MOLECULAR BIOTECHNOLOGY

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Preface

Recombinant DNA technology, or genetic engineering, has revolutionised our understanding of life at the molecular level—giving us a detailed picture of the living cells functions, spurning diverse biotechnologies that use molecules, cells, tissues and even entire organisms. With the field of biotechnology becoming increasingly stratified and specialised, molecular biotechnology has assumed new proportions in its significance, and currently holds position as one of the fields of biotechnology with immense potential.

'Molecular Biotechnology' has been designed keeping in mind the need for a practical, up-to-date guide on this rapidly growing field. A very detailed introduction to the fundamentals in molecular biology is followed by an overview of standard techniques applied in molecular biotechnology—including chromatography and electrophoresis, cloning techniques, gene expression systems, immunological methods, labelling of proteins and in-situ techniques, microscopy and laser systems. Also under focus are the key topics ranging from functional genomics, proteomics and bioinformatics to drug targeting recombinant antibodies, structural biology and knock mice. Moreover, patenting issues and market opportunities also provide a more thorough understanding of this field. By focusing on an all-encompassing exploration of the subject, this volume exemplifies the convergence of biological, chemical, and informational advances in the discoveries in molecular biotechnology.

Lucid, comprehensive and insightful, the both should prove to be a useful reference for biotechnology professionals, researchers, physicians and others interested in learning more about the field of molecular biotechnology.

Mahadev Sharma
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Principle of Cell Culture

Cell culture is the process by which either prokaryotic or eukaryotic cells are grown under controlled conditions. In practice the term "cell culture" has come to refer to the culturing of cells derived from multicellular eukaryotes, especially animal cells. The historical development and methods of cell culture are closely interrelated to those of tissue culture and organ culture. Animal cell culture became a routine laboratory technique in the 1950s, but the concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century.

Cell culture techniques were advanced significantly in the 1940s and 1950s to support research in virology. Growing viruses in cell cultures allowed preparation of purified viruses for the manufacture of vaccines. The Salk polio vaccine was one of the first products mass-produced using cell culture techniques.

KINDS OF CELL CULTURE

Primary Explantation and Disaggregation

When cells are isolated from donor tissue, they may be maintained in a number of different ways. A simple small fragment of tissue that adheres to the growth surface, either spontaneously or aided by mechanical means, a plasma clot, or an extracellular matrix constituent, such as collagen, will usually give rise to an outgrowth of cells. This type of culture is known as a *primary explant*, and the cells migrating out are known as the *outgrowth* (Figures 1 and 2). Cells in the outgrowth are selected, in the first instance, by their ability to migrate from the explant and subsequently, if subcultured, by their ability to proliferate.

When a tissue sample is disaggregated, either mechanically or enzymatically (Figure 1), the suspension of cells and small aggregates that is generated will contain a proportion of cells capable of attachment to a solid substrate, forming a

monolayer. Those cells within the monolayer that are capable of proliferation will then be selected at the first subculture and, as with the outgrowth from a primary explant, may give rise to a *cell line*.

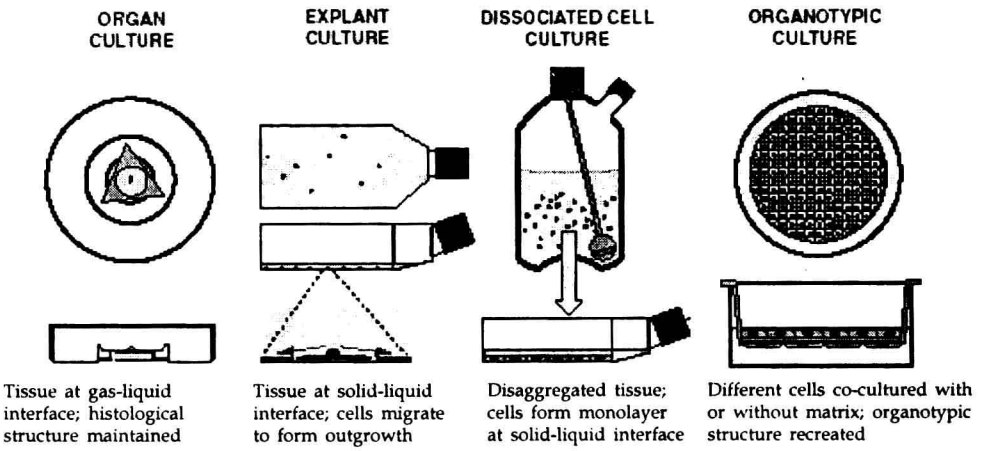

Figure 1: Types of culture.

Tissue disaggregation is capable of generating larger cultures more rapidly than explant culture, but explant culture may still be preferable where only small fragments of tissue are available or the fragility of the cells precludes survival after disaggregation.

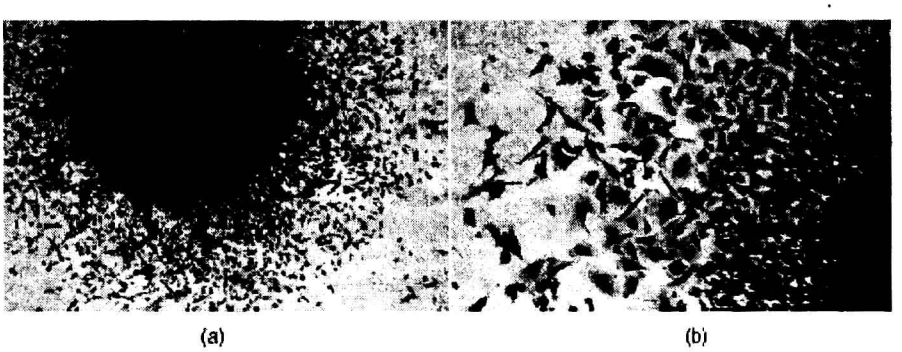

Figure 2: Primary explant and outgrowth. a) Low-power (4× objective) photograph of explant (top left) and radial outgrowth. b) Higher-power detail (10× objective) showing the center of the explant to the right and the outgrowth to the left.

Proliferation and Differentiation

Generally, the differentiated cells in a tissue have limited ability to proliferate.

Therefore, differentiated cells do not contribute to the formation of a primary culture, unless special conditions are used to promote their attachment and preserve their differentiated status. Usually it is the proliferating committed precursor compartment of a tissue (Figure 3), such as fibroblasts of the dermis or the basal epithelial layer of the epidermis, that gives rise to the bulk of the cells in a primary culture, as, numerically, these cells represent the largest compartment of proliferating, or potentially proliferating, cells.

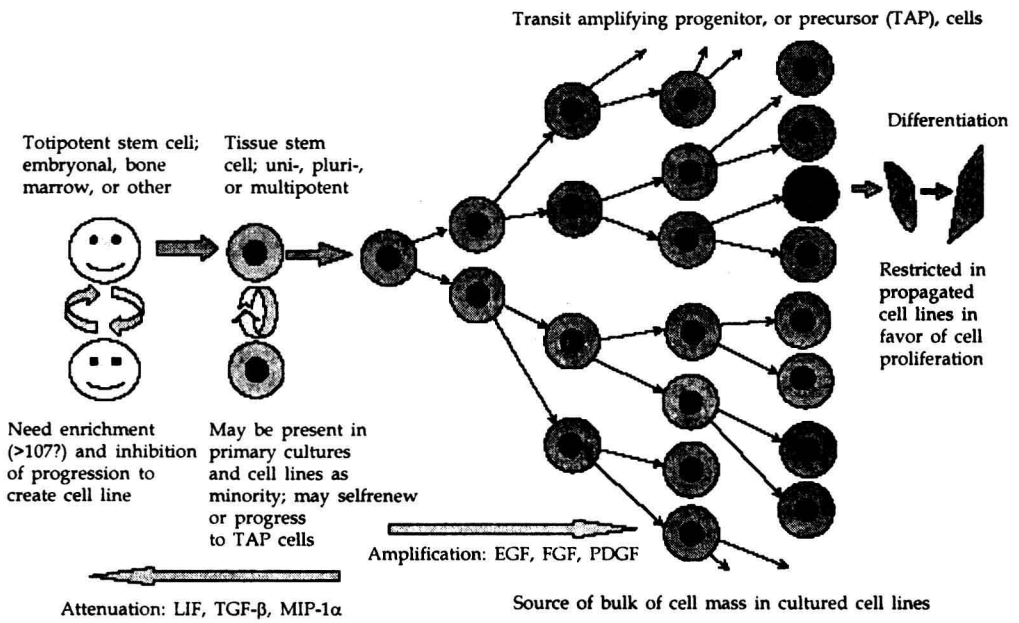

Figure 3: Origin of cell lines.

However, it is now clear that many tissues contain a small population of regenerative cells which, given the correct selective conditions, will also provide a satisfactory primary culture, which may be propagated as stem cells or mature down one of several pathways toward differentiation. This implies that not only must the correct population of cells be isolated, but the correct conditions must be defined to maintain the cells at an appropriate stage in maturation to retain their proliferative capacity if expansion of the population is required. This was achieved fortuitously in early culture of fibroblasts by the inclusion of serum that contained growth factors, such as plateletderived growth factor (PDGF), that helped to maintain the proliferative precursor phenotype.

However, this was not true of epithelial cells in general, where serum growth factors such as transforming growth factor β (TGF-β) inhibited epithelial

proliferation and favoured differentiation. It was not until serum-free media were developed that this effect could be minimised and factors positive to epithelial proliferation, such as epidermal growth factor and cholera toxin, used to maximum effect. Although undifferentiated precursors may give the best opportunity for expansion in vitro, transplantation may require that the cells be differentiated or carry the potential to differentiate. Hence, two sets of conditions may need to be used, one for expansion and one for differentiation.

In general, differentiation will probably require a selective medium for the cell type, supplemented with factors that favour differentiation, such as retinoids, hydrocortisone, and planar-polar compounds, such as sodium butyrate (NaBt). In addition, the correct matrix interaction, homotypic and heterotypic cell interaction, and, for epithelial cells, the correct cellular polarity will need to be established, usually by using an organotypic culture. This assumes, of course, that tissue replacement will require the graft to be completely or almost completely differentiated, as is likely to be the case where extensive tissue repair is carried out. However, there is also the option that cell culture will only be required to expand a precursor cell type and the process of implantation itself will then induce differentiation, as appears to be the case with stem cell transplantation.

Organotypic Culture

Dispersed cell cultures clearly lose their histologic characteristics after disaggregation and, although cells within a primary explant may retain some of the histology of the tissue, this will soon be lost because of flattening of the explant with cell migration and some degree of central necrosis due to poor oxygenation. Retention of histologic structure, and its associated differentiated properties, may be enhanced at the air/medium interface, where gas exchange is optimised and cell migration minimised, as distinct from the substrate/medium interface, where dispersed cell cultures and primary outgrowths are maintained. This so-called organ culture will survive for up to 3 weeks, normally, but cannot be propagated.

An alternative approach, with particular relevance to tissue engineering, is the amplification of the cell stock by generation of cell lines from specific cell types and their subsequent recombination in organotypic culture. This allows the synthesis of a tissue equivalent or construct on demand for basic studies on cell-cell and cell-matrix interaction and for in vivo implantation. The fidelity of the construct in terms of its real tissue equivalence naturally depends on identification of all the participating cell types in the tissue in vivo and the ability to culture and recombine them in the correct proportions with the correct matrix and juxtaposition. So far this has worked best for skin, but even then, melanocytes have only recently been added to the construct, and islet of Langerhans cells are

still absent, as are sweat glands and hair follicles, although some progress has been made in this area.

There are a great many ways in which cells have been recombined to try to simulate tissue, ranging from simply allowing the cells to multilayer by perfusing a monolayer to highly complex perfused membrane (Membroferm) or capillary beds. These are termed *histotypic cultures* and aim to attain the density of cells found in the tissue from which the cells were derived. It is possible, using selective media, cloning, or physical separation methods, to isolate purified cell strains from disaggregated tissue or primary culture or at first subculture.

These purified cell populations can then be combined in organotypic culture to recreate both the tissue cell density and, hopefully, the cell interactions and matrix generation found in the tissue. Filter well inserts provide the simplest model system to test such recombinants, but there are many other possibilities including porous matrices, perfused membranes, and concentric double microcapillaries.

Substrates and Matrices

Initially, cultures were prepared on glass for ease of observation, but cells may be made to grow on many different charged surfaces including metals and many polymers. Traditionally, a net negative charge was preferred, such as found on acidwashed glass or polystyrene treated by electric ion discharge, but some plastics are also available with a net positive charge (e.g., Falcon Primaria), which is claimed to add some cell selectivity. In either case, it is unlikely that the cell attaches directly to synthetic substrates and more likely that the cell secretes matrix products that adhere to the substrate and provide ligands for the interaction of matrix receptors such as integrins.

Hence it is a logical step to treat the substrate with a matrix product, such as collagen type IV, fibronectin, or laminin, to promote the adhesion of cells that would otherwise not attach. Suffice it to say at this stage that scaffolds have the same requirements as conventional substrates in terms of low toxicity and ability to promote cell adhesion, often with the additional requirement of a three-dimensional geometry. If the polymer or other material does not have these properties, derivatisation and/or matrix coating will be required.

Most studies suggest that cell cultivation on a three-dimensional scaffold is essential for promoting orderly regeneration of engineered tissues in vivo and in vitro. Scaffolds investigated to date vary with respect to material chemistry (e.g., collagen, synthetic polymers), geometry (e.g., gels, fibrous meshes, porous sponges, tubes), structure (e.g., porosity, distribution, orientation, and connectivity of the

pores), physical properties (e.g., compressive stiffness, elasticity, conductivity, hydraulic permeability), and degradation (rate, pattern, products).

In general, scaffolds should be made of biocompatible materials, preferentially those already approved for clinical use. Scaffold structure determines the transport of nutrients, metabolites, and regulatory molecules to and from the cells, whereas the scaffold chemistry may have an important role in cell attachment and differentiation. The scaffold should biodegrade at the same rate as the rate of tissue assembly and without toxic or inhibitory products. Mechanical properties of the scaffold should ideally match those of the native tissue being replaced, and the mechanical integrity should be maintained as long as necessary for the new tissue to mature and integrate.

APPLICATIONS OF CELL CULTURE

Mass culture of animal cell lines is fundamental to the manufacture of viral vaccines and many products of biotechnology. Biologicals produced by recombinant DNA (rDNA) technology in animal cell cultures include enzymes, hormones, immunobiologicals (monoclonal antibodies, interleukins, lymphokines), and anticancer agents. Although many simpler proteins can be produced using rDNA in bacterial cultures, more complex proteins that are glycosylated (carbohydrate-modified), currently must be made in animal cells. An important example of such a complex protein is the hormone erythropoietin. The cost of growing mammalian cell cultures is high, so research is underway to produce such complex proteins in insect cells or in higher plants.

Vaccines

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

ISOLATION OF CULTURED CELLS

Tissue Collection and Transportation

The first, and most important, element in the collection of tissue is the cooperation and collaboration of the clinical staff. This is best achieved if a member of the surgical team is also a member of the culture project, but even in the absence of

this, time and care must be spent to ensure the sympathy and understanding of those who will provide the clinical material. It is worth preparing a short handout explaining the objectives of the project and spending some time with the person likely to be most closely involved with obtaining samples. This may be the chief surgeon (who will need to be informed anyway), or it may be a more junior member of the team willing to set up a collaboration, one of the nursing staff, or the pathologist, who may also require part of the tissue.

Whoever fulfils this role should be identified and provided with labeled containers of culture medium containing antibiotics, bearing a contact name and phone number for the cell culture laboratory. A refrigerator should be identified where the containers can be stored, and the label should also state clearly do not freese! The next step is best carried out by someone from the laboratory collecting the sample personally, but it is also possible to leave instructions for transportation by taxi or courier.

If a third party is involved, it is important to ensure that the container is well protected, preferably double wrapped in a sealed polythene bag and an outer padded envelope provided with the name, address, and phone number of the recipient at the laboratory. Refrigeration during transport is not usually necessary, as long as the sample is not allowed to get too warm, but if delivery will take more than an hour or two, then one or two refrigeration packs, such as used in picnic chillers, should be included but kept out of direct contact.

If the tissue sample is quite small, a further tissue sample (any tissue) or a blood sample should be obtained for freezing. This will be used ultimately to corroborate the origin of any cell line that is derived from the sample by DNA profiling. A cell line is the culture that is produced from subculture of the primary, and every additional subculture after this increases the possibility of cross-contamination, so verification of origin is important. In addition, the possibility of misidentification arises during routine subculture and after recovery from cryopreservation.

Biosafety and Ethics

All procedures involved in the collection of human material for culture must be passed by the relevant hospital ethics committee. A form will be required for the patient to sign authorising research use of the tissue, and preferably disclaiming any ownership of any materials derived from the tissue. The form should have a brief layman's description of the objectives of the work and the name of the lead scientist on the project. The donor should be provided with a copy.

All human material should be regarded as potentially infected and treated with caution. Samples should be transported securely in double-wrapped waterproof containers; they and derived cultures should be handled in a Class II biosafety cabinet and all discarded materials autoclaved, incinerated, or chemically disinfected. Each laboratory will its own biosafety regulations that should be adhered to, and anyone in any doubt about handling procedures should contact the local safety committee (and if there is not one, create it!). Rules and regulations vary among institutions and countries, so it is difficult to generalise, but a good review can be obtained in Caputo.

Recording

When the sample arrives at the laboratory, it should be entered into a record system and assigned a number. This record should contain the details of the donor, identified by hospital number rather than by name, tissue site, and all information regarding collection medium, time in transit, treatment on arrival, primary disaggregation, and culture details, etc. This information will be important in the comparison of the success of individual cultures, and if a long-term cell line is derived from the culture, this will be the first element in the cell line's provenance, which will be supplemented with each successive manipulation or experimental procedure.

Such records are best maintained in a computer database where each record can be derived from duplication of the previous record with appropriate modifications. There may be issues of data protection and patient confidentiality to be dealt with when obtaining ethical consent.

Disaggregation and Primary Culture

Disaggregated tissue will contain a variety of different cell types, and it may be necessary to go through a separation technique, such as density gradient separation or immunosorting by magnetisable beads (MACS), using a positive sort to select cells of interest or a negative sort to eliminate those that are not required, or by using fluorescence-activated cell sorting (FACS). The cell population can then be further enriched by selection of the correct medium (e.g., keratinocyte growth medium (KGM) or MCDB 153 for keratinocytes, many of which are now available commercially, and supplementing this with growth factors. Survival and enrichment may be improved in some cases by coating the substrate with gelatin, collagen, laminin, or fibronectin.

SUBCULTURE

Frequently, the number of cells obtained at primary culture may be insufficient to create constructs suitable for grafting. Subculture gives the opportunity to expand the cell population, apply further selective pressure with a selective medium, and achieve a higher growth fraction and allows the generation of replicate cultures for characterisation, preservation by freezing, and experimentation. Briefly, subculture involves the dissociation of the cells from each other and the substrate to generate a single-cell suspension that can be quantified.

Reseeding this cell suspension at a reduced concentration into a flask or dish generates a secondary culture, which can be grown up and subcultured again to give a tertiary culture, and so on. In most cases, cultures dedifferentiate during serial passaging but can be induced to redifferentiate by cultivation on a 3D scaffold in the presence of tissue-specific differentiation factors (e.g., growth factors, physical stimuli). However, the cell's ability to redifferentiate decreases with passaging. It is thus essential to determine, for each cell type, source, and application, a suitable number of passages during subculture.

Most normal cell lines will undergo a limited number of subcultures, or passages, and are referred to as *finite cell lines*. The limit is determined by the number of doublings that the cell population can go through before it stops growing because of senescence. Senescence is determined by a number of intrinsic factors regulating cell cycle, such as Rb and p53, and is accompanied by shortening of the telomeres on the chromosomes. Once the telomeres reach a critical minimum length, the cell can no longer divide.

Telomere length is maintained by telomerase, which is down regulated in most normal cells except germ cells. It can also be higher in stem cells, allowing them to go through a much greater number of doublings and avoid senescence. Transfection of the telomerase gene hTRT into normal cells with a finite life span allows a small proportion of the cells to become immortal, although this probably involves deletion or inactivation of other genes such as p53 and *myc*.

Growth Cycle

Each time that a cell line is subcultured it will grow back to the cell density that existed before subculture (within the limits of its finite life span). This process can be described by plotting a growth curve from samples taken at intervals throughout the growth cycle (Figure 4), which shows that the cells enter a latent period of no growth, called the *lag period*, immediately after reseeding. This period lasts from a few hours up to 48 h, but is usually around 12-24 h, and allows the

cells to recover from trypsinisation, reconstruct their cytoskeleton, secrete matrix to aid attachment, and spread out on the substrate, enabling them to reenter cell cycle. They then enter exponential growth in what is known as the *log phase*, during which the cell population doubles over a definable period, known as the *doubling time* and characteristic for each cell line.

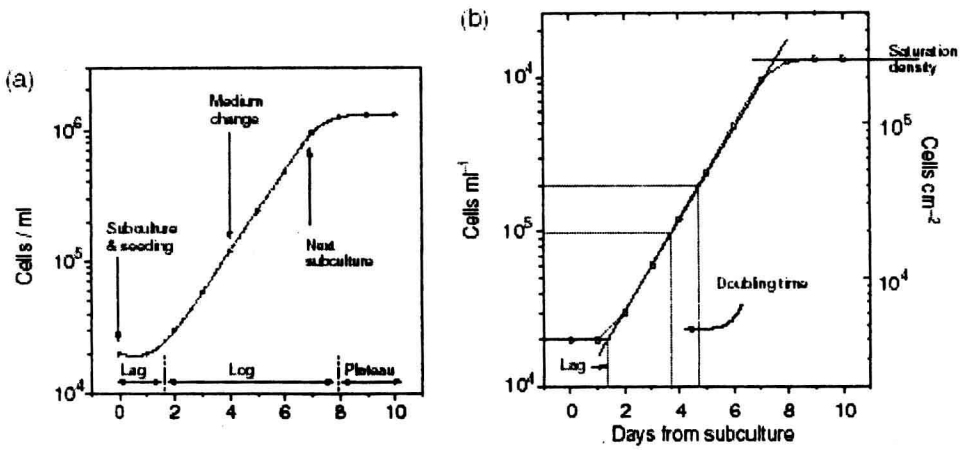

Figure 4: Growth curve. a) Defines the lag, log (exponential), and plateau phases, and when culture should be fed and subcultured after the indicated seeding time.
b) Shows the kinetic parameters that can be derived from the growth curve.

As the cell population becomes crowded when all of the substrate is occupied, the cells become packed, spread less on the substrate, and eventually withdraw from the cell cycle. They then enter the *plateau* or *stationary phase*, where the growth fraction drops to close to zero. Some cells may differentiate in this phase; others simply exit the cell cycle into G0 but retain viability. Cells may be subcultured from plateau, but it is preferable to subculture before plateau is reached, as the growth fraction will be higher and the recovery time (lag period) will be shorter if the cells are harvested from the top end of the log phase.

Reduced proliferation in the stationary phase is due partly to reduced spreading at high cell density and partly to exhaustion of growth factors in the medium at high cell concentration. These two terms are not interchangeable. Density implies that the cells are attached, and may relate to monolayer density (two-dimensional) or multilayer density (three-dimensional). In each case there are major changes in *cell shape*, cell surface, and extracellular matrix, all of which will have significant effects on *cell proliferation* and differentiation.

A high density will also limit nutrient perfusion and create local exhaustion of peptide growth factors. In normal cell populations this leads to a withdrawal from the cycle, whereas in transformed cells, cell cycle arrest is much less effective and the cells tend to enter apoptosis. Cell concentration, as opposed to cell density, will exert its main effect through nutrient and growth factor depletion, but in stirred suspensions cell contact mediated effects are minimal, except where cells are grown as aggregates.

Cell concentration per se, without cell interaction, will not influence proliferation, other than by the effect of nutrient and growth factor depletion. High cell concentrations can also lead to apoptosis in transformed cells in suspension, notably in myelomas and hybridomas, but in the absence of cell contact signaling this is presumably a reflection of nutrient deprivation.

Each time the culture is subcultured the growth cycle is repeated. The number of doublings should be recorded (Figure 5) with each subculture, simplified by reducing the cell concentration at subculture by a power or two, the so-called *split ratio*. A split ratio of two allows one doubling per passage, four, two doublings, eight, three doublings, and so on.

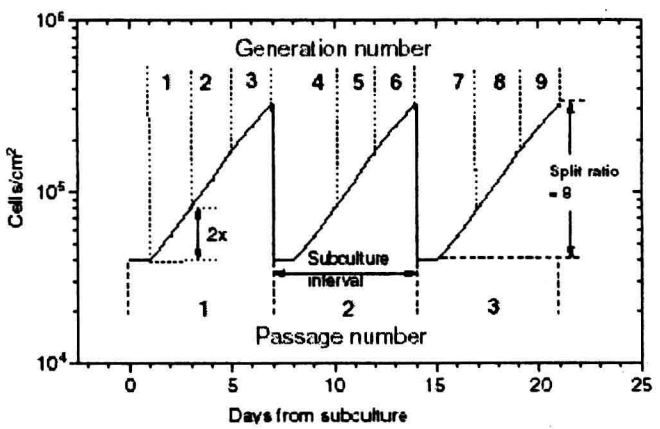

Figure 5: Serial subculture.

The number of elapsed doublings should be recorded so that the time to senescence can be predicted and new stock prepared from the freezer before the senescence of the existing culture occurs.

CRYOPRESERVATION

If a cell line can be expanded sufficiently, preservation of cells by freezing will

allow secure stocks to be maintained without aging and protect them from problems of contamination, incubator failure, or medium and serum crises. Ideally, 1×10^6-1×10^7 cells should be frozen in 10 ampoules, but smaller stocks can be used if a surplus is not available. The normal procedure is to freese a token stock of one to three ampoules as soon as surplus cells are available, then to expand remaining cultures to confirm the identity of the cells and absence of contamination, and freese down a seed stock of 10-20 ampoules. One ampoule, thawed from this stock, can then be used to generate a using stock.

In many cases, there may not be sufficient doublings available to expand the stock as much as this, but it is worth saving some as frozen stock, no matter how little, although survival will tend to decrease below 1×10^6 cells/ml and may not be possible below 1×10^5 cells/ml. Factors favouring good survival after freezing and thawing are:

- High cell density at freezing (1×10^6-1×10^7 cells/ml).
- Presence of a preservative, such as glycerol or dimethyl sulfoxide (DMSO) at 5-10%.
- Slow cooling, 1.C/min, down to -70.C and then rapid transfer to a liquid nitrogen freezer.
- Rapid thawing.
- Slow dilution, ~20-fold, in medium to dilute out the preservative.
- Reseeding at 2- to 5-fold the normal seeding concentration. For example, if cells are frozen at 5×10^6 cells in 1 ml of freezing medium with 10% DMSO and then thawed and diluted 1:20, the cell concentration will still be 2.5×10^5 cells/ml at seeding, higher than the normal seeding concentration for most cell lines, and the DMSO concentration will be reduced to 0.5%, which most cells will tolerate for 24 h.
- Changing medium the following day (or as soon as all the cells have attached) to remove preservative. Where cells are more sensitive to the preservative, they may be centrifuged after slow dilution and resuspended in fresh medium, but this step should be avoided if possible as centrifugation itself may be damaging to freshly thawed cells.

There are differences of opinion regarding some of the conditions for freezing and thawing, for example, whether cells should be chilled when DMSO is added or diluted rapidly on thawing, both to avoid potential DMSO toxicity. In the author's experience, chilling diminishes the effect of the preservative, particularly with glycerol, and rapid dilution reduces survival, probably due to osmotic shock. Culturing in diluted DMSO after thawing can be a problem for some cell lines if