficiency of virally transformed cells, although they will often do so with low plating efficiencies.

The difference between transformed and untransformed cells is not as clear with early-passage tumor cell lines, as plating efficiencies can be quite low; normal glia and fetal skin fibroblasts also form colonies in suspension with similar efficiencies (<1%) . Cell cloning

and the use of selective conditions have a significant advantage over physical cell separation techniques, in that contaminating cells are either eliminated entirely by clonal selection or repressed by constant or repeated application of selective conditions. Even the best physical cell separation techniques still allow some overlap between cell populations, such that overgrowth recurs. A steady state cannot be achieved, and the constitution of the culture changes continuously.

Cell separation

Although cloning and using selective culture conditions are the preferred methods for purifying a culture there are occasions when cells do not plate with a high enough efficiency to make cloning possible or when appropriate selection conditions are not available. It may then be necessary to resort to a physical or immunological separation technique. Separation techniques have the advantage that they give a high yield more quickly than cloning, although not with the same purity.

The more successful separation techniques depend on differences in:

(1) cell density (specific gravity)

(2) affinity of antibodies to cell surface epitopes

(3) cell size

(4) light scatter or fluorescent emission as sorted by flow cytometry.

The most effective separations often employ two or more parameters to obtain a high level of purity:

1- Cell density and isopyknic sedimentation:

Separation of cells by density can be performed by centrifugation at low g with conventional equipment. The density medium should be nontoxic and nonviscous at high densities (1.10 g/mL) and should exert little osmotic pressure in solution. Serum albumin, dextran, Ficoll (Pharmacia) ,metrizamide (Nygaard) and Percoll (colloidal silica).

A gradient may be generated :

(1) by layering different densities of Percoll with a pipette, syringe, or pump

(2) with a special gradient or

(3) by high-speed spin : one spin is required, spinning the cells at such a high-g force may damage them.

Ficoll is one of the most popular media because, like Percoll, it can be autoclaved. Ficoll is a little more viscous than Percoll at high densities and may cause some cells to agglutinate. Metrizamide (Nycomed), a nonionic derivative of metrizoate, which is a radiopaque iodinated substance used in radiography (Isopaque, Hypaque, Renografin) and in lymphocyte purification , is less viscous than Ficoll at high densities but may be taken up by some cells ,so it is preferable to layer cells on top of a preformed gradient.

2- Cell size and sedimentation velocity

Sedimentation of cells is also influenced by cell size (cross sectional area), which becomes the major determinant of sedimentation velocity at 1 g and a significant component at higher sedimentation rates at elevated g. The relationship between the particle size and sedimentation rate at 1 g, although complex for submicron-sized particles, is fairly simple for cells and can be expressed approximately as

$$v \approx \frac{r^2}{4}$$

where v is the sedimentation rate in mm/h and r is the radius of the cell in μm.

3- antibody –based techniques:

There are a number of techniques that rely on the specific binding of an antibody to the cell surface. These include immune lysis by an antibody against unwanted cells, e.g., fibroblasts in an epithelial population, fluorescence-activated cell sorting , immune panning and sorting with antibody conjugated magnetizable beads immune targeting of a cytotoxin. These techniques all depend on the specificity of the selecting antibody and the presentation of the correct epitope on the cell surface of living cells, as confirmed by immune staining or flow cytometry.

Lecture 9-11 **Dr. Rasha Al-Sahlanee**

March, 2019

4- Fluorescence- activated cell sorting:

Fluorescence-activated cell sorting operates by projecting a single stream of cells through a laser beam in such a way that the light scattered from the cells is detected by one or more photomultipliers and recorded .

a- Aflow cytometry

is an analytical instrument that processes the output of the photomultipliers to analyze the constitution of a cell population (e.g., to determine the proportion of cells in different phases of the cell cycle, measured by a combination of DNA fluorescence and cell size measurements).

b- a fluorescence- activated cell sorter (FACS)

is an instrument that uses the emission signals from each cell to sort the cell into one of two sample collection tubes and a waste reservoir. If specific coordinates are set to delineate sections of the display, the cell sorter will divert those cells with properties that would place them within these coordinates (e.g., high or low light scatter, high or low fluorescence) into the appropriate receiver tube, placed below the cell stream. The stream itself is broken up into droplets by a high-frequency vibration applied to the flow chamber, and the droplets containing single cells with specific attributes are charged as they leave the chamber. These droplets are deflected, left or right according to the charge applied, as they pass between two oppositely charged plates. The charge is applied briefly and at a set time after the cell has cut the laser beam such that the droplet containing one specifically marked cell is deflected into the receiver. The concentration in the cell stream must be low enough that the gap between cells is sufficient to prevent two cells from inhabiting one droplet. All cells having similar properties are collected into the

same tube.

This method may be used to separate cells according to any differences that may be detected by light scatter (e.g., cell size) or fluorescence (e.g., DNA, RNA, or protein content; enzyme activity; specific antigens) and has been applied to a wide range of cell types. It has probably been utilized most extensively for hematopoietic cells , for which disaggregation into the obligatory single-cell suspension is relatively simple, but has also been used for solid tissues (e.g., lung , skin and gut). It is an extremely powerful tool but is limited by the cell yield (about 1 × 107 cells is a reasonable maximum number of cells that can be processed at one time).

5- Other techniques:

Electrophoresis: is performed either in a Ficoll gradient or by curtain electrophoresis; used to separate kidney tubular epithelium.

Affinity chromatography: uses antibodies or plant lectins that are bound to nylon fiber or Sephadex useful for fresh blood cells.

Countercurrent distribution: has been utilized to purify murine ascites tumor cells with reasonable viability.

<u>Quantitation</u>

Quantitation in cell culture is required for the characterization of the growth properties of different cell lines for experimental analyses and to establish reproducible culture conditions.

1- Cell counting

a- Haemocytometer: The concentration of a cell suspension may be determined by placing the cells in an optically flat chamber under a microscope , The cell number within a defined area of known depth (i.e., within a defined volume) is counted, and the cell concentration is derived from the count. Hemocytometer counting is cheap and gives you the opportunity to see what you are counting. If the cells were previously mixed with an

equal volume of a viability stain, a viability determination may be performed at the same time. However, the procedure is rather slow and prone to error both in the method of sampling and in the total number of cells counted; it also requires a minimum of 1×10^6 cells/mL.

b- Electronic counting : The main suppliers of electronic cell counters are Beckman Coulter (Coulter Z1 and Z2) and Scharfe Systems (CASY 1). Both use the system devised originally by Coulter Electronics (now Beckman Coulter) in which cells, drawn through a fine orifice, increase the electrical resistance to the current flowing through the orifice, in proportion to the volume of the cells, producing a series of pulses that are sorted and counted.

2- Cell weight

Wet weight is seldom used unless very large cell numbers are involved, because the amount of adherent extracellular liquid gives a large error. As a rough guide, however, there are about 2.5 × 108 HeLa cells (14–16 µm in diameter) per gram wet weight, about 8–10 × 108 cells/g for murine leukemias, e.g., L5178Y murine lymphoma, Friend murine erythroleukemia, myelomas, and hybridomas (11–12 µm in diameter), and about 1.8 × 108 cells/g for human diploid fibroblasts (16–18 µm in diameter). Similarly, dry weight is seldom used, because salt derived from the medium contributes to the weight of unfixed cells, and fixed cells lose some of their low-molecular-weight intracellular constituents and lipids. However, an estimate of dry weight can be derived by interferometry .

3- DNA content

In practice, besides the cell number, DNA and protein are the two most useful measurements for quantifying the amount of cellular material. DNA may be assayed by several fluorescence methods, including reaction with DAPI , PicoGreen (assay kit from Molecular Probes), or Hoechst 33258 . The fluorescence emission of Hoechst 33258 at 458 nm is increased by interaction of the dye with DNA at pH 7.4 and in high salt to dissociate the chromatin protein. This method gives a sensitivity of 10 ng/mL, but requires intact double-stranded DNA. DNA can also be measured by its absorbance at 260 nm,

where 50 μg/mL has an optical density (O.D.) of 1.0. Because of interference from other cellular constituents, the direct absorbance method is useful only for purified DNA.

4- Protein

The protein content of cells is widely used for estimating total cellular material and can be used in growth experiments or as a denominator in expressions of the specific activity of enzymes, the receptor content, or intracellular metabolite concentrations. The amount of protein in solubilized cells can be estimated directly by measuring the absorbance at 280 nm, with minimal interference from nucleic acids and other constituents. The absorbance at 280 nm can detect down to 100 μg of protein, or about 2 × 105 cells. Colorimetric assays are more sensitive than measurements of absorption, and among these assays, the Bradford reaction with Coomassie blue is one of the most widely used.

5- Rates of synthesis

a- DNA Synthesis

Measurements of DNA synthesis are often taken to be representative of the amount of cell proliferation. [3H]thymidine ([3H]-TdR) or [3H]deoxycytidine is the usual precursor that is employed. Exposure to one of these precursors may be for short periods (0.5–1 h) for rate estimations or for longer periods (24 h or more) to measure accumulated DNA synthesis when the basal rate is low (e.g., in high-density cultures). [3H]-TdR should not be used for incubations longer than 24 h or at high specific activities, as radiolysis of DNA will occur, because of the short path length of β-emission (∼1 μm) from decaying tritium; the β-emission releases energy within the nucleus and causes DNA strand breaks.

b- Protein Synthesis: Colorimetric assays measure the total amount of protein present at any one time. Sequential observations over a period of time may be used to measure the net protein accumulation or loss (i.e., protein synthesized—protein degraded), while the rate of protein synthesis may be determined by incubating cells with a radio-isotopically labelled amino acid, such as [3H]leucine or [35S]methionine, and measuring (e.g., by

scintillation counting) the amount of radioactivity incorporated into acid-insoluble material per 10^6 cells or per milligram of protein over a set period of time.

6- Preparation of samples for enzyme and immunoassay cytometry

As the amount of cellular material available from cultures is often too small for efficient homogenization, other methods of lysis are required to release soluble products and enzymes for assay. It is convenient either to set up cultures of the necessary cell number in sample tubes or multiwell plates or to trypsinize a bulk culture and place aliquots of cells into assay tubes. In either case, the cells should be washed in HBSS or D-PBSA to remove the serum, and lysis buffer should be added to the cells. The lysis buffer should be chosen to suit the assay, but, if the particular lysis buffer is unimportant, 0.15 M NaCl or D-PBSA may be used. If the product to be measured is membrane bound, add 1% detergent (Na deoxycholate, Nonidet P40) to the lysis buffer. If the cells are pelleted, resuspend them in the buffer by vortex mixing. Freeze and thaw the preparation three times by placing it in EtOH containing solid CO2 (∼−90◦C) for 1 min and then in 37◦C water for 2 min (longer for samples greater than 1 mL). Finally, spin the preparation at 10,000 g for 1 min (e.g., in an Eppendorf centrifuge), and collect the supernatant for assay.

7- CYTOMETRY

a- *In Situ* Labeling : Fluorescence labeling, either directly with a fluorescent dye (e.g.,Hoechst 33258 for DNA) or with a conjugated antibody for detection of an antigen or molecular probe , can measure the amounts of enzyme, DNA, RNA, protein, or other cellular constituents in situ with a CCD camera. This process allows qualitative as well as quantitative analyses to be made, but is slow if large numbers of cells are to be scanned.

b- Flow Cytometry: Flow cytometry of a cell suspension while losing the relationship between cytochemistry and morphology, samples up to 1×10^7 cells, can measure multiple cellular constituents and activities and enables correlation of these measurements with other cellular parameters, such as cell size, lineage, DNA content, or viability .

8- Replicate sampling

three replicates are sufficient, and for many simple observations (e.g., cell counts), duplicates may be sufficient. Many types of culture vessel are available for replicate monolayer cultures and the choice of which vessel to use is determined

(1) by the number of cells required in each sample and

(2) by the frequency or type of sampling.

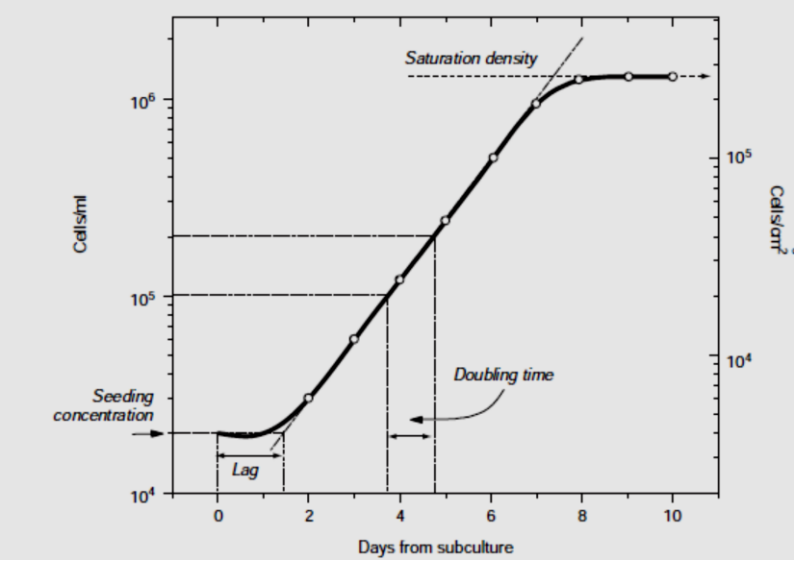
For example, if the incubation time is not a variable, replicate sampling is most readily performed in multiwell plates, such as microtitration plates or 24-well plates.

9- Cell proliferation

Measurements of cell proliferation rates are often used to determine the response of cells to a particular stimulus or toxin . Quantitation of culture growth is also important in routine maintenance, as it is a crucial element for monitoring the consistency of the culture and knowing the best time to subculture ,the optimum dilution, and the estimated plating efficiency at different cell densities. Testing medium, serum, new culture vessels or substrates, and so forth all require quantitative assessment.

a- Growth cycle

after subculture, cells progress through a characteristic growth pattern of lag phase, exponential, or log phase, and stationary, or plateau phase. The log and plateau phases give vital information about the cell line, the population doubling time (PDT) during exponential growth, and the maximum cell density achieved in the plateau phase (i.e., the saturation density).



The measurement of the PDT is used to quantify the response of the cells to different inhibitory or stimulatory culture conditions, such as variations in nutrient concentration, hormonal effects, or toxic drugs. It is also a good monitor of the culture during serial passage and enables the calculation of cell yields and the dilution factor required at subculture. Growth curves are particularly useful for the determination of the saturation density.

The PDT derived from a growth curve should not be confused with the cell cycle or generation time. The PDT is an average figure that applies to the whole population, and it describes the net result of a wide range of division rates, including a rate of zero, within the culture. The cell cycle time or generation time is measured from one point in the cell cycle until the same point is reached again.

A new growth cycle begins each time the culture is subcultured and can be analyzed Using flasks for a growth curve is more labor intensive, limiting the number of replicates to two per day for 10 days, requiring 20 flasks, with an additional 4 flasks for staining or to act as back-up. A cell concentration of 2×10^4 cells/mL should be chosen for a rapidly growing line and 1×10^5 cells/mL for a slower-growing finite cell line. Repeating the growth curve with higher or lower seeding concentrations should then allow the correct seeding concentration and subculture interval to be established.

phases of the growth cycle derivatives from the growth curve.

The lag phase: This phase is the time after subculture and reseeding during which there is little evidence of an increase in the cell number. It is a period of adaptation during which the cell replaces elements of the cell surface and extracellular matrix lost during trypsinization, attaches to the substrate, and spreads out. During spreading, the cytoskeleton reappears, an integral part of the spreading process. The activity of enzymes, such as DNA polymerase, increases, followed by the synthesis of new DNA and structural proteins. Some specialized cell products may disappear and not reappear until the cessation of cell proliferation at a high cell density.

The log phase: This phase is the period of exponential increase in the cell number following the lag period and terminating one or two population doublings after confluence is reached. The length of the log phase depends on the seeding density, the growth rate of the cells, and the density that inhibits cell proliferation. In the log phase, the growth fraction is high (usually 90–100%), and the culture is in its most reproducible form. It is the optimal time for sampling, because the population is at its most uniform and the viability is high. However, the cells are randomly distributed in the cell cycle and, for some purposes, may need to be synchronized.

The plateau phase: Toward the end of the log phase, the culture becomes confluent—i.e., all of the available growth surface is occupied and all of the cells are in contact with surrounding cells. After confluence, the growth rate of the culture is reduced, and in some cases, cell proliferation ceases almost completely after one or two further population doublings . At this stage, the culture enters the plateau, or stationary, phase, and the growth fraction falls to between 0 and 10%. The cells may become less motile; some fibroblasts become oriented with respect to one another, forming a typical parallel array of cells. ‘‘Ruffling’’ of the plasma membrane is reduced, and the cell both occupies less surface area of substrate and presents less of its own surface to the medium. There may be a relative increase in the synthesis of specialized versus structural proteins, and the constitution and charge of the cell surface may be changed**.**

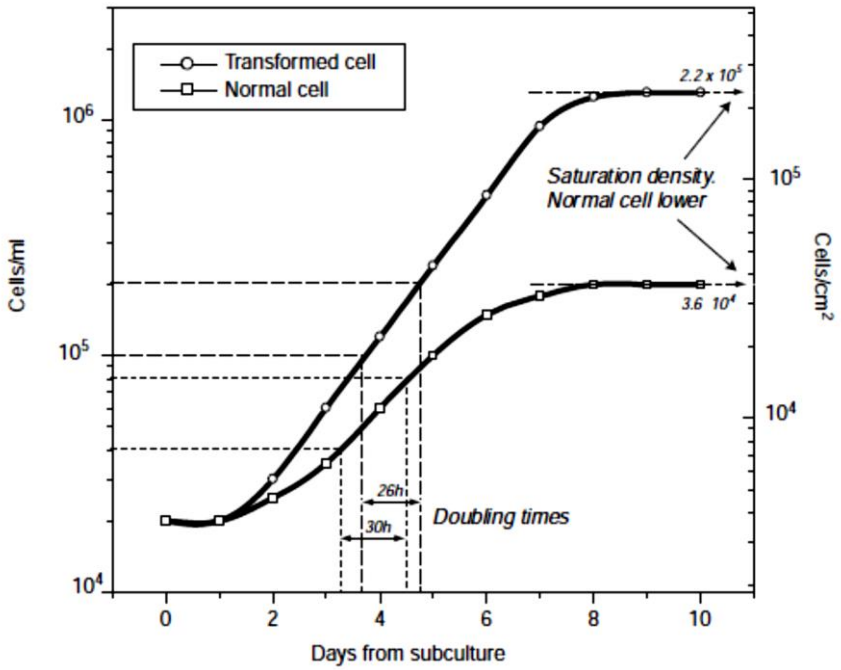


Figure: Saturation Density. Transformation produces an increase in the saturation density of transformed cells, relative to that found in the equivalent normal cells. This increase is often accompanied by a shorter PDT.

Cultures of normal simple epithelial and endothelial cells stop growing after reaching confluence and remain as a monolayer. Most cultures, however, with regular replenishment of medium, will continue to proliferate (although at a reduced rate) well beyond confluence, resulting in multilayers of cells.

Human embryonic lung and adult skin fibroblasts, which express contact inhibition of movement, will continue to proliferate, laying down layers of collagen between the cell layers until multilayers of six or more cells can be reached under optimal conditions. Cultures that have transformed spontaneously or have been transformed by virus or chemical carcinogens will usually reach a higher cell density in the plateau phase than their normal counterparts.