BASIC TECHNIQUES IN ANIMAL CELL CULTURE

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Introduction

One of the characteristics of most animals is that they are multicellular–in other words– they are composed of many cells. With this multicellularity comes the specialization of cells. In a multicellular organism, each cell does not have to carry out all the activities necessary for the life of the organism. Although most cells of any higher organism have many organelles and metabolic pathways in common, each cell is also unique in expressing some of these components to an enhanced degree in order to fulfill a specific function within the organism. Each cell type has its own role–to secrete a specific product, to contract, to transmit an electrical impulse, and so on. The result of this cellular specialization is that animals consist of a number of different types of cells - each with a characteristic size, shape, structure and function. Such cells are said to have differentiated. A vertebrate has more than 100 different types of cells. These cells associated in very organized patterns to perform specialized functions.

Many animal cells can, with special care, be induced to grow outside of their organ or tissue of origin. Isolated cells, tissues or organs can be grown in plastic dishes when they are kept at defined temperatures using an incubator and supplemented with a medium containing cell nutrients and growth factors. The *in vitro* cultivation of organs, tissues and cells is collectively known as tissue culture, and is used in many areas of science.

The partial list of different cell types which can be grown in culture includes connective tissue elements such as fibroblasts, skeletal tissue (bone and cartilage), skeletal, cardiac and smooth muscle, epithelial tissue (liver, lung, breast, skin, bladder and kidney), neural cells (glial cells and neurons, although neurons do not proliferate *in vitro*), endocrine cells (adrenal, pituitary, pancreatic islet cells), melanocytes and many different types of tumor cells. The development of these tissue culture techniques owes much to two major branches of medical research: cancer research and virology.

Historical background

Tissue culture is not a new technique – in the scientific literature there are references to its use dating back to 1885 (Table 1). An embryologist, called Roux, was able to maintain the medullary plate of a chick embryo in warm saline for a few days. This was the first recorded example of successful explantation. In 1903, Jolly made detailed observations on *in vitro* cell survival and cell division using salamander leukocytes. In the early experiments, fragments of tissue were studied, and this gave rise to the name *'tissue culture'*. Officially, the term tissue culture is used when cells are maintained *in vitro* for more than 24 hours.

The development of animal cell culture can be traced back to the work of Ross Harrison in 1907 on cell entrapment and growth from explants of frog embryo tissue. The cell growth that he observed in clotted lymph fluid in a depression slide is often regarded as the foundation of animal cell culture as a science. In his technique ('the hanging drop') the isolated tissue was suspended on the underside of a coverslip which was sealed over a depression in a microscope slide. Burrows continued the development of this technique with the use of plasma clots which were found to be more efficient for the growth of cells from warm-blooded animals. The matrix of insoluble protein provided the necessary anchor for cell growth, and nutrients were provided by the enclosed fluid.

One of the major difficulties of this work was the maintenance of the cultures free from contamination. The difference in growth rates between animal and bacterial cells is such that a low-level contamination in an animal cell culture can quickly lead to bacterial overgrowth. This led Alexis Carrel, who was trained as a surgeon, to apply strict aseptic techniques to cell culture *in vitro*. He introduced the 'Carrel flask' which facilitated subculture under aseptic conditions and became the forerunner of modern tissue culture flasks. However, the procedures were elaborate and difficult to repeat and so cell culture was not adopted as a routine laboratory technique until much later.

In 1912, Carrel initiated a culture of chick embryo heart cells which were passaged for a reported period of 34 years. This led to the erroneous belief that, given the appropriate conditions, isolated cells could be cultured indefinitely. Later, analysis of Carrel's work showed that, as cell growth was maintained by the use of embryo extracts, new cells were being continuously added to the culture during medium replenishment. The finite capacity for growth of 'normal' cells was eventually established from the work of Hayflick and Moorhead in 1961.

Another significant advance was the use of trypsin (a proteolytic enzyme) by Rous and Jones in 1916 to free cells from tissue matrix. It was subsequently used for the subculture of adherent cells and in the 1950s, the technique of trypsinization was exploited to produce homogeneous cell strains (as opposed to tissue cultures which contain a mixture of cell types) and this marked the start of animal cell culture techniques. Trypsinization is the term applied to the treatment of cells by the proteolytic enzyme trypsin to change their adhesiveness. The term *'cell culture'* refers to cultures derived from dispersed cells taken from the original tissue. In cell culture the cells are no longer organized into tissues.

To meet the nutritional requirements of cells, embryo extracts or animal blood serum were added to the cells. These were particularly vulnerable to contamination but the addition of the antibiotics, penicillin and streptomycin, to the cell culture medium from the 1940s onwards alleviated this problem. The problems of microbial contamination have also been greatly reduced by the use of laminar air flow cabinets, which minimize the possibility of contamination by air-borne microbes. This encouraged the more widespread use of cell culture as a laboratory technique, particularly after the isolation of a variety of cell types which showed good growth characteristics *in vitro*. These included the chemically transformed mouse L cells and the human carcinoma cell line, HeLa.

These cell lines were the focus of Earle and Eagle's work in the 1950s on the development of chemically defined media. The nutrient formulations developed replaced the undefined biological extracts previously used. This had the advantages of consistency between batches, ease of sterilization, and reduced chance of contamination.

The impetus for the application of the techniques of cell culture on a laboratory scale to large industrial scale processes came with the capability of virus propagation on cell culture. The polio vaccine which was produced for mass vaccination in the 1950s became one of the first major commercial products of cultured animal cells. Since the 1950s a range of other products synthesized from animal cells have found commercial application. Consequently, the study of the optimization of culture conditions to maintain consistently high productivity from such animal cells *in vitro* has become of increased importance.

Nowadays, animal cell culture becomes a reasonable alternative for animal experiments in the process of drug discovery and development. Overall, an aspect of pharmaceutical research which promisingly employs cell culture models is the study of *in vitro* drug transport/absorption and metabolism.

Table 1. Historical events in the development of cell culture

1878:	Claude Bernard proposed that physiological systems of an organism can be maintained in a living system after the death of an organism.
1885:	Roux maintained embryonic chick cells in a saline culture.
1897:	Loeb demonstrated the survival of cells isolated from blood and connective tissue in serum and plasma.
1903:	Jolly observed cell division of salamander leucocytes *in vitro*.
1907:	Harrison cultivated frog nerve cells in a lymph clot held by the 'hanging drop' method and observed the growth of nerve fibers *in vitro* for several weeks. He was considered by some as the father of cell culture.
1910:	Burrows succeeded in long term cultivation of chicken embryo cell in plasma clots. He made detailed observation of mitosis.
1911:	Lewis and Lewis made the first liquid media consisted of sea water, serum, embryo extract, salts and peptones. They observed limited monolayer growth.
1913:	Carrel introduced strict aseptic techniques so that cells could be cultured for long periods.
1916:	Rous and Jones introduced proteolytic enzyme trypsin for the subculture of adherent cells.
1923:	Carrel and Baker developed 'Carrel' or T-flask as the first specifically designed cell culture vessel. They employed microscopic evaluation of cells in culture.
1927:	Carrel and Rivera produced the first viral vaccine - Vaccinia.
1933:	Gey developed the roller tube technique.
1940s:	The use of the antibiotics penicillin and streptomycin in culture medium decreased the problem of contamination in cell culture.
1948:	Earle isolated mouse L fibroblasts which formed clones from single cells. Fischer developed a chemically defined medium, CMRL 1066.
1949:	Enders reported that polio virus could be grown on human embryonic cells in culture.
1952:	Gey established a continuous cell line from a human cervical carcinoma known as HeLa (Helen Lane) cells. Dulbecco developed plaque assay for animal viruses using confluent monolayers of cultured cells.
1954:	Abercrombie observed contact inhibition: motility of diploid cells in monolayer culture ceases when contact is made with adjacent cells.
1955:	Eagle studied the nutrient requirements of selected cells in culture and established the first widely used chemically defined medium.
1961:	Hayflick and Moorhead isolated human fibroblasts (WI-38) and showed that they have a finite lifespan in culture.
1964:	Littlefield introduced the HAT medium for cell selection.
1965:	Ham introduced the first serum-free medium which was able to support the growth of some cells.
1965:	Harris and Watkins were able to fuse human and mouse cells by the use of a virus.
1975:	Kohler and Milstein produced the first hybridoma capable of secreting a monoclonal antibody.
1978:	Sato established the basis for the development of serum-free media from cocktails of hormones and growth factors.
1982:	Human insulin became the first recombinant protein to be licensed as a therapeutic agent.
1985:	Human growth hormone produced from recombinant bacteria was accepted for therapeutic use.
1986:	Lymphoblastoid γIFN licensed.
1987:	Tissue-type plasminogen activator (tPA) from recombinant animal cells became commercially available.
1989:	Recombinant erythropoietin in trial.
1990:	Recombinant products in clinical trial (HBsAG, factor VIII, HIVgp120, CD4, GM-CSF, EGF, mAbs, IL-2).

Basic characteristics of tissue culture

Tissue culture

Tissue culture is used as a generic term to include the *in vitro* cultivation of organs, tissues and cells. Originally, the term is not limited to animal cells, but includes the *in vitro* cultivation of plant cells. Tissue culture can be subdivided into three major categories; organ culture, explant culture, and cell culture.

Organ culture

Organ culture refers to a three-dimensional culture of tissue retaining some or all of the histological features of the tissue *in vivo*. The whole organ or part of the organ is maintained in a way that allows differentiation and preservation of architecture, usually by culturing the tissue at the liquid-gas interface on a grid or gel.

There are disadvantages to organ cultures. Organs cannot be propagated so each piece of tissue can only be used once, which makes it difficult to assess the reproducibility of a response. And, of course, the particular cells of interest may be very small in number in a given piece of tissue so the response produced may be difficult to detect and quantify. It may not be possible to supply adequate oxygen and nutrients throughout the tissue because of the absence of a functioning vascular system, so necrosis of some cells occurs fairly rapidly. This problem may be ameliorated to some extent by keeping the organ in stirred cultures or in roller bottles which alternately provide air and soluble nutrients.

Explant (or organotypic) culture

In explant culture, small pieces of the tissue of interest are simply allowed to attach to an appropriate substrate, usually one that has been coated with collagen, and are cultured in a rich medium, usually one containing serum. Following attachment, cell migration is promoted in the plane of the solid substrate. Traditionally, explants have been maintained in Maximov chambers in which cells are grown on coverslips sealed over a depression in a thick glass slide, and this approach is still in use. More recently, it has become common to use regular culture dishes, which are much more convenient since they do not need to be disassembled and reassembled at each feeding. As with dissociated cell culture, immature tissue grows best, and explants are generally prepared from embryonic or neonatal tissue. Typically, the tissue is cut with scalpels into slices 0.5 to 1.0 mm thick, but in some cases it is simply fragmented by passing through a nylon mesh. The need for diffusion of nutrients and oxygen to the center of the explant limits thickness to about a millimeter.

In experienced hands, explant cultures can be maintained for months, and cells within the explant continue their development more or less appropriately. One of the principal advantages of this method is that some aspects of the tissue's architecture can be preserved within the explant.

(Dissociated) Cell culture

Cell culture refers to cultures derived from dissociated cells taken from the original tissue ('primary cell culture'). Cells are dispersed (mechanically and/or enzymatically) into a cell suspension which may then be cultured as a monolayer on a solid substrate, or as a suspension in the culture medium. These cultures have lost their histotypic architecture and often some of the biochemical properties associated with it. However, they can be propagated and hence expanded and divided to give rise to replicate cultures. Cell cultures can be characterized and a defined population can be preserved by freezing.

The most obvious advantage of cell culture, and of dissociated cell culture in particular, is that it makes individual living cells accessible. All in all, primary dissociated cell cultures are particularly amenable to study using morphological and physiological techniques, which can be applied on a cell by cell basis. They are obviously less well suited to traditional biochemical approaches because the quantity of material obtainable

from these cultures is usually limited and they contain a heterogeneous population of cells.

One final drawback of working with primary cell cultures is that success is not automatic. Finding the conditions that permit good cell growth and maturation, getting culture to grow reproducibly, and documenting that you have accomplished all of this entails plenty of hard work.

Basic equipment and facilities in animal cell culture

Some of the specific equipment and techniques are required for the maintenance of cell cultures. A rule of thumb is that the more equipment you get, the more efficient cell culturing you perform.

Sterile work area

Where possible, a separate room should be made available for clean cell culture work. This room should be free of through traffic and, if possible, equipped with an air flow cabinet which supplies filtered air around the work surface. A HEPA (High Efficiency Particle Air Filter) filtered air supply is desirable but not always affordable. Primary animal tissue and micro-organisms must not be cultured in or near the cell culture laboratory and the laboratory must be specifically designated for clean cell culture work. Clean laboratory coats should be kept at the entrance and should not be worn outside of this laboratory and brought back in.

If strict sterility is needed, a laminar flow hood offers the best sterile protection available. If a hazardous chemical is to be handled a Class II Biohazard Cabinet which has a vertical laminar flow should be used. However, for primary cultures and also if no laminar flow hood or sterile room is available, an area for sterile work should be set aside, where there is no thoroughfare. If aseptic techniques are adhered to and the area kept clean and tidy, sterility can be easily maintained.

All work surfaces, benches and shelves and the base of the airflow cabinets must be kept clean by frequent swabbing with 70% ethanol or an alternative disinfectant. If an airflow cabinet cannot be provided, the culture work may be done on a clean bench using a Bunsen burner to create a sterile 'umbrella' under which the work can be done.

Incubation facilities

In addition to an airflow cabinet and benching which can be easily cleaned, the cell culture laboratory will need to be furnished with an incubator or hot room to maintain the cells at 30-40 °C. The incubation temperature will depend on the type of cells being cultivated. Insect cells will grow best at around 30 °C while mammalian cells require a temperature of 37 °C. It may be necessary to use an incubator which has been designed to allow CO_2 to be supplied from a main supply or gas cylinder so that an atmosphere of between 2-5% CO_2 is maintained in the incubator.

In general, many cell lines can be maintained in an atmosphere of 5% CO_2:95% air at 99% relative humidity. The concentration of CO_2 is kept in equilibrium with sodium bicarbonate in the medium. Different media have differing buffering capacity. If a CO_2 controlled incubator is not available, or cultures must be kept sealed in flasks (i.e., after treatment with some volatile substances), then cells may be maintained in flasks sealed after gassing with 5% CO_2:95% air, or vessels kept in boxes gassed and then sealed with pressure sensitive tape. In the case of boxes, the humidity must be maintained with a dish of water.

Figure 1. A CO_2 controlled incubator.

Various media may be used so that a controlled CO_2 atmosphere is not required and in this case a CO_2 incubator is not necessary. Hepatocytes in primary culture are often maintained in Leibovitz L-15 medium which does not require a CO_2 atmosphere, however, flasks must not be sealed (as the hepatocytes require a high O_2 tension which is reduced with time in sealed ungassed vessels). Most cell lines are maintained at 36.5 °C, although some cultures, such as skin cultures may require lower temperatures. Cultured cells can generally survive lower temperatures, but rarely survive temperatures greater than 2 °C above normal, and therefore the incubator should be set to cut out at approximately 38.5 °C to prevent cell death. Incubators are designed to regulate an even temperature and this is more important than accuracy, i.e., temperature should be ±0.5 ° C. Most incubators have areas of differing temperature, therefore fan assisted incubators are preferable to help maintain even temperature distribution.

Refrigerators and freezer (-20 °C)

Both items are very important for storage of liquid media at 4 °C and for enzymes (e.g., trypsin) and some media components (e.g., glutamine and serum) at -20 °C.

A refrigerator or cold room is required to store medium and buffers. A freezer will be needed for keeping pre-aliquoted stocks of serum, nutrients and antibiotics. Reagents may be stored at a temperature of -20 °C but if cells are to be preserved it may be necessary to provide liquid nitrogen or a -70 °C freezer.

Microscopes

A simple inverted microscope is essential so that cultures can be examined in flasks and dishes. It is vital to be able to recognize morphological changes in cultures since these may be the first indication of deterioration of a culture. A very simple light microscope with x100 magnification will suffice for routine cell counts in a hemocytometer, although a microscope of much better quality will be required for chromosome analysis or autoradiography work.

A microscope with normal Kohler illumination will be needed for cell counting. An inverted microscope will also be needed for examining flasks and multi-well dishes from underneath. Both microscopes should be equipped with a x10 and a x20 objective and it may be useful to provide a x40 and a x100 objective for the normal microscope. Additional features such as a camera, CCD video camera, adapter and attachments and UV facility may also be required for some purposes.

Figure 2. An inverted microscope equiped with bright field, Normaski, phase-contrast, and fluorescence optics. Attached CCD video camera connects microscopic visual fields to the TV monitor and computer.

Tissue culture ware

A variety of tissue culture plasticware is available, the most common being specially treated polystyrene. Although all tissue culture plasticware should support cell growth adequately, it is essential when using a new supplier or type of dish to ensure that cultures grow happily in it. The tests to ensure this, such as growth curves and time of reaching a confluent monolayer, are similar, to those used to ensure that serum batches are satisfactory.

Cells can be maintained in Petri dishes or flasks (25 cm^2 or 75 cm^2) which have the added advantage that the flasks can be gassed and then sealed so that a CO_2 incubator need not be used. This is particularly useful if incubators fail. Tissue cultureware is always chosen to match the procedure.

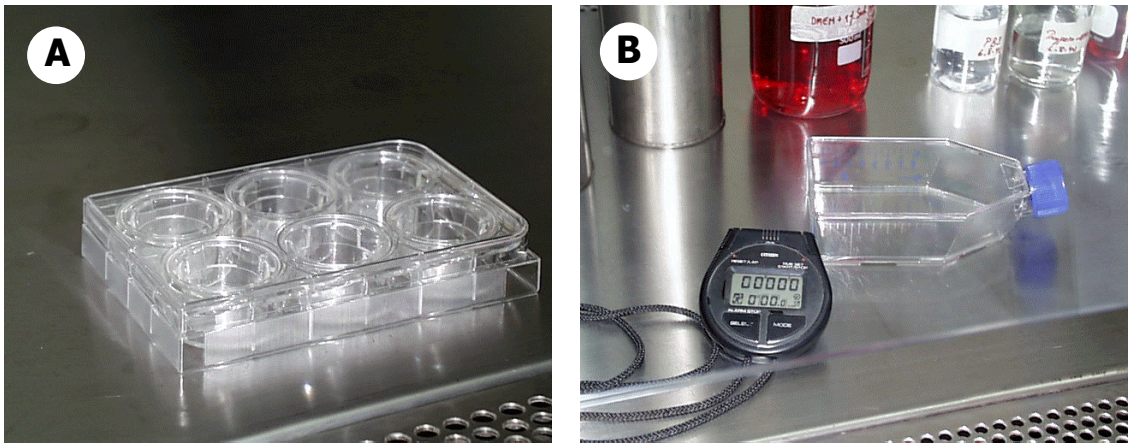

Figure 3. The disposable plasticware used for culturing cells. A: Multi-well plate (6 wells). B: Flask (75 cm^2).

Sometimes it may be necessary to condition a surface by pretreatment with 'spent' medium which has been used with another culture (conditioned medium). The choice of

vessel depends on several factors: whether the culture is in suspension or grows as a monolayer; the cell yield; whether it needs CO_2 or not; and what form of sampling is to be taken place. Cost can also be a limiting factor. Cell yield is proportional to available surface areas. It is important to ensure that an even monolayer can grow, especially in the currently popular multi-well dishes (24, 48 and 96 wells).

For adherent cells to which histological stains may be applied, cover slips fitted into multi-well dishes which can be removed and treated with various organic solvent in staining are required. Commercially available multi-well slide-chamber dishes are also a convenient, but costly, alternative. Normal tissue cultureware is not resistant to organic solvents.

Washing up and sterilizing facilities

Availability of a wide range of plastic tissue culture reduces the amount of necessary washing up. However, glassware such as pipettes should be soaked in a suitable detergent, then passed through a stringent washing procedure with thorough soaking in distilled water prior to drying and sterilizing. Pipettes are often plugged with non-absorbent cotton wool before putting into containers for sterilizing.

Glassware, such as pipettes, conical flasks, beakers (covered with aluminum foil) are sterilized in a hot air oven at 160 °C for one hour. All other equipment, such as automatic pipette tips and bottles (lids loosely attached) are autoclaved at 121 °C for 20 min. Sterilizing indicators such as sterile test strip are necessary for each sterilizing batch to ensure that the machine is operating effectively. Autoclave bags are available for loose items. Aluminum foil also makes good packaging material.

Liquid N_2 / deep freezer

Invariably for continuous and finite cell lines, samples of cultures will need to be frozen down for storage. It is important to maintain continuity in cells to prevent genetic drift and to guard against loss of the cell line through contamination and other disasters. The procedure for freezing cells is general for all cells in culture. They should be frozen in exponential phase of growth with a suitable preservative, usually dimethylsulfoxide (DMSO). The cells are frozen slowly at 1 °C/min to -50 °C and then kept either at -196 °C immersed in liquid N_2 (in sealed glass ampoules) or above the liquid surface in the gas phase (screw top ampoules). Deterioration of frozen cells has been observed at -70 °C, therefore, -196 °C (liquid N_2) seems to be necessary.

To achieve slow freezing rates a programmable freezer or an adjustable neck plug or freezing tray for use in a narrow-necked liquid nitrogen freezer can be used. Alternatively, ampoules may be frozen in a polystyrene box with 1" thick walls. This will insulate the ampoules to slow the freezing process to 1 °C/min in a -70 °C freezer.

Water still or reverse osmosis apparatus

A double distilled or reverse osmosis water supply is essential for preparation of media, and rinsing glassware. The pH of the double distilled water should be regularly checked as in some cases this can vary. Variations in the quality of water used may account for variation in results, therefore water from one source should be used. Water is sterilized by autoclaving at 121 °C for 20 min. The distilled water must be glass distilled and stored in glass if it is to be used for the preparation of media. Storage in plastic may result in leaching of toxic substances from the plastic into the water.

Filter sterilization

Media that cannot be autoclaved must be sterilized through a 0.22 μm pore size membrane filter. These are obtainable in various designs to allow a wide range of volumes to be filtered (e.g., Millipore, Gelman). They can be purchased as sterile, disposable filters, or they may be sterilized by autoclaving in suitable filter holders.

Culture media, enzymes, hormones, cofactors and bicarbonate buffers are examples of non-autoclavable substances.

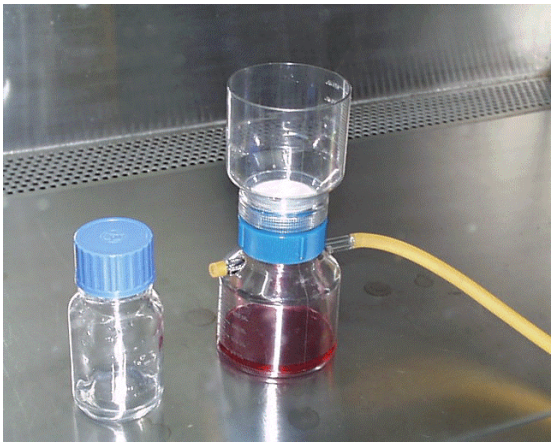

Figure 4. Media sterilization through a 0.22 μm membrane filter assembled in a filter holder.

Facilities for counting cells

It is possible to monitor cell growth by eyes (looking for confluency), however, more accurate cell counts are required for most experimental purposes.

The most commonly used device is the Improved Neubauer hemocytometer originally designed for counting blood cells. It consists of a thickened slide with a central chamber of known depth. A grid is etched out of the silvered chamber bottom. The counting chamber is prepared and loaded with a suspension of single cells for counting. It is important to aspirate the cell suspension adequately before loading the chamber in order to break up clumps of cells which are difficult to count accurately. The counting chamber is examined under a microscope using a x10 objective. The cells in the grid are counted. Since the cells are distributed randomly over the grid, then, in principle the more cells we count the more accurate our result will be.

An electronic particle counting method using a Coulter counter can also be used. In this method the cells are drawn through a small orifice which changes the current flow through the orifice thereby producing a series of pulses which are sorted and counted. The counter will need calibrating for the size of cell being used.

General small items of equipment

A number of small items of equipment are useful for performing cell culture. A water bath and a centrifuge with sealed buckets is also necessary. A vacuum pump is helpful to enable quick aspiration of the medium from cultures. The pump should be adequately protected from back movement of liquid.

Additional requirements include graduated pipettes of various sizes, centrifuge tubes and universal containers, disposable Pasteur pipettes, rubber bulbs for use with Pasteur pipettes. In addition, precisely calibrated automatic pipettes (small volumes of 1-1,000 μ l) such as Gilsons, Oxfords and Finnpipette are suitable for treating cultures with chemicals as they utilize disposible, autoclavable, plastic tips. For larger volumes, using glass or plastic pipettes, some form of suction aid such as 'Pipet-aid' or 'pi-pump' is necessary. More specialized equipment may be needed for particular experiments.

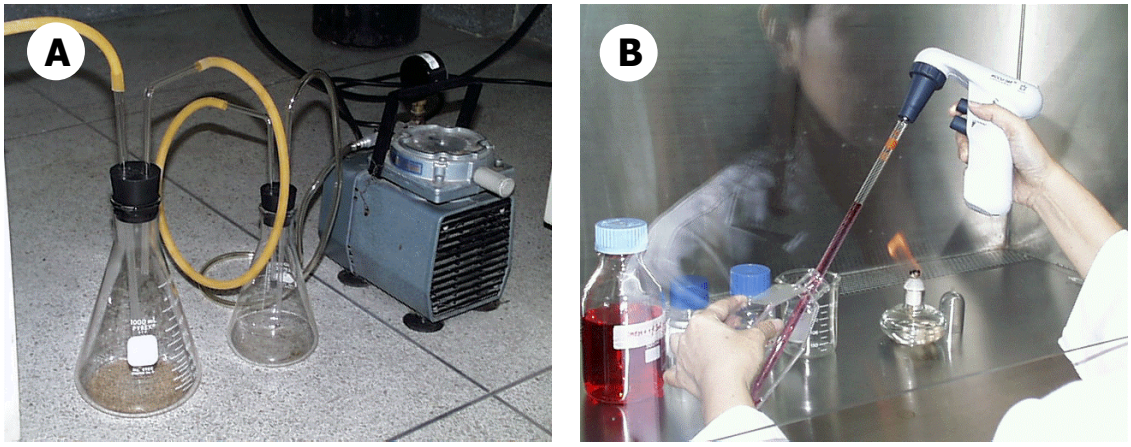

Figure 5: A: The vacuum pump with a 'trapping flask' protection. B: Glass or plastic pipettes with some form of suction aid are practical for transferring larger volumes.

Table 2. A variety of equipment used in animal cell culture

Essential equipment	Beneficial equipment	Useful additional equipment
Incubator	Laminar Flow Hood	Low-Temperature Freezer
Sterilizer	Cell Counter	Glassware Washing Machine
Microscope	Vacuum Pump	Closed-Circuit TV
Washing-up Equipment	CO_2 Incubator	Colony Counter
Sterilizing & Drying Oven	Preparation & Quality Control	Cell Sizing
Water Purification	Upright Microscope	Time-Lapse Cinemicrography
Centrifuge	Temperature Recording	Controlled-Rate Cooler
Cell Freezing	Bulk Culture	Centrifugal Elutriator
	Pipette Aids & Automatic Pipetting	Fluorescence-Activated Cell Sorter

Culture media

Today, with standardized media and sophisticated incubation conditions, culturing animal cells is considerably easier than it used to be. Since 1950s, tissue culture media were developed and conditions were worked out which closely simulate the situation *in vivo*. In particular, the environment is regulated with regard to the temperature, osmotic pressure, pH, essential metabolites (such as carbohydrates, amino acids, vitamins, proteins and peptides), inorganic ions, hormones and extracellular matrix. Among the biological fluids that proved successful for culturing cells, serum is the most significant. Five to twenty percents of serum is usually added to media for optimal cell growth. Serum is an extremely complicated mixture of compounds including undefined components, therefore much work has gone towards creating a chemically defined alternative to serum.

A wide variety of culture media is currently available. The choice of culture media is dependent on the requirements of cells. The components of suitable culture media include:

Basic media

The most basic media are balanced salt solutions (BSS), e.g., phosphate-buffered saline (PBS), which may be used for washing cells and for short incubations in suspension. More complex defined media are used for growth and maintenance. Defined media can also vary in complexity, by the addition of a number of constituents, e.g., from Eagle's minimum essential medium (MEM) which contains essential amino acids, vitamins and salts, to McCoy's medium, which contains a larger number of different amino acids, vitamins, minerals and other extra metabolites (such as nucleosides).

Buffering capacity

A number of supplements to the basic media are necessary to enable them to be used for culturing cells. Cel cultures have an optimum pH for growth, generally between pH 7.4-7.7. The type of buffering that is used for the media depends on the growth conditions. When cells are incubated in a CO_2 atmosphere an equilibrium is maintained between the medium and the gas phase. A bicarbonate–CO_2 buffering system is most often used due to its low toxicity towards the cells. HEPES, a much stronger buffer, may also be used, however, in this case much greater concentrations of HEPES than bicarbonate are required when used in a CO_2 atmosphere.

Each type of media has a recommended bicarbonate concentration and CO_2 tension to achieve the correct pH and osmolarity. Nevertheless, this may vary slightly among laboratories, therefore, a sample of media should be left under the normal incubating conditions and monitored overnight, the buffering can then be adjusted accordingly.

HEPES buffer should normally be used in conjunction with bicarbonate for which a relationship between the HEPES and bicarbonate exists for differing CO_2 levels, although, HEPES alone can maintain pH in the absence of exogenous CO_2. The addition of 1-5 mM pyruvate to the medium increases the endogenous cellular production of CO_2 and limits the need for a CO_2 atmosphere. Some defined media have been devised for this purpose, e.g., Leibovitz L-15 medium. Cells which produce large amounts of endogenous CO_2 under certain incubation conditions may require HEPES to buffer this CO_2 product.

The density of the culture may affect the CO_2 requirement, however, in general phenol red in the medium will indicate the state of the pH at any given time.

Glutamine and amino acids

In addition to buffering the medium, there are other growth requirements including amino acids, the requirement for which may vary with cell culture type. Commonly the necessary amino acids include cysteine and tyrosine, but some non-essential amino acids may be needed. Glutamine is also required by most cell lines and it has been suggested that cultured cells use glutamine as an energy and carbon source in preference to glucose, although glucose is present in most defined media. Glutamine is usually added at a final concentration of 2 mM, however, once added to the medium the glutamine is only stable for about 3 weeks at 4 °C.

Serum

Although there is much research aimed at attempting to reduce the requirement of cells for serum, by alternative supplementation of the media, it is apparent that most cell lines still require serum for adequate growth. Various sources of serum may be used such as calf, fetal calf, and horse. Many continuous cultures utilize calf serum, but often fetal calf serum provides the best growing conditions. The level of serum used depends on the particular cell line and should be determined empirically.

Batches of serum may vary considerably in their ability to support cellular growth. It is therefore important to test batches of serum and have sufficient quantities of a batch that shows suitable growth supporting characteristics stored at -20 °C. To check these

properties, cloning efficiency and growth characteristics (morphology, growth patterns) should be carried out over a concentration range of 2% to 20% serum. This wide concentration range will disclose if the alteration in the concentration of serum is possible to give optimal growth characteristics for a particular cell line.

Table 3. Some quality control tests for high-quality serum using in mammalian cell culture.

Test	Desired result
Sterility	No growth on a variety of media at room temperature or 37 °C
Mycoplasmas and viruses	Absent
Efficacy of cell growth	Compare cell growth in T-flask with control serum lot
Total protein content and immunoglobulin pattern	Same as content/pattern of control serum
pH value after filtration	7.2 - 7.8
Osmolality	290 - 310 mOsm/l
Hemoglobin content	Less than 20 mg%

Antibiotics and antimycotics

Unless good sterile conditions can be maintained (e.g., using laminar flow hoods) it is necessary to incorporate antibiotics and antimycotics into the media. A wide range of suitable preparations are available from relatively specific antibiotics, e.g., penicillin/streptomycin solutions, to broader spectrum antibacterial/antimycotic agents such as kanamycin or amphotericin B. The antibiotics chosen should clearly not to be toxic to the cells in culture and may depend on the type of contamination experienced in the individual laboratory.

Supply and preparation of culture media

The choice of culture media used will depend on the type of primary cell, cell line, and the incubation conditions. However, it is best to start with the medium recommended by the original supplier of the cells. Changing the medium necessitates the investigation on growth characteristics such as growth curves and cloning efficiency. To render a change in culture conditions from one medium to another it is advisable to 'condition' the cells, by increasing the ratio of new to old medium with successive passages.

Culture media have a limited storage life and the recommendations indicated by the supplier should be followed. Liquid defined media may have a storage life at 4 °C of up to one year, while glutamine lasts only 3 weeks at 4 °C. Serum lasts for about one year at -20 °C.

Culture media can be supplied in powdered form which requires dissolving and filter sterilizing, as a 10x concentrate liquid which requires dilution prior to use, or as a 1x liquid media. Media preparation, in all cases, requires high quality water.

Bottles of media should be prepared in small batches, for instance two weeks supply at a time. This will ensure that constituents such as glutamine do not have time to deteriorate and also that if contamination should occur it is confined to a few bottles.

Culturing animal cells

Selecting sources of tissue for culture

Adult or embryonic tissue

Cultures can be derived from adult tissue or from embryonic tissue. Cultures derived from embryonic tissue generally survive and grow better than those taken from adult tissue. Tissues from almost all parts of the embryo are easy to culture, whereas tissues from adult are often difficult or even impossible to culture. This presumably reflects the lower level of specialization and presence of replicating precursor or stem cells in the embryo. Adult tissues will usually have a lower growth fraction and a high proportion of non-replicating specialized cells, often within a more structured, and less readily disaggregated, extracellular matrix. Initiation and propagation are more difficult, and the lifespan of the culture is often shorter.

Embryonic or fetal tissue has many practical advantages, but it must always be remembered that in some instances the cells will be different from adult cells and it cannot be assumed that they will mature into adult-type cells unless this can be confirmed by appropriate characterization.

Examples of widely used embryonic cell lines are the various 3T3 lines (mouse embryo fibroblasts) and MRC-5 and other human fetal lung fibroblasts. Mesodermally derived cells (fibroblasts, endothelium, myoblasts) are also easier to culture than epithelium, neurons or endocrine tissue but this may reflect the extensive use of fibroblast cultures during the early years of the development of culture media together with the response of mesodermally derived cells to mitogenic factors present in serum. A number of new selective media have now been designed for epithelial and other cell types and with some of these it has been shown that serum is inhibitory to growth and may promote differentiation.

Embryonic stem cells

A more recent development has been the removal of embryonic stem cells (ES-cells) from the embryo during the blastocyst stage of development. These cells can be grown in culture for many generations and are of particular interest because they can be manipulated in culture and then re-introduced into embryos.

Normal or neoplastic tissue

Normal tissue usually gives rise to cultures with a finite lifespan while cultures from tumors can give continuous cell lines, although there are several examples of continuous cell lines (MDCK dog kidney, 3T3 fibroblasts) which are non-tomorigenic.

Normal cells will generally grow as an undifferentiated stem cell or precursor cell and the onset of differentiation is accompanied by a cessation in cell proliferation which may be permanent. Some normal cells, e.g., fibrocytes or endothelium, are able to differentiate and still dedifferentiate and resume proliferation and in turn redifferentiate, while others, e.g., squamous epithelium and many hemopoietic cells, once initiated into differentiation are incapable of resuming proliferation.

Cells cutured from neoplasms, however, can express at least partial differentiation, e.g., B16 mouse melanoma, while retaining the capacity to divide. Many studies of differentiation have taken advantage of this fact and used differentiated tumors such as the minimal deviation hepatomas of the rat and human and rodent neuroblastomas, although whether this can be taken as normal differentiation is always in doubt.

Tumor tissue can often be passaged in the syngeneic host, providing a cheap and simple method of producing large numbers of cells, albeit with lower purity. Where the natural host is not available, tumors can also be propagated in athymic mice with greater difficulty but similar advantages.

Many other differences between normal and neoplastic cells are similar to those between finite and continuous cell lines and indeed the importance of immortalization in neoplastic transformation has been recognized.

Selecting types of animal cell culture

Organ culture or cell culture

Early attempts at culturing tissues relied upon the explantation of whole tissue or organ which could be maintained *in vitro* for only very short periods. Nowadays it is more usual to grow specific cell types from tissues, although there are still some situations where it is necessary to grow a whole organ (or a part of it).

In adopting a particular type of culture the following points should be taken into account. Organ culture will preserve cell interaction, retain histological and biochemical differentiation for longer, and, after the initial trauma of explantation and some central necrosis will generally remain in a non-growing steady state for a period of several days and even weeks. They cannot be propagated, generally incur greater experimental variation between replicates, and tend to be more difficult to use for quantitative determinations due to minor variations in geometry and constitution.

Adherent or suspension culture

Cells may grow as an adherent monolayer or in suspension. Adherent cells are said to be anchorage-dependent and attachment to a substratum is a prerequisite for proliferation. They are generally subjected to contact inhibition, which means they grow as an adherent monolayer and stop dividing when they reach such a density that they touch each other. Most cells, with the exception of mature hemopoietic cells and transformed cells, grow in this way.

In contrast to anchorage-dependent cells, cells cultured from blood, spleen or bone marrow adhere poorly if at all to the culture dish. In the body, these cells are held in suspension or are only loosely adherent. It is important to realize this if you are working with this category of cells, since the methods used to propagate these cells are very different to those for adherent cells.

Suspension cultures are easier to propagate, since subculture only requires dilution with medium. Cultures in which cells grow attached to each other or to a substratum have to be treated by a proteolytic enzyme to break the bond between cells and substratum. The most commonly used enzyme is trypsin (other enzymes, e.g., collagenase, papain, dispase, and pronase, are also used). Clearly, freely suspended cultures do not require trypsinization. They are, therefore, also easier to harvest.

Primary cultures or continuous cell lines

If you remove tissue from an embryo, dissociate it into a suspension of single cells, and plate them out onto a culture dish, a series of characteristic events occurs. Firstly, cells are in a lag phase, usually no more than 1-2 days in length, during which there is little or no increase in cell number. During this time, cells are "conditioning" the medium, undergoing internal cytoskeletal and enzyme changes and adjusting to the new medium. Secondly, the cells undergo a period of rapid division, so-called log phase growth. Then, as they approach confluency and form contacts with one another, their rate of division slows and they begin to express a program of differentiation characteristic of their tissue of origin. Muscle cells fuse and acquire cross-striation, epithelial cells from the kidney or gut become linked by junctional complexes and transport ions from one surface to another, heart cells begin to beat spontaneously.

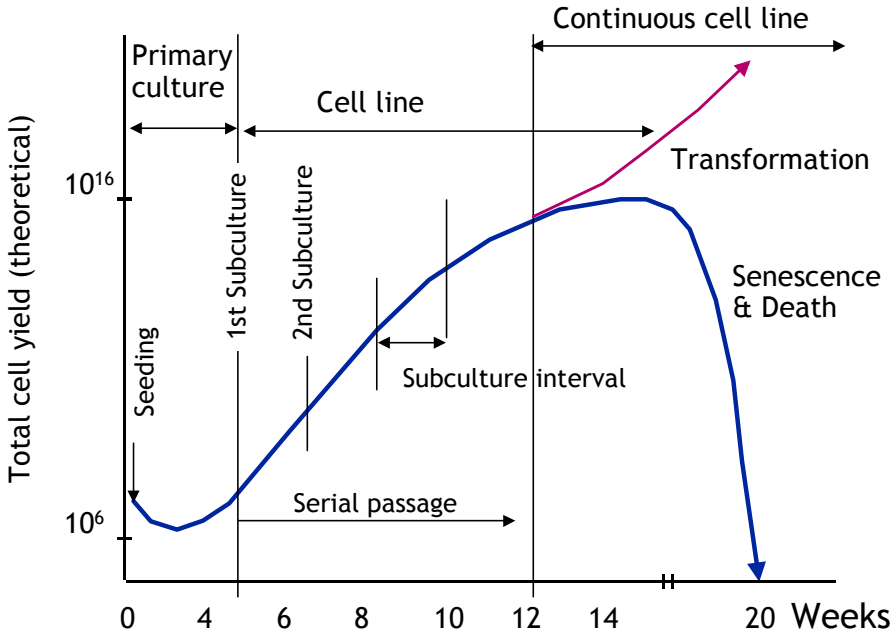

Figure 6. Growth of animal cells in culture

Cultures such as those just described are referred to as *primary cultures*, because they are prepared from cells taken directly from the animal. The cells divide or not (depending on what they are accustomed to), acquire differentiated characteristics, and ultimately die. For the next experiment, it's back to the animal again to obtain new tissue and prepare new culture.

Alternatively, in the case of cells that divide in culture, it is possible to 'passage' or 'subculture' them by inducing them to detach from the substrate, 'splitting' them (i.e., diluting them several-fold in medium and replating them into new dishes), and allowing them to reenter log phase growth. However, the properties of the cultured cells often change gradually with passaging, as more rapidly dividing cell populations come to predominate and more 'differentiated' cells, which divide more slowly, are lost.

When cells are repeatedly subcultured, most cease division after a finite number of generations, typically between 20 and 80. This is thought to reflect the same process of senescence that occurs in cells *in situ*. However, it is possible to develop populations of cells that can be passaged indefinitely and that express a reasonably stable phenotype. These are referred to as *established or continuous cell lines*. Some cell lines have arisen spontaneously in normal cells being passaged in culture, but the majority has been obtained by culturing tumor cells. In addition to their infinite life span (their 'immortality'), such cell lines frequently share several additional properties that distinguish them from 'normal' cells in culture. They divide more rapidly, they do not require attachment to the substratum for growth, and when reintroduced into animals, they form tumors. Cell lines with these properties are sometimes referred to as *transformed cell lines*.

Once a continuous cell line has been established, it is customary to clone the cells in order to obtain a genetically homogeneous population. Many separate clonal cell lines, each exhibiting a unique set of properties, can be obtained from a single tumor, or tumor cell line. For example, dozens have been derived from the original mouse neuroblastoma, glioma, and pheochromocytoma cells have been derived from neural tumors, some from tumors that arose spontaneously, others from tumors induced by chemical carcinogens.

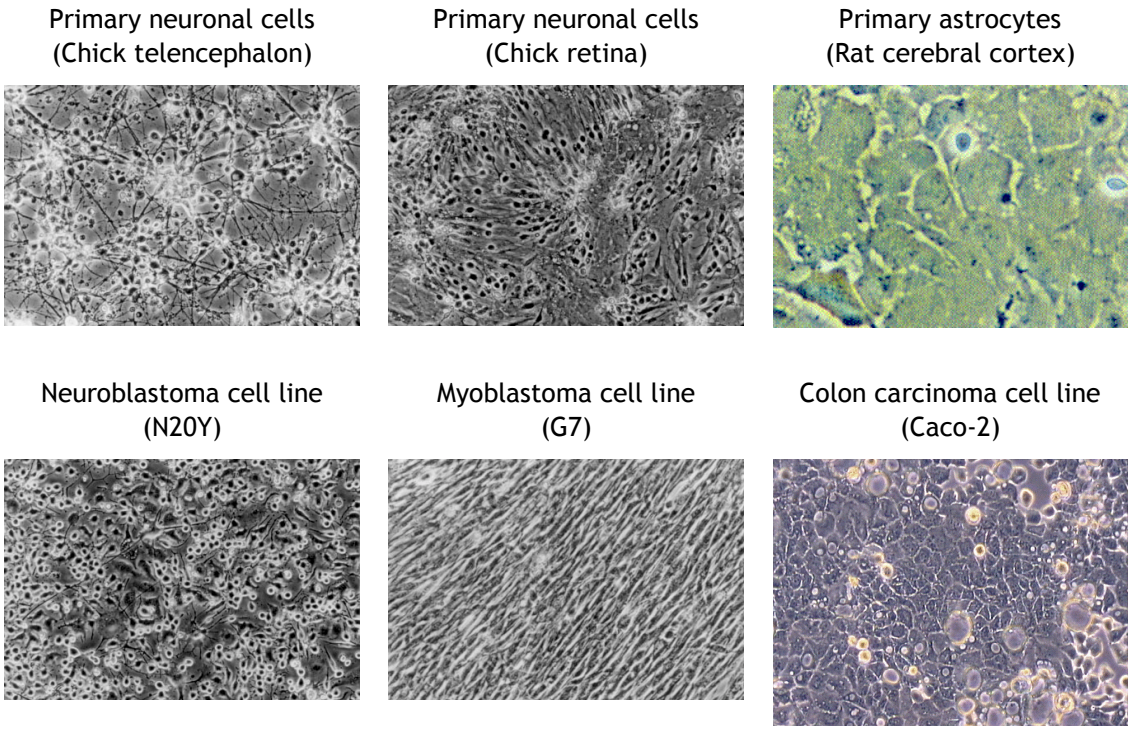

Figure 7. Examples of primary cultures and continuous cell line cultures

Primary cultures or clonal cell lines? This is the first and most important choice that must be made after deciding to culture cells. Since I am a pharmacologist who work with primary cultures, my bias is already apparent. But both approaches have advantages as well as drawbacks; both have their ardent advocates and their opponents.

Primary cultures: Advantages and limitations

As previously described, a primary culture is defined as one 'started from cells, tissues or organs taken directly from organisms'. The major advantages of primary cultures are the retention of : (1) the capacity for biotransformation; and (2) tissue-specific functions.

In many cases, the metabolic profile generated by a primary cell culture such as hepatocytes has greater similarity to *in vivo* than does the pattern seen with subcellular fractions used as an exogenous source for biotransformation. Hepatocytes possess the ability for conjugation reactions that may be absent in subcellular fractions. In fact, metabolic studies in hepatocytes have resulted in the identification of previously undetected *in vivo* products. Studies in hepatocytes lead to the discovery of ketoethinimate as a metabolite of ethinimate. Subsequent investigation revealed this metabolite was also formed *in vivo*.

The second advantage of primary cultures is the retention of tissue specific functions. For example, primary cultures of rat myocardial cells consisting of synchronously beating cells can be prepared. When these cultures were exposed to tricyclic antidepressants that are cardiotoxic, both arrhythmias and cessation of beating were observed.

One limitation of primary cultures is the necessity to isolate cells for each experiment. Procedures to isolate cells require the disruption of the tissue, often with proteolytic enzymes. This may result in the loss or damage of specific membrane receptors, damage to the integrity of the membrane, and loss of cellular products. During the interval necessary to establish monolayer cultures, damage is often repaired. An example of this phenomenon is the loss of Ca^{++} during the isolation of hepatocytes. There is a 60%

decrease in the concentration of Ca^{++} in freshly isolated cells; however, following 24 hours in culture, the level is restored to that of the intact liver.

Primary cultures have a limited life span and changes in metabolism and tissue specific functions will occur with time in culture. Many alterations can be postponed depending upon the culture conditions. It has been shown using hepatocytes that P-450 levels can be maintained with appropriate medium, and inducibility can be altered by the substrate upon which the cells are cultured.

In summary, primary cultures offer a cell system which can often provide a better approximation of the intact tissue than can cell lines. However, care must be taken to minimize the changes that occur with increasing time in culture.

Continuous (Clonal) cell lines: Advantages and limitations

The following important considerations should be taken into account before undertaking any experimental study with a cell line:

1. Many cell lines have been available for extended time periods, having undergone many *in vitro* passages. Before use, cultures should be carefully examined for homogeneity of cell type, and if necessary, must be re-cloned. Cell lines are usually more resistant to toxic insult than freshly isolated short-term cultures.
2. Investigators should be aware of population doubling time since this may play a role in sensitivity to the test agents (i.e., cells may have to undergo mitosis to reflect drug effect). Replication time may also affect the number of cells seeded in order to obtain a required density.
3. Type of cell and culture conditions used at the time of exposure to the test agent should best serve the particular assay at hand (i.e., suspension versus adherent cultures; sparsely seeded versus dense cultures). For certain experimental procedures, sparsely seeded cultures maintained over several days might be most suitable. Other experiments might be more informative when cells are in exponential growth or are confluent. Types of seeding must be kept constant for all experiments within a given study.
4. Information concerning normal or tumorigenic characteristics should be noted. This may also be reflected in chromosome number and ability to replicate in medium with low serum concentration. (Tumor cells can grow more readily in low serum medium.)
5. Some cell lines maintain or secrete tissue-specific proteins and enzymes which can be used for examination as biological markers. Specific biomarkers should be noted, such as special receptor sites or responses to specific growth factors.
6. If defined medium for a particular line is not available, serum concentrations should be kept as low as possible without affecting cell viability. Serum can adsorb xenobiotics, and thus reduce the concentration of test agent available.
7. Temperature effects may yield important information which can be obtained from cultures adapted to lower temperature or cell lines. For example, it may be more appropriate to expose keratinocyte cell lines, such as the XB-2 to the test agent at the lower temperatures found at the skin surface.
8. For assays involving test agents which are in need of metabolic activation to convert to a toxic intermediate, cell lines that are known to contain P-450 microsomal enzymes, or can be induced to make them, should be used. Alternatively, preparations of S-9 mixtures which contain the mixed functions oxygenase, can be added to the incubation medium.
9. Cell lines must be clean of contaminating mycoplasma, bacteria or viruses and sterile techniques must be strictly adhered to.
10. Frequent microscopic examination and monitoring of stock cultures is mandatory to detect changes in growth patterns and morphology.

11. It is essential that all experiments are repeated several times, using the same environmental conditions to determine variability.
12. Immortalized epithelial cells (nontumorigenic) derived from some organs, such as the lung and liver, are now available (due to transfection of viral components) for long term studies.
13. Stable lymphocytic lines (mostly of neoplastic origin) which maintain the functions of macrophages and other cells of the hematopoietic system are available. Similar stable cell lines maintaining the function of cells from other organ systems are not easily available, with some exceptions (for example, the PC12 neuroblastoma cell line).

Basic techniques in animal cell culture

Aseptic techniques

In culturing animal cells, it is essential that all procedures are carried out using aseptic or sterile techniques. As mentioned ealier, laminar flow facilities or sterile rooms provide a suitable environment, but even then aseptic techniques should be employed. If a non-sterile environment is used, the the reliance on aseptic techniques is very high. Basic aseptic technique is to ensure that the work area is clear, swabbed down regularly with 70% ethanol and that all the equipment used has been sterilized. Clean laboratory coats are also essential.

You have probably already some experience of aseptic techniques from culturing micro-organisms. Similar techniques may be used in culturing animal cells. However, it is not possible to describe in detail all aseptic techniques, these are better learnt by watching an experienced worker.

The basic rules for aseptic techniques which should be used even if an airflow cabinet is available include:

- If working on the bench, use a Bunsen flame to heat the air surrounding the Bunsen. This causes the movement of air and contaminants upwards and reduces the chance of contamination entering open vessels. Open all bottles and perform all maneuvers in this area only;
- Swab all bottle tops and necks with 70% ethanol to clean them before opening;
- Flame all bottle necks and pipettes by passing very quickly through the hottest part of the flame. This is not necessary with sterile, individually wrapped, plastic flasks and pipettes;
- Avoid placing caps and pipettes down on the bench; practice holding bottle tops with the little finger while holding the bottles for pouring or pipetting;
- Work either left to right or *vice versa*, so that all material to be used is on one side and, once finished, is placed on the other side of the Bunsen burner. (This may also stop the operator using the same reagent twice!);
- Manipulate bottles and flasks carefully. The tops of bottles and flasks must not be touched by the operator. Touching of open vessels should also be prevented when pouring. If necessary practice pouring from one container to another keeping a distance of 5 mm between the two vessels;
- Clear up spills immediately and always leave the work area clean and tidy. Dispose of glassware in appropriate bins and discard used plasticware in marked polythene bags for autoclaving or incineration. All glassware or plasticware used for infectious work must always be autoclaved before incineration. Re-usable glassware should be immersed in disinfectant whilst awaiting transfer to an autoclave.

The rules listed above are by no means exhaustive. The way in which they are implemented are, however, slightly different in different laboratories. Learning to apply these rules depends upon gaining 'hand on' experience within a laboratory. You should not attempt to carry out culture transfers without being shown how to do it properly by an experienced operator.

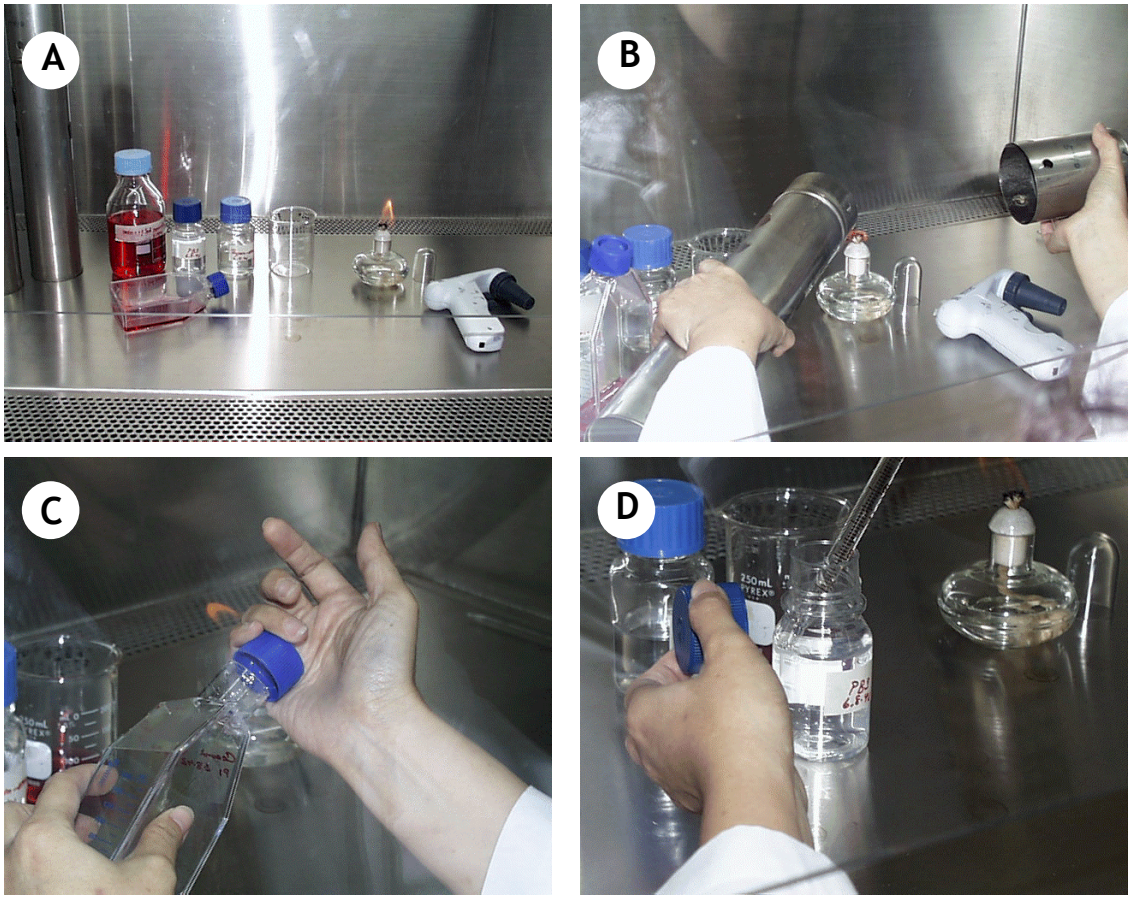

Figure 8. Basic aseptic techniques. A: Performing all maneuvers in a laminar flow hood. B: Using flames to fix microorganisms on container necks. C: Holding a bottle cap with the little finger. D: Avoid touching tops of open vessels while transferring their content.

Checking and prevention for contamination

Perhaps the biggest pre-occupation of the cell culture biologist is how to prevent contamination of the tissue cultures. The media used to grow the cells also provide excellent nutrition for unwanted organisms. Contamination of cell cultures by bacteria, fungi and mycoplasma often results in the loss of a great deal of time and money. An additional problem is the cross contamination of cells from other cell lines. Before we examine how to ensure contamination is kept at a minimum we need to briefly consider how we would detect contamination.

Regularly checking aliquots from culture flasks using a microscope can reveal some types of contamination, for example, yeast and fungi can be seen easily. Bacteria can be Gram stained or plated out on blood agar plates and general cloudiness of medium also indicates yeast or bacterial contamination. Mycoplasma are more insidious, the medium is not cloudy, the organisms cannot be seen under an ordinary microscope and most mycoplasma species are very difficult to grow on agar plates. Testing for mycoplasma is time consuming but must be done regularly because cross contamination is very easy.

DNA stains or molecular probes may be used to detect their presence in cells, those cells which turn out to contain DNA in the cytoplasm must be discarded.

Much of the contamination comes from the culture medium and its components or from inadequately cleaned glassware. Routine sterility checks on the medium, serum and nutrients are recommended. To do this, a small aliquot of the medium should be incubated at room temperature and another similar aliquot simultaneously incubated at 37 °C for up to one week before the medium is used for culturing cells. If these small samples are found to be contaminated, the medium should be autoclaved and discarded.

The use of antibiotics in the medium helps to contain the problem to some extent but it should never be seen as an alternative to good aseptic technique and careful monitoring. Generally, if contamination occurs, affected cultures should be disposed of into 2.5% hyperchlorite solution. Media bottles known, or suspected, to be contaminated should be disposed of as well. Some basic preventive measures may be taken: checking sterilizing procedures (autoclave and oven procedures); checking sterility of laminar flow hoods; regular checks on cultures; each worker having his or her own set of media; and disposal of contaminated cultures rather than attempting to decontaminate them.

Preparation of primary cultures

It is not always necessary to disaggregate tissue before culturing. Some embryonic tissues can be cultured simply by leaving the whole tissue on the flask surface and individual cells will simply grow out from the whole tissue and proliferate. After a few days the original tissue can be removed and the culture medium replenished to allow the new cells to continue growing. This method works for some tissues but it is not suitable for growing specific cells from a piece of tissue.

Tissues that do not require enzymatic disaggregation

Tissue for primary explant can be treated as follows:

- Excess blood is removed by rinsing with a sterile balanced salt solution (BSS). If the tissue is to be transported it can be kept in BSS or medium;
- Unwanted material such as fat and cartilage is cut off and the rest is chopped finely using a pair of crossed scalpels. It is important to make clean cuts, the use of tearing actions or scissors may damage the cells;
- The cell suspension is transferred to a sterile 50 ml centrifuge tube together with the buffered saline and the cells are allowed to settle out;
- The medium is carefully pipetted off and the pellet is washed in fresh BSS. The cells are allowed to settle again or the suspension is gently centrifuged at about 1,000 rpm for 5 min;
- The cell pellet is resuspended in 10-15 ml of medium and the suspension is aliquoted into 2 or 3 of 25 cm^2 flasks. If the pieces of tissue are still quite large the suspension may be passed through a sieve before culturing, the cultures are incubated at the appropriate temperature for 18-24 hours. (Note that in describing the cultivation of adherent cells, it is usual to describe the size of the vessels in terms of surface area rather than volume);
- If the pieces of tissue have adhered, the medium should be changed weekly until a significant outgrowth of cells has occurred;
- The original tissue pieces which show outgrowth can then be picked off and transferred to a fresh flask;
- The medium in the original flask is replaced and the cells cultivated until they cover at least 50% of the surface available for growth. They can then be sub-cultured if required.

All steps must, of course, be done in such a way as to reduce the chances of the tissue, media and vessels becoming contaminated. Thus all dissection tools, centrifuge tubes and reagents need to be sterilized before use.

This method is useful for small pieces of tissue such as skin biopsies. Fibroblasts, glial cells, epithelium and myoblasts all migrate out of the tissue very successfully. Some selection occurs because not all cells will adhere to the same extent and some cells rapidly outgrow the others.

Tissues that require enzymatic disaggregation

The tissue may be kept whole or may be cut into small pieces as described above. However, densely packed tissue is more difficult to digest and prolonged contact with digestive enzymes such as trypsin often causes destruction of viable cells. Large pieces of tissue for enzymatic disaggregation should be cut into smaller pieces as for the primary explant and digested with trypsin for about 30 min at 37 °C. Then the trypsin is neutralized by the addition of serum. The amount of trypsin that is required to achieve satisfactory release of cells is tissue-dependent and also depends upon the activity of the enzyme preparation. Trypsin suppliers often provide useful guidance for the use of their own preparations. Typically 0.25% w/v trypsin solution is satisfactory.

Trypsin is a proteolytic enzyme which hydrolyzes proteins. By adding a lot of protein (in the form of serum), the enzyme begins to hydrolyze these proteins rather than the proteins which bind cells together. What we are really doing is 'diverting' the enzyme to an alternative substrate so that it is no longer available to attack the extracellular matrix.

Thus once trypsinization is complete; medium containing serum should be added to neutralize enzyme activity. If trypsin is used to disaggregate whole tissue, the dissociated cells should be harvested after about 30 min and washed to remove trypsin. The remaining whole tissue may be retrypsinized. An alternative method is to soak the piece of tissue in cold trypsin at 4 °C overnight. The cold trypsin penetrates throughout the whole tissue but will have minimal activity at this temperature. The tissue can be disaggregated by increasing the temperature to 37 °C for 30-40 min, then enzyme activity is neutralized by the addition of medium with serum as before.

Collagenase and versene (phosphate buffered saline + EDTA) are also used to disaggregate tissues. Both are gentler in action than trypsin but expensive because larger amounts are required. Collagenase or versene can also be used together with trypsin to allow trypsin to be used at lower concentrations.

A variety of other hydrolytic enzymes (for example pronase and dispase) have also been employed to disaggregate tissues in some laboratories.

Once disaggregation is complete, the cells can be washed in fresh BSS or medium and seeded out in 24 cm^2 flasks. The cell layer can be supplied with fresh medium after 48 hours. This culture is referred to as a primary culture.

Removing non-viable cells from the primary culture

If the primary culture is of anchorage-dependent or adherent cells, the non-viable cells can be removed by pouring off the medium and rinsing the cell layer with buffer before adding fresh medium. Cells which are not viable will not be able to adhere to the substratum.

If the cells are to be cultured in suspension, the non-viable cells gradually become diluted as the viable cells proliferate. If it is necessary to remove the non-viable cells from the suspension, the cells may be layered onto Ficoll or 'lymphoprep' and be centrifuged at 2,000 rpm for 15-20 min. The non-viable cells will sink to the bottom and the viable cells can be collected from the medium-Ficoll interface.

Maintaining the culture

If a primary culture is not to be used as such, it may be sub-cultured to produce a cell line. Cell lines may be of a very limited lifespan or they may be passaged several times before the cells become senescent. Some cells, such as macrophages and neurons do not divide *in vitro* and can only be used as primary cultures.

Sub-culturing from primary to secondary cell culture

A primary culture contains a very heterogeneous population of cells from the original explant. Some of these cells will die, some will fail to grow, others will grow quickly and become the dominant cell type present. On sub-culturing the primary cell culture, the dominant types will become even more dominant. We can, therefore, foresee that sub-culturing enables us to produce more homogeneous cell populations.

We call the culture produced after sub-culture of a primary culture, a secondary culture. Sub-culturing, therefore, enables us to produce cell lines from our original explant. These cell lines may be further sub-cultured, characterized and cloned.

Producing a cell line has certain obvious advantages. A homogeneous population of characterized cells can be grown to a large scale, replicates are uniform and this makes designing experiments much easier. There are, however, disadvantages. In establishing a cell line only those cells best suited to the *in vitro* conditions are selected for. These cells may lose some of the differentiated characteristics they had while growing *in vivo* and are prone to genetic instability, particularly if they divide rapidly.

Producing cell lines of a particular cell type

We really have only two main options here. Firstly, of course, we must choose the appropriate tissue in the primary explant stage. It is no good attempting to produce a particular epithelial cell line if we use tissues which do not contain the appropriate cell type. Secondly, not all cells will grow equally well in the same medium. So, in principle, by selecting our medium carefully we may provide the condition most suited to our cells of interest.

Propagating a cell line

Once a cell line is established, it needs to be propagated in order to produce sufficient cells for characterization and storage, as well as for particular experiments. A cell line is given a name or code which identifies its source (for example HuT, Human T cells) and, if more than one line was developed from the same source, a cell line number is also given (for example HuT 78). If cells from this line are cloned, then a clone number need to be given, e.g., HuT 78 clone 6D5.

If the cell line is likely to be viable for only a few sub-culturings or generations each generation should be noted, for example HuT 78 clone 6D5/2 or HuT 78 clone 6D5/3.

Once a culture is confluent (i.e., the cells cover 60-70% of the growth surface available) it can be transferred into a holding or maintenance medium which provides just enough nutrients to keep the cells alive and healthy but reduces the replication rate so that the cells do not overgrow. The usual method is to use the same basic medium and reduce the amount of serum it contains.

If cells are required for experiments or storage the contents of the flask can be split or divided to seed 2-4 new flasks depending on the vigor of the cell growth.

The content of a flask of cells grown in suspension is very simple to split. This is done in the following way:

- Stand the flask upright for 1-5 min to allow cells to settle;

- Aseptically remove as much medium as possible without disturbing the cells. A 10 ml graduated pipette is best. Discard used medium into a waste pot for autoclaving;
- Resuspend cells in the remaining volume, measure and divide between the required number of flasks;
- Top up each flask with fresh growth medium and incubate as normal;
- Record the cell line code, clone number and the passage number.

Cells which grow as monolayer adhering to the flask surface are a little more difficult to split. The steps are:

- Remove medium by pouring it off aseptically;
- Rinse the monolayer with pre-warmed (37 °C) phosphate buffered saline (PBS) or BSS containing 1 mM EDTA; pour the medium off into discard pot;
- Add 10 ml of a 0.25% (w/v) solution of trypsin in saline. Tilt the flask so that all the cells are covered with trypsin. Other enzymes may also be used in the same way;
- Pour off the trypsin and incubate the flask at 37 °C for 1-10 min. Cells which are very susceptible to trypsin should be checked after 1 min;
- Once the cells have begun to round up and are sliding off the flask surface (it may help to tip the side of the flask gently), the trypsin must be neutralized by the addition of 5-10 ml of medium containing serum;
- Wash all the cells down from the sides of the flask and aspirate gently using a pipette. This should break up any aggregates and allow for easier cell counting if required. Avoid creating froth as this can lead to contamination and cell damage;
- A small aliquot (0.2 ml) of the cell suspension may be removed for counting if required. Otherwise, measure the total volume of the cell suspension and divide between the required number of flasks and top up with fresh medium and incubate as before;
- Remember to record the cell line code, clone number and the number of times it has been divided (the passage number).

The intervals between sub-culturing or changing medium will depend on the cell line and the rate at which it grows. Rapidly dividing cell lines such as HeLa and VERO need to be sub-cultured at least once a week with a medium change in between. Fibroblast HEL cells may be sub-cultured once every 10-14 days. The rate of growth of fibroblasts may be increased, if more frequent sub-culturing is required, by increasing the serum concentration in the medium.

Quantitation of cells in cell culture

For properly run experiments, it may be necessary to count the cell numbers before, after and even during the experiment. Day to day maintenance of cell lines also requires quantitative assessment of cell growth so that optimum cell densities for sub-culturing and storing can be determined.

We can divide the methods available for determining cell growth into two sub-groups. These are:

- Direct methods;
- Indirect methods.

In the direct method, cell numbers are determined directly either by counting using a counting chamber or by using an electronic particle counter. In the indirect methods

measurement of some parameters such as DNA content or protein content related to cell number is used as the method of estimating biomass.

Direct methods for quantitation of cells in culture

Counting chambers

A hemocytometer (Improved Neubaur) is the simplest and cheapest method of counting. A coverslip slightly wet at the edges is placed over the counting grid such that interference patterns ('Newtons rings') formed when glass attaches to glass indicate that it is securely in place. Introduce the medium containing cells under the coverslip from a Pasteur pipette by capillary action. Fluid will run into the grooves of the chamber to take away the excess. The cells in the central grid area can then be counted. The hemocytometer is designed such that when properly set up an area of 1 mm^2 and 0.1 mm deep is filled – this is a central area of 25 smaller squares bounded by triple parallel lines. Each of the 25 squares is further subdivided into 16 to aid counting. For routine subculture about 100-300 cells should be counted, but 500-1,000 for more precise counts. For the Improved Neubaur chamber, the cell concentration, calculated from the number of cells (n) in the central 25 square area (1 mm^2, depth 0.1 mm), is $n \times 10^4$ cells/ml. Viability determinations can be easily carried out after diluting cells with dye. This counting method is open to error in a number of ways. Errors may be introduced by incorrect preparation of the chamber, incorrect sampling of cells, and aggregation of cells. However, it has the advantage of allowing visual inspection of the morphology of the cells.

Coulter counters

Electronic particle counters consist of two electrodes separated by a small orifice. If a potential is applied to the electrodes, current will pass between them through the buffer in the orifice. The amount of current will be dependent upon the conductance (dielectric constant) of the buffer. As the particle enters the orifice, the conductance of the solution between electrodes would be reduced. Thus the current flowing would be reduced and this could be detected electronically.

The size of the change in current flow depends on the size of the particle and the difference in the dielectric constant (conductivity) of the particle and the suspending buffer. This is the principle upon which cell counting using a Coulter counter is carried out.

Cells in suspension are drawn through a fine orifice in the Coulter counter and, as each cell passes through, it produces a change in the current flowing across the orifice. Each change is recorded as a pulse and it is these pulses that are sorted and counted. The size of the pulse is proportional to the volume of the particle passing through, so signals of varying size are produced. The pulse height threshold can be set, therefore, to eliminate electronic noise and weak pulses produced by debris.

Electronic counters, of which the Coulter counter is the most widely used, provide rapid results, but high cell numbers are required to give an accurate count. Another disadvantage is that they cannot distinguish between dead and viable cells and clumps of cells may register as a single pulse thus leading to inaccurate counts.

Indirect methods for determining cells in culture

Other methods of quantitation such as radioisotope labelling and estimation of total DNA or protein are used less frequently. They are useful when cells are grown in microwell plates or as hanging drop cultures. The cells can be mixed *in situ*, stained and counted by eye under a microscope. DNA and protein assays are inaccurate, particularly if cells are multinucleated. They do not distinguish viable and non-viable cells.

Cell viability determination

When cells are freshly isolated from a tissue or confluent monolayers are subcultured, the proportion of living, or viable, cells should be determined before they are used. This is most often determined by assessment of membrane permeability, under the assumption that a cell with a permeable membrane has suffered severe, irreversible damage.

Trypan blue exclusion

This is a rapid test for gross damage which is conveniently combined with determining cell number.

- Mix a small aliquot of cell suspension with an equal volume of 0.4% trypan blue solution.
- Then, within 1-5 min, introduce the suspension into a hemocytometer chamber.
- Non-viable cells appear blue, with the nucleus staining particularly darker.
- Count the viable (unstained) cells and the total number of cells.
- Express the number of viable cells as a percentage of the total. Calculate the cell number after multiplying by 2 to allow for the dilution with dye.

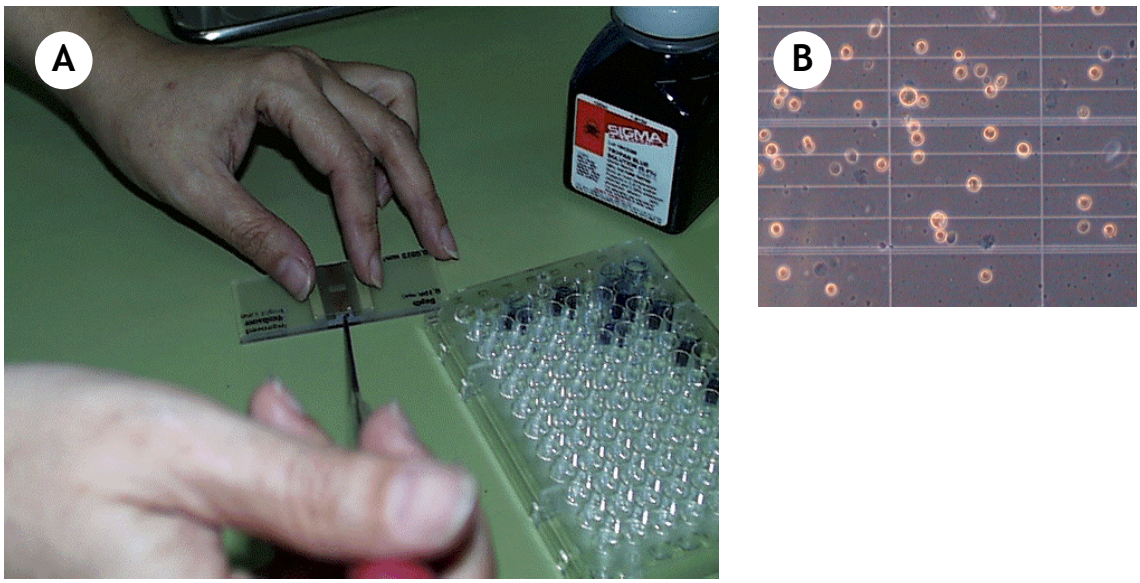

Figure 9. Quantitation of cell viability by trypan blue exclusion. A: Filling the counting chamber with a trypan blue-stained cell suspension. B: Counting viable and non-viable cells under a 100x microscopic field.

LDH leakage

Leakage of cytosolic enzymes such as lactate dehydrogenase (LDH) provides a test that is similar in sensitivity to dye exclusion and can be more accurately quantitated but takes rather longer to perform.

- Centrifuge an aliquot of cell suspension at a speed suitable for sedimenting all the cells without causing cell damage (e.g., 50 x g for 2 min)
- Transfer the supernatant medium to a clean tube and keep on ice
- Add an equal volume of solubilizing agent to the cell pellet and vortex mix. Samples should be kept on ice and the assays performed on the same day.

- To a cuvette, add 3 ml of substrate at 37 °C, 50 µl of NADH and 25-200 µl of sample (depending on the cell type).
- Mix rapidly and follow the increase in absorbance at 340 nm at 37 °C.
- Calculate the initial change in absorbance per min for the medium and for the solubilized cells and express the activity of the medium as a percentage of the total (i.e., to give percent leakage).

Table 5. Reagents and equipment for determining of LDH leakage

1. Solubilizing agent
   0.9% NaCl
   0.1% Triton X-100
   0.1% Bovine serum albumin
2. Substrate
   3.5 g K_2HPO_4
   0.45 g KH_2PO_4
   31.0 mg sodium pyruvate
   in 450 ml distilled water (must be stored at -20 °C)
3. NADH
   42 mg in 4.5 ml 1% $NaHCO_3$, freshly prepared.
4. Recording spectrophotometer with cell thermostat equilibrated to 37 °C.

Cell Morphometry

Specialized morphological characteristics of cultured cells can be determined and analyzed by using computerized image analysis, e.g., neurite length and soma size of cultured neurons.

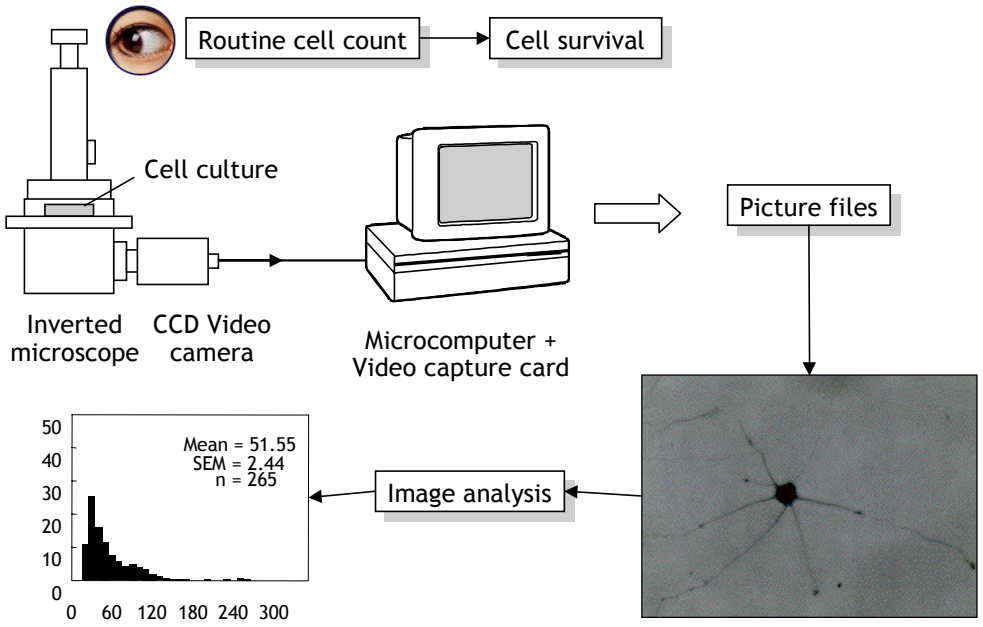

Figure 10. Morphometric analysis of cultured hippocampal neurons.

Illustrated examples of cell culture preparation

As I have mentioned that I use primary cultures in the neuropharmacological research. Therefore, I would like to show diagrammatic illustrations of how to prepare primary neuronal cell cultures. These methods have been adopted in my research for many years and they seem to be reproducible. The only reason for providing these examples is to give you a conceptual framework for the preparation of Caco-2 cell culture in a laboratory session.

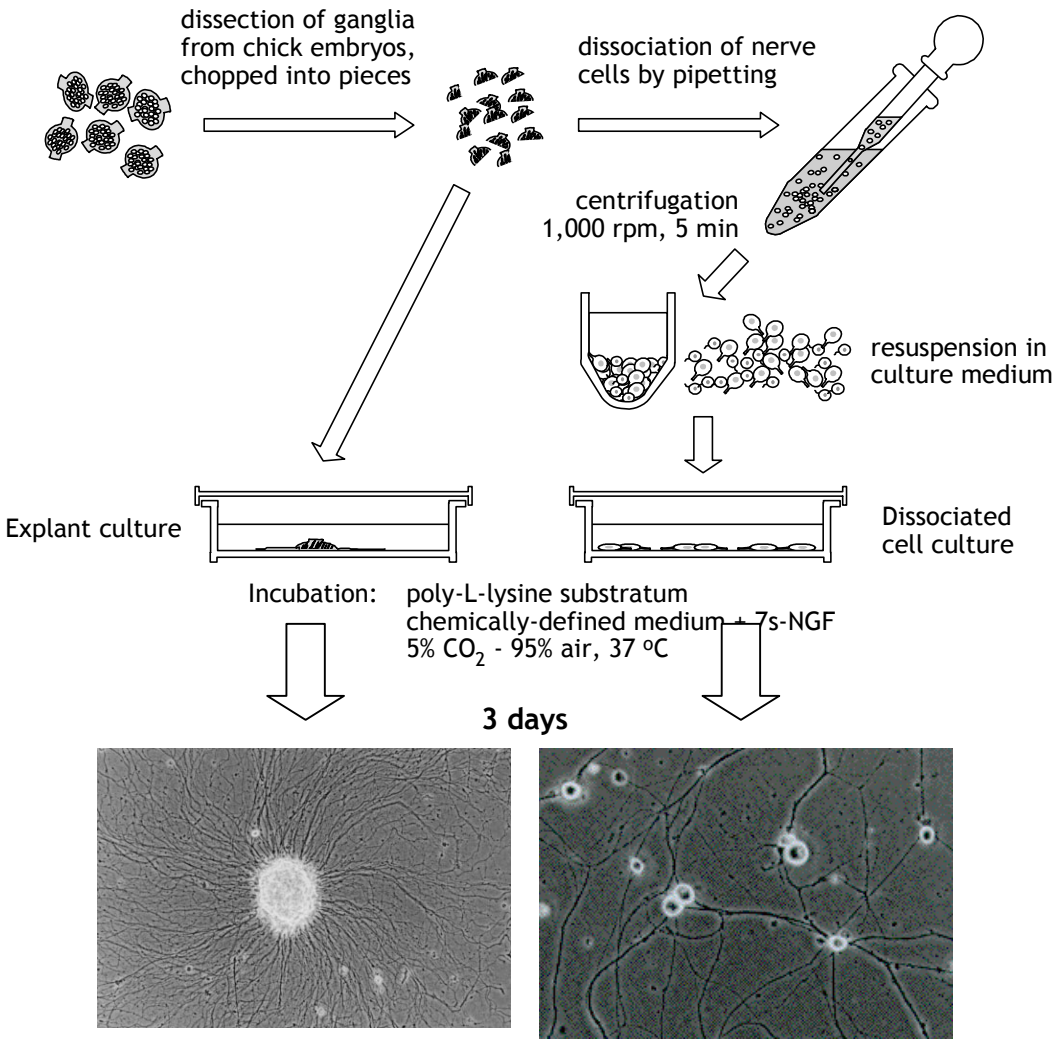

Figure 11. Simplified method for the preparation of chick embryonic dorsal root ganglion (DRG) cultures

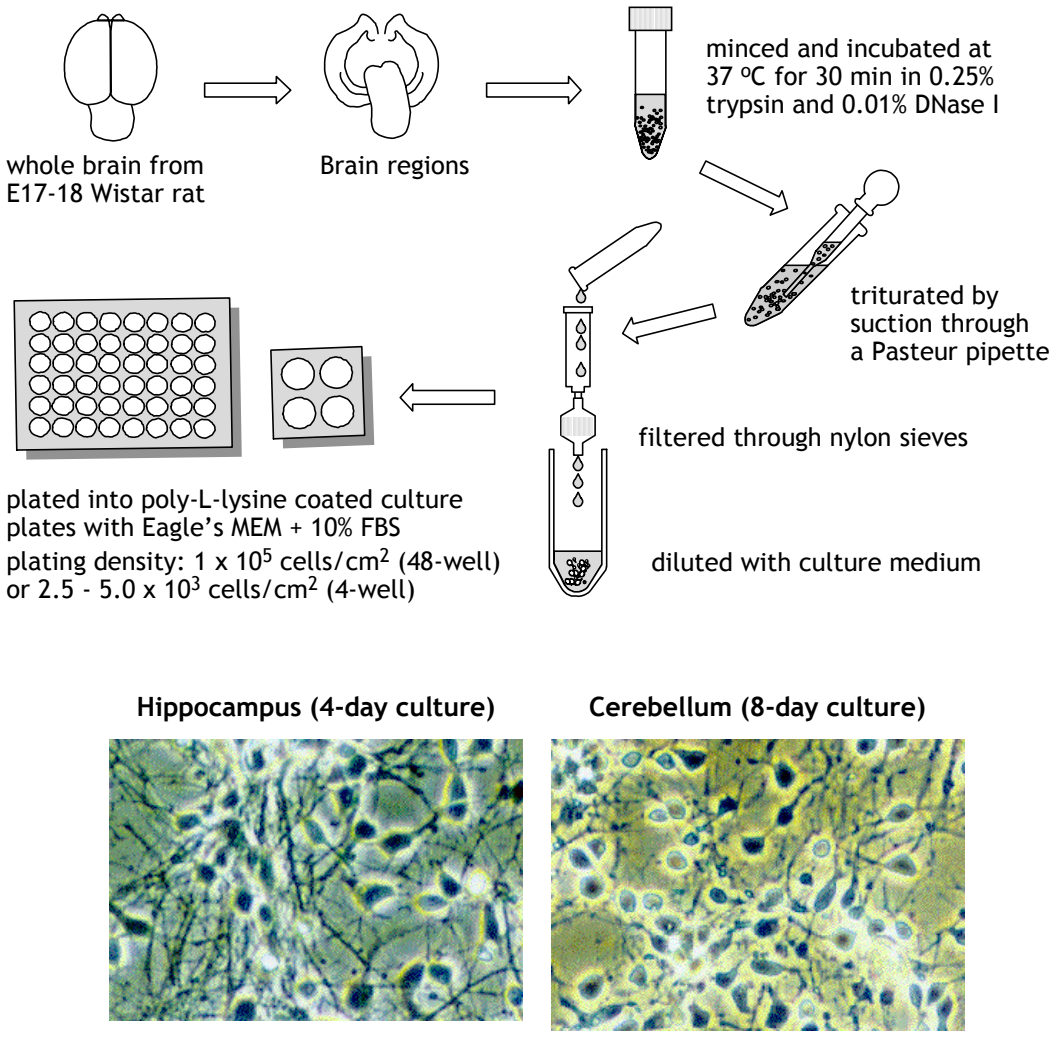

Figure 12. Preparation of neuronal cell cultures from the embryonic rat brain.

Specific example of cell culture application in pharmaceutical research

The main reason why you are here is to learn the basic background and technique of using Caco-2 cell culture as an *in vitro* model for studying intestinal drug transport and metabolism. The following figure summarizes what you will encounter in the next 2 days.

Cell Culture Models for Intestinal Transport & Metabolism	
Caco-2:	Human intestinal epithelial cell line (colon carcinoma)
T84:	Human intestinal epithelial cell line (colon adenocarcinoma)
IEC-6:	Rat small intestinal crypt cell line (normal intestine)
Important characteristics: **Monolayer, Tight junction and Polarity**	

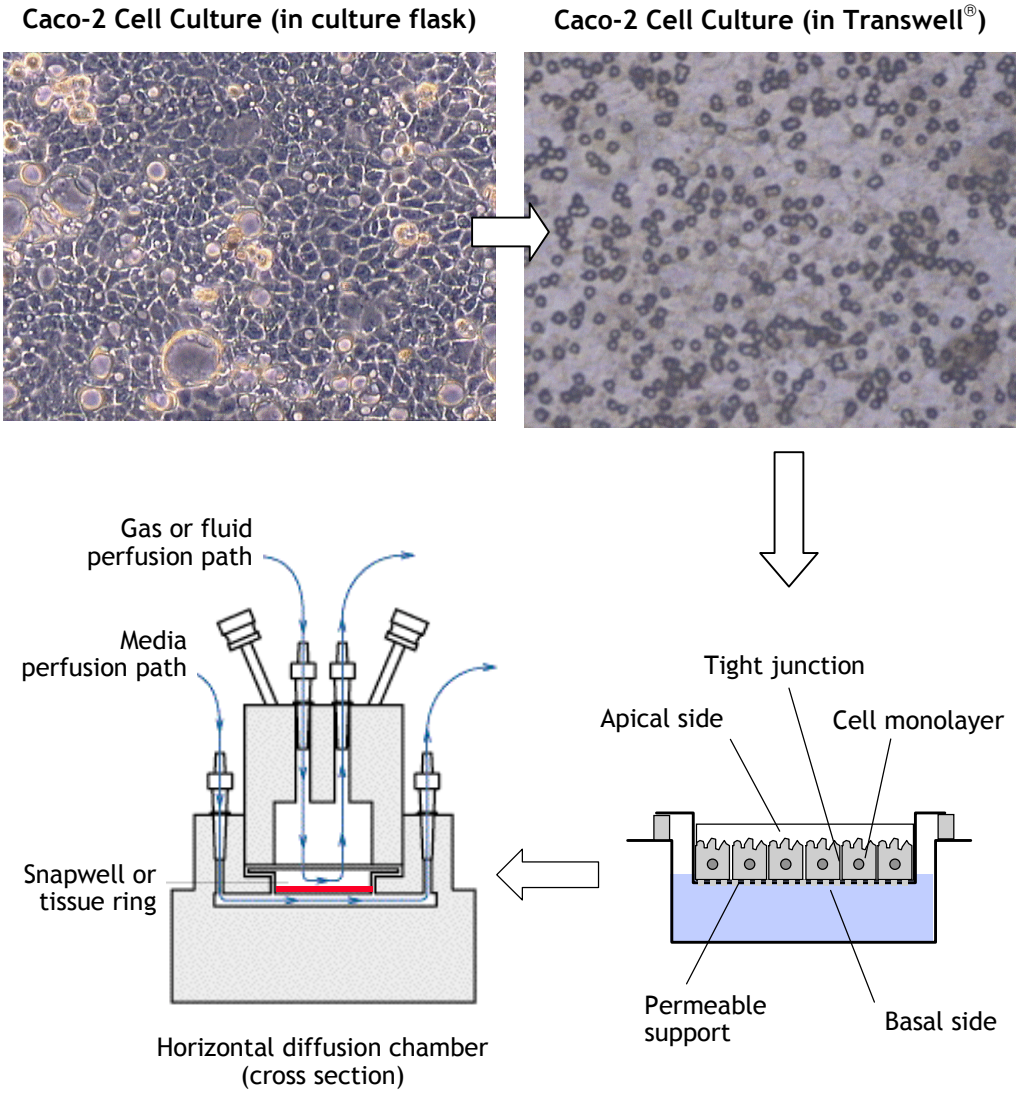

Figure 13. Using Caco-2 cell culture as an *in vitro* model for the study of intestinal drug transport/metabolism.

Conclusion

In recent years the use of animal cell culture has undergone a major expansion from being a purely experimental procedure to become an accepted technological component of many aspects of biological research. This chapter summarizes theoretical background and basic techniques of culturing animal cells in a format that is readily accessible to all researchers in the field. Thereafter, the Caco-2 cell culture laboratory session will be a practical exercise for the newcomers to 'touch and feel' with these techniques.

Bibliography

Benford, D.J. and Hubbard, S.A. (1987) Preparation and culture of mammalian cells, in *Biochemical Toxicology: A Practical Approach* (Snell, K. and Mullock, B. eds) pp 57-82, IRL Press, Oxford.

Butler, M. (1991) *Mammalian Cell Biotechnology: A Practical Approach*. Oxford University Press, Oxford.

Conn, P.M. (1990) *Methods in Neuroscience Volume 2: Cell Culture*. Academic Press, California.

Darling, D.C. and Morgan, S.J. (1994) *Animal Cells: Culture and Media*. John Wiley & Sons, New York.

Doyle, A., Griffiths, J.B. and Newell, D.G. (1993) *Cell and Tissue Culture: Laboratory Procedures*. John Wiley & Sons, Chichester.

Freshney, R.I. (1992) *Animal Cell Culture: A Practical Approach*. 2nd edition, Oxford University Press, Oxford.

Freshney, R.I. (1994) *Culturing of Animal Cells: A Manual of Basic Technique*. 3rd edition, Wiley-Liss, Inc., New York.

Pollard, J.W. and Walker, J.M. (1990*) Methods in Molecular Biology Volume 5: Animal Cell Culture*. Humana Press, New Jersey.