2 Cell culture techniques

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2.1 INTRODUCTION

Cell culture is a technique that involves the isolation and maintenance *in vitro* of cells isolated from tissues or whole organs derived from animals, microbes or plants. In general, animal cells have more complex nutritional requirements and usually need more stringent conditions for growth and maintenance. By comparison, microbes and plants require less rigorous conditions and grow effectively with the minimum of needs. Regardless of the source of material used, practical cell culture is governed by the same general principles, requiring a sterile pure culture of cells, the need to adopt appropriate aseptic techniques and the utilisation of suitable conditions for optimal viable growth of cells.

Once established, cells in culture can be exploited in many different ways. For instance, they are ideal for studying intracellular processes including protein synthesis, signal transduction mechanisms and drug metabolism. They have also been widely used to understand the mechanisms of drug actions, cell–cell interaction and genetics. Additionally, cell culture technology has been adopted in medicine, where genetic abnormalities can be determined by chromosomal analysis of cells derived, for example, from expectant mothers. Similarly, viral infections can be assayed both qualitatively and quantitatively on isolated cells in culture. In industry, cultured cells are used routinely to test both the pharmacological and toxicological effects of pharmaceutical compounds. This technology thus provides a valuable tool to scientists, offering a user-friendly system that is relatively cheap to run and the

exploitation of which avoids the legal, moral and ethical questions generally associated with animal experimentation. More importantly, cell culture also presents a tremendous potential for future exploitation in disease treatment, where, for instance, defective or malfunctioning genes could be corrected in the host's own cells and transplanted back into the host to treat a disease. Furthermore, successful development of culture techniques for stem cells will provide a much needed cell-based strategy for treating diseases where organ transplant is currently the only available option.

In this chapter, fundamental information required for standard cell culture, together with a series of principles and outline protocols used routinely in growing animal and bacterial cells are discussed. Additionally, a section has been dedicated to human embryonic stem cell culture, an emerging field where protocols to be used routinely are still being developed. The discussion in this chapter is thus limited to techniques that are now becoming routine for stem cell culture and should therefore provide the basic knowledge for those new to the field of cell culture and act as a revision aid for those with limited experience in the field. Throughout the chapter, particular attention is paid to the importance of the work environment, outlining safety considerations together with adequate description and hints on the essential techniques required for tissue culture work.

2.2 THE CELL CULTURE LABORATORY AND EQUIPMENT

2.2.1 The cell culture laboratory

The design and maintenance of the cell culture laboratory is perhaps the most important aspect of cell culture, since a sterile surrounding is critical for handling of cells and culture media, which should be free from contaminating microorganisms. Such organisms, if left unchecked, would outgrow the cells being cultured, eventually resulting in culture-cell demise owing to the release of toxins and/or depletion of nutrient from the culture medium.

Where possible, a cell culture laboratory should be designed in such a way that it facilitates preparation of media and allows for the isolation, examination, evaluation and maintenance of cultures under controlled sterile conditions. In an ideal situation, there should be a room dedicated to each of the above tasks. However, many cell culture facilities, especially in academia, form part of an open-plan laboratory and as such are limited in space. It is not unusual therefore to find an open-plan area where places are designated for each of the above functions. This is not a serious problem as long as a few basic guidelines are adopted. For instance, good aseptic techniques (discussed below) should be used at all times. There should also be adequate facilities for media preparation and sterilisation, and all cell culture materials should be maintained under sterile conditions until used. In addition, all surfaces within the culture area should be non-porous to prevent adsorption of media and other materials that may provide a good breeding ground for microorganisms, resulting in the infection of the cultures. Surfaces should also be easy to clean and all waste

generated should be disposed of immediately. The disposal procedure may require prior autoclaving of the waste, which can be carried out using pressurised steam at 121 °C under 105 kPa for a defined period of time. These conditions are required to destroy microorganisms.

For smooth running of the facilities, daily checks should be made of the temperature in incubators, and of the gas supply to the incubators by checking the CO_2 cylinder pressure. Water baths should be kept clean at all times and areas under the work surfaces of the flow cabinets cleaned of any spills.

2.2.2 Equipment for cell culture

Several pieces of equipment are essential. These include a tissue culture hood, incubator(s), autoclave and microscope. A brief description will be given of these and other essential equipments.

Cell culture hoods

The cell culture hood is the central piece of equipment where all the cell handling is carried out and is designed not only to protect the cultures from the operator but in some cases to protect the operator from the cultures. These hoods are generally referred to as laminar flow hoods as they generate a smooth uninterrupted streamlined flow (laminar flow) of sterile air which has been filtered through a **high-efficiency particulate air** (HEPA) filter. There are two types of laminar flow hood classified as either **vertical** or **horizontal**. The horizontal hoods allow air to flow directly at the operator and as a result are generally used for media preparation or when one is working with non-infectious materials, including those derived from plants. The vertical hoods (also known as **biology safety cabinets**) are best for working with hazardous organisms, since air within the hood is filtered before it passes into the surrounding environment.

Currently, there are at least three different classes of hood used which all offer various levels of protection to the cultures, the operator or both and these are described below.

Class I hoods

These hoods, as with the class II type, have a screen at the front that provides a barrier between the operator and the cells but yet allows access into the hood through an opening at the bottom of the screen (Fig. 2.1). This barrier prevents too much turbulence to air flow from the outside and, more importantly, provides good protection for the operator. Cultures are also protected but to a lesser extent when compared to the class II hoods as the air drawn in from the outside is sucked through the inner cabinet to the top of the hood. These hoods are suitable for use with low-risk organisms and when operator protection only is required.

Class II hoods

Class II hoods are the most common units found in tissue culture laboratories. These hoods offer good protection to both the operator and the cell culture. Unlike class I hoods, air drawn from the outside is passed through the grill in the front of the work area and filtered through the HEPA filter at the top of the hood before streaming down

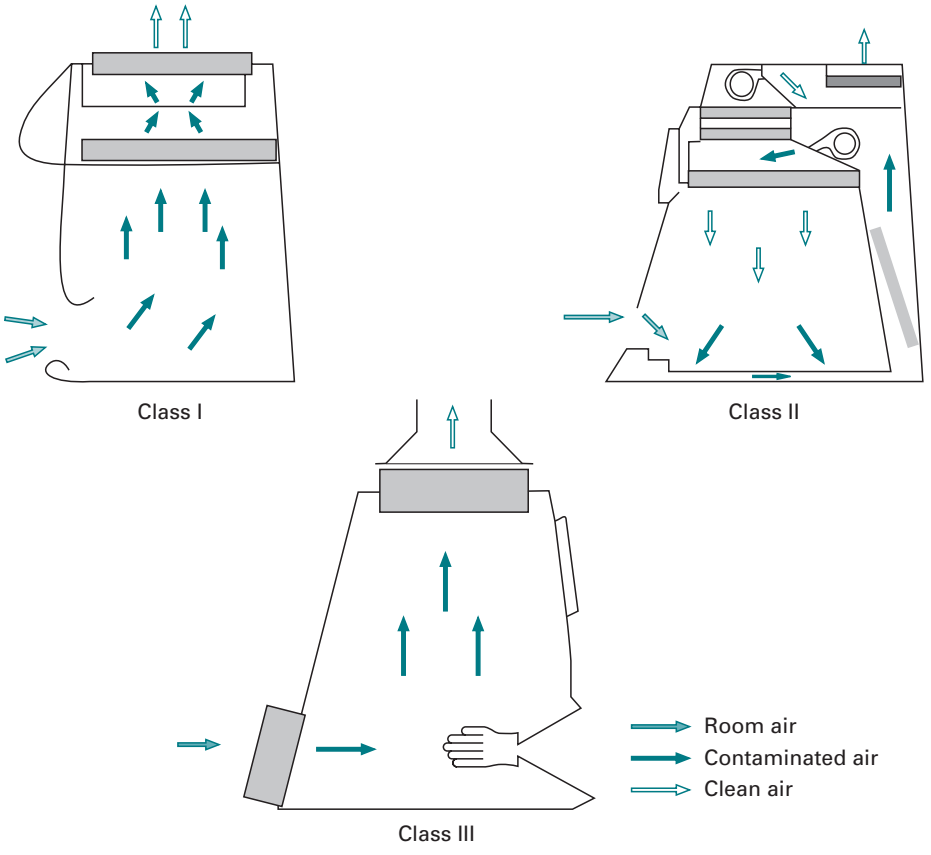

Fig. 2.1 Schematic representation of tissue culture cabinets.

over the tissue culture (Fig. 2.1). This mechanism protects the operator and ensures that the air over the cultures is largely sterile. These hoods are adequate for animal cell culture, which involves low to moderate toxic or infectious agents, but are not suitable for use with high-risk pathogens, which may require a higher level of containment.

Class III hoods

Class III safety cabinets are required when the highest levels of operator and product protection are required. These hoods are completely sealed, providing two glove pockets through which the operator can work with material inside the cabinet (Fig. 2.1). Thus the operator is completely shielded, making class III hoods suitable for work with highly pathogenic organisms including tissue samples carrying known human pathogens.

Practical hints and safety aspects of using cell culture hoods

All hoods must be maintained in a clutter-free and clean state at all times as too much clutter may affect air flow and contamination will introduce infections. Thus, as a rule of thumb, put only items that are required inside the cabinet and clean all work surfaces before and after use with industrial methylated spirit (IMS). The latter is used at an effective concentration of 70% (prepared by adding 70% v/v IMS to 30% Milli-Q

water), which acts against bacteria and fungal spores by dehydrating and fixing cells, thus preventing contamination of cultures.

Some cabinets may be equipped with a short-wave ultraviolet light that can be used to irradiate the interior of the hood to kill microorganisms. When present, switch on the ultraviolet light for at least 15 min to sterilise the inside of the cabinet, including the work area. Note, however, that ultraviolet radiation can cause adverse damage to the skin and eyes and precaution should be taken at all times to ensure that the operator is not in direct contact with the ultraviolet light when using this option to sterilise the hood. Once finished, ensure that the front panel door (class I and II hoods) is replaced securely after use. In addition always turn the hood on for at least 10 min before starting work to allow the flow of air to stabilise. During this period, monitor the air flow and check all dials in the control panel at the front of the hood to ensure that they are within the safe margin.

CO_2 incubators

Water-jacketed incubators are required to facilitate optimal cell growth under strictly maintained and regulated conditions, normally requiring a constant temperature of 37 °C and an atmosphere of 5–10% CO_2 plus air. The purpose of the CO_2 is to ensure that the culture medium is maintained at the required physiological pH (usually pH 7.2–7.4). This is achieved by the supply of CO_2 from a gas cylinder into the incubator through a valve that is triggered to draw in CO_2 whenever the level falls below the set value of 5% or 10%. The CO_2 that enters the inner chamber of the incubator dissolves into the culture medium containing bicarbonate. The latter reacts with H^+ (generated from cellular metabolism), forming carbonic acid, which is in equilibrium with water and CO_2, thereby maintaining the pH in the medium at approximately pH 7.2.

$$HCO_3^- + H^+ \rightleftharpoons H_2CO_3 \rightleftharpoons CO_2 + H_2O$$

These incubators are generally humidified by the inclusion of a tray of sterile water on the bottom deck. The evaporation of water creates a highly humidified atmosphere, which helps to prevent evaporation of medium from the cultures.

An alternative to humidified incubators is the dry non-gassed unit which is not humidified and relies on the use of alternative buffering systems such as 4(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid (Hepes) or morpholinopropane sulphonic acid (Mops) for maintaining a balanced pH within the culture medium. The advantage of this system is that it eliminates the risk from infections that can be posed by the tray of water in the humidified unit. The disadvantage, however, is that the culture medium will evaporate rapidly, thereby stressing the cells. One way round this problem is to place the cell culture plate in a sandwich box containing little pots of sterile water. With the sandwich box lid partially closed, evaporation of water from the pots will create a humidified atmosphere within the sandwich box, thus reducing the risk of evaporation of medium from the culture plate.

Practical hints and safety aspects of using cell culture incubators

The incubator should be maintained at 37 °C and supplied with 5% CO_2 at all times. A constant temperature can be maintained by keeping a thermometer in the incubator,

preferably on the inside of the inner glass door. This can then be checked on a regular basis and adjustments made as required. CO_2 levels inside the unit can be monitored and adjusted by using a gas analyser such as the Fyrite Reader. Regular checks should also be made on the levels of CO_2 in the gas cylinders that supply CO_2 to the incubators and these should be replaced when levels are very low. Most incubators are designed with an inbuilt alarm that sounds when the CO_2 level inside the chamber drops. At this point the gas cylinder must be replaced immediately to avoid stressing or killing the cultures. It is now possible to connect two gas cylinders to a cylinder changeover unit that switches automatically to the second source of gas supply when the first is empty. It is advisable therefore to use this device where possible.

When one is using a humidified incubator, it is essential that the water tray is maintained and kept free from microorganisms. This can be achieved by adding various agents to the water such as the antimicrobial agent Roccal at a concentration of 1% (w/v). Other products such as Thimerosal or SigmaClean from Sigma-Aldrich can also be used. Proper care and maintenance of the incubator should, however, include regular cleaning of the interior of the unit using any of the above reagents then swabbing with 70% IMS. More recently, copper-coated incubators have been introduced which, due to the antimicrobial properties of copper, are reported to reduce microbial contamination.

Microscopes

Inverted phase contrast microscopes (see Chapter 4) are routinely used for visualising cells in culture. These are expensive but easy to operate, with a light source located above and the objective lenses below the stage on which the cells are placed. Visualisation of cells by microscopy can provide useful information about the morphology and state of the cells. Early signs of cell stress may be easily identified and appropriate action taken to prevent loss of cultures.

Other general equipment

Several other pieces of equipment are required in cell culture. These include a centrifuge to spin down cells, a water bath for thawing frozen samples of cells and warming media to 37 °C before use, and a fridge and freezer for storage of media and other materials required for cell culture. Some cells need to attach onto a surface in order to grow and are therefore referred to as adherent. These cells are cultured in non-toxic polystyrene plastics that contain a biologically inert surface on which the cells attach and grow. Various types of plastics are available for this purpose and include Petri dishes, multi-well plates (with either 96, 24, 12 or 6 wells per plate) and screwcap flasks classified according to their surface areas: T-25, T-75, T-225 (cm^2 of surface area). A selection of these plastics is shown in Fig. 2.2.

2.3 SAFETY CONSIDERATIONS IN CELL CULTURE

Because of the nature of the work, safety in the cell culture laboratory must be of a major concern to the operator. This is particularly the case when one is working with pathogenic

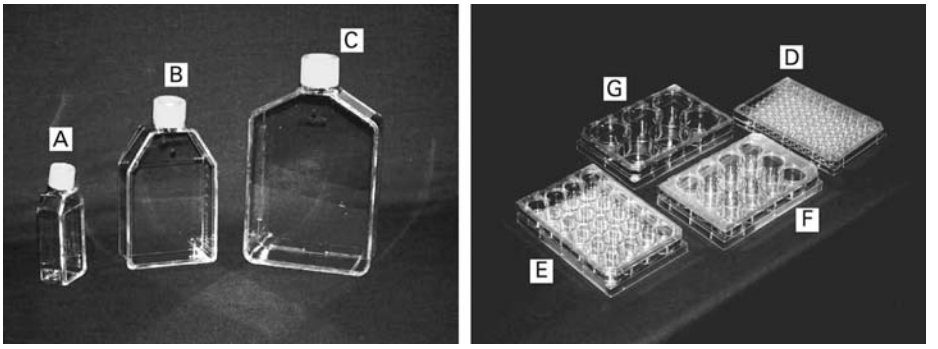

Fig. 2.2 Tissue culture plastics used generally for cell culture. (A–C) T-flasks; (D–G) representative of multi-well plates. (A) T–25 (25 cm^2), (B) T-75 (75 cm^2), (C) T-225 (225 cm^2), (D) 96-well plate, (E) 24-well plate, (F) 12-well plate and (G) 6-well plate.

microbes or with fresh primate or human tissues or cells which may contain agents that use humans as hosts. One very good example of this would be working with fresh human lymphocytes, which may contain infectious agents such as the human immunodeficiency virus (HIV) and/or hepatitis B virus. Thus, when one is working with fresh human tissue, it is essential that the infection status of the donor is determined in advance of use and all necessary precautions taken to eliminate or limit the risks to which the operator is exposed. A recirculation class II cabinet would be a minimum requirement for this type of cell culture work and the operator should be provided with protective clothing including latex gloves and a face mask if required. Such work should also be carried out under the guidelines laid down by the UK Advisory Committee on Dangerous Pathogens (ACDP).

Apart from the risks posed by the biological material being used, the operator should also be aware of his or her work environment and be fairly conversant with the equipment being used, as these may also pose a serious hazard. The culture cabinet should be serviced routinely and checked (approximately every 6 months) to ensure its safety to the operator. Additionally the operator could ensure his or her own safety by adopting some common precautionary measures such as refraining from eating or drinking whilst working in the cabinet and using a pipette aid as opposed to mouth pipetting to prevent ingestion of unwanted substances. Gloves and adequate protective clothing such as a clean laboratory coat should be worn at all times and gloves must be discarded after handling of non-sterile or contaminated material.

2.4 ASEPTIC TECHNIQUES AND GOOD CELL CULTURE PRACTICE

2.4.1 Good practice

In order to maintain a clean and safe culture environment, adequate aseptic or sterile technique should be adopted at all times. This simply involves working under

conditions that prevent contaminating microorganisms from the environment from entering the cultures. Part of the precaution taken involves washing hands with antiseptic soap and ensuring that all work surfaces are kept clean and sterile by swabbing with 70% IMS before starting work. Moreover, all procedures, including media preparation and cell handling, should be carried out in a cell culture cabinet that is maintained in a clean and sterile condition.

Other essential precautions should include avoiding talking, sneezing or coughing into the cabinet or over the cultures. A clean pipette should be used for each different procedure and under no circumstance should the same pipette be used between different bottles of media, as this will significantly increase the risk of cross-contamination. All spillages must be cleaned quickly to avoid contamination from microorganisms that may be present in the air. Failing to do so may result in infections to the cultures, which may be reduced by using antibiotics. However, this is not always guaranteed and good aseptic techniques should eliminate the need for antibiotics. In the event of cultures becoming contaminated, these should be removed immediately from the laboratory, disinfected and autoclaved to prevent the contamination spreading. Under no circumstance can an infected culture be opened inside the cell culture cabinet or incubator. Moreover, all waste generated must be decontaminated and disposed of immediately after completing the work. This should be carried out in accordance with the national legislative requirements, which state that cell culture waste including media be inactivated using a disinfectant before disposal and that all contaminated materials and waste be autoclaved before being discarded or incinerated.

The risk from infections is the most common cause for concern in cell culture. Various factors can contribute to this, including poor work environment, poor aseptic techniques and indeed poor hygiene of the operator. The last of these is important, since most of the common sources of infections such as bacteria, yeast and fungus originate from the worker. Maintaining a clean environment and adopting good laboratory practice and aseptic techniques should, therefore, help to reduce the risks of infection. However, should infections occur, it is advisable to address this immediately and eradicate the problem. To do this, it helps to know the types of infection to expect and what to look for.

In animal cell cultures, bacterial and fungal infections are relatively easy to identify and isolate. The other most common contamination originates from mycoplasma. These are the smallest (approximately 0.3 mm in diameter) self-replicating prokaryotes in existence. They lack a rigid cell wall and generally infect the cytoplasm of mammalian cells. There are at least five species known to contaminate cells in culture: *Mycoplasma hyorhinis, Mycoplasma arginini, Mycoplasma orale, Mycoplasma fermentans* and *Acholeplasma laidlawii*. Infections caused by these organisms are more problematic and not easily identified or eliminated. Moreover, if left unchecked, mycoplasma contamination will cause subtle but adverse effects on cultures, including changes in metabolism, DNA, RNA and protein synthesis, morphology and growth. This can lead to non-reproducible, unreliable experimental results and unsafe biological products.

2.4.2 Identification and eradication of bacterial and fungal infections

Both bacterial and fungal contaminations are easily identified as the infective agents are readily visible to the naked eye even in the early stages. This is usually made noticeable by the increase in turbidity and the change in colour of the culture medium owing to the change in pH caused by the infection. In addition, bacteria can be easily identified under microscopic examination as motile round bodies. Fungi on the other hand are distinctive by their long hyphal growth and by the fuzzy colonies they form in the medium. In most cases the simplest solution to these infections is to remove and dispose of the contaminated cultures. In the early stages of an infection, attempts can be made to eliminate the infecting microorganism using repeated washes and incubations with antibiotics or antifungal agents. This is however not advisable as handling infected cultures in the sterile work environment increases the chances of the infection spreading.

As part of the good laboratory practice, sterile testing of cultures should be carried out regularly to ensure that cultures are free from microbial organisms. This is particularly important when preparing cell culture products or generating cells for storage. Generally, the presence of these organisms can be detected much earlier and necessary precautions taken to avoid a full-blown contamination crisis in the laboratory. The testing procedure usually involves culturing a suspension of cells or products in an appropriate medium such as tryptone soya broth (TSB) for bacterial or thioglycollate medium (TGM) for fungal detection. The mixture is incubated for up to 14 days but examined daily for turbidity, which is used as an indication of microbial growth. It is essential that both positive and negative controls are set up in parallel with the sample to be tested. For this purpose a suspension of bacteria such as *Bacillus subtilis* or fungus such as *Clostridium sporogenes* is used instead of the cells or product to be tested. Uninoculated flasks containing only the growth medium are used as negative controls. Any contamination in the cell cultures will result in the broth appearing turbid, as would the positive controls. The negative controls should remain clear. Infected cultures should be discarded, whilst clear cultures would be safe to use or keep.

2.4.3 Identification of mycoplasma infections

Mycoplasma contaminations are more prevalent in cell culture than many workers realise. The reason for this is that mycoplasma contaminations are not evident under light microscopy nor do they result in a turbid growth in culture. Instead the changes induced are more subtle and manifest themselves mainly as a slowdown in growth and in changes in cellular metabolism and functions. However, cells generally return to their native morphology and normal proliferation rates relatively rapidly after eradication of mycoplasma.

The presence of mycoplasma contamination in cultures has, until recently, been difficult to determine and samples had to be analysed by specialist laboratories. There are, however, improved techniques now available for detection of mycoplasma in cell culture laboratories. These include microbiological cultures of infected cells, an

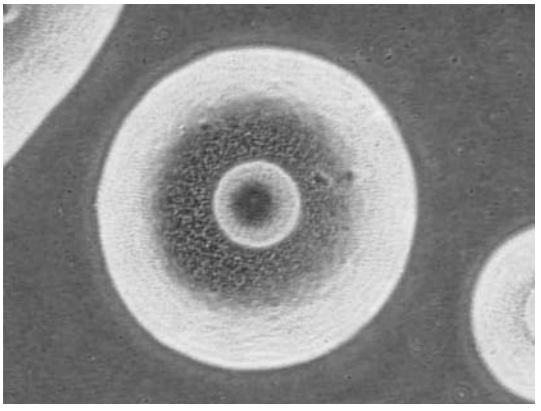

Fig. 2.3 Photograph of mycoplasma, showing the characteristic opaque granular central zone surrounded by a translucent border, giving a 'fried egg' appearance.

indirect DNA staining technique using the fluorochrome dye Hoechst 33258, enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR).

With the microbiological culture technique, cells in suspension are inoculated into liquid broth and then incubated under aerobic conditions at 37 °C for 14 days. A non-inoculated flask of broth is used as a negative control. Aliquots of broth are taken every 3 days and inoculated onto an agar plate, which is incubated anaerobically as above. All plates are then examined under an inverted microscope at a magnification of 300× after 14 days of incubation. Positive cultures will show the typical mycoplasma colony formation, which has an opaque granular central zone surrounded by a translucent border, giving a 'fried egg' appearance (Fig. 2.3). It may be necessary to set up positive controls in parallel, in which case plates and broth should be inoculated with a known strain of mycoplasma such as *Mycoplasma orale* or *Mycoplasma pneumoniae*.

The DNA binding method offers a rapid alternative for detecting mycoplasma and works on the principle that Hoechst 33258 fluoresces under ultraviolet light once bound to DNA. Thus, in contaminated cells, the fluorescence will be fairly dispersed in the cytoplasm of the cells owing to the presence of mycoplasma. In contrast, uncontaminated cells will show localised fluorescence in their nucleus only.

The Hoechst 33258 assay, although rapid, is relatively less sensitive when compared with the culture technique described above. For this assay, an aliquot of the culture to be tested is placed on a sterile coverslip in a 35-mm culture dish and incubated at 37 °C in a cell culture incubator to allow cells to adhere. The coverslip is then fixed by adding a fixative consisting of 1 part glacial acetic acid and 3 parts methanol, prepared fresh on the day. A freshly prepared solution of Hoechst 33258 stain is added to the fixed coverslip, incubated in the dark at room temperature to allow the dye to bind to the DNA and then viewed under ultraviolet fluorescence at 1000×. All positive cultures will show fluorescence of mycoplasma DNA, which will appear as small cocci or filaments in the cytoplasm of the contaminated cells (Fig. 2.4b, see also colour section). Negative cultures will show only fluorescing nuclei of

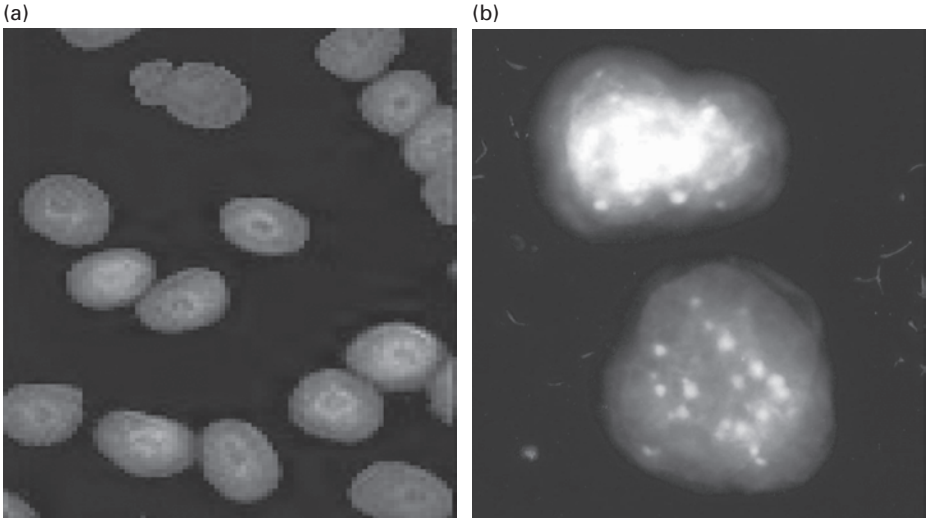

Fig. 2.4 Hoechst 33258 staining of mycoplasma in cells. (a) A Hoechst-negative stain, with the dye staining cellular DNA in the nucleus and thus showing nuclear fluorescence. (b) A Hoechst-positive stain, showing staining of mycoplasma DNA in the cytoplasm of the cells. (See also colour plate.)

uncontaminated cells against a dark cytoplasmic background (Fig. 2.4a, see also colour section). However, this technique is prone to errors, including false-negative results. To avoid the latter, cells should be cultured in antibiotic-free medium for two to three passages before being used. A positive control using a strain of mycoplasma seeded onto a coverslip is essential. Such controls should be handled away from the cell culture laboratory to avoid contaminating clean cultures of cells. It is also important to ensure that the fluorescence detected is not due to the presence of bacterial contamination or debris embedded into the plastics during manufacture. The former normally appear larger than the fluorescing cocci or filaments of mycoplasma. Debris, on the other hand, would show a non-uniform fluorescence owing to the variation in size of the particles usually found in plastics.

ELISA detection of mycoplasma is now becoming more commonly used and can be carried out using specifically designed kits following the manufacturer's protocol and reagents supplied. In this assay, 96-well plates are coated with the antibodies against different mycoplasma species. Each plate is then incubated at 37 °C for 2 h with the required antibody or antibodies before blocking with the appropriate blocking solution and incubating with the test sample(s). A negative control, which is simply media with sample buffer, and a positive control normally provided with the kits, should also be included in each assay. A detection antibody is subsequently added to the samples, incubated for a further 2 h at 37 °C before washing and incubating with a streptavidin solution for 1 h at 37 °C. Each plate is then detected for mycoplasma by adding the substrate solution and read on a plate reader at 405 nm after a further 30 min incubation at room temperature. This method is apparently suitable for detecting high levels of mycoplasma and could also be used to identify several species in one assay.

As with the ELISAs, commercial kits are also available for PCR detection of mycoplasma which contain the required primers, internal control template, positive control template and all the relevant buffers. Samples are generated and set up in a reaction mix as instructed in the manufacturer's protocol. The PCR is performed, again using the defined conditions outlined in the manufacturer's protocol, and the products generated analysed by electrophoresis on a high-grade 2% agarose gel. Although sensitive, PCR detection of mycoplasma is not always the protocol of choice because it has been shown to be prone to false-negative results, presumably due to the presence of ingredients in the kit which may inhibit PCR amplification of the target gene. In addition, this method is time-consuming and expensive.

2.4.4 Eradication of mycoplasma

Until recently, the most common approach for eradicating mycoplasma has been the use of antibiotics such as gentamycin. This approach is, however, not always effective, as not all strains of mycoplasma are susceptible to this antibiotic. Moreover antibiotic therapy does not always result in long-lasting successful elimination and most drugs can be cytotoxic to the cell culture. More recently, a new generation of bactericidal antibiotic preparation referred to as Plasmocin™ was introduced and has been shown to be effective against mycoplasma even at relatively low, non-cytotoxic concentrations. The antibiotics contained in this product are actively transported into cells, thus facilitating killing of intracellular mycoplasma but without any adverse effects on actual cellular metabolism.

Apart from antibiotics, various products have also been introduced into the cell culture market that the manufacturers claim eradicate mycoplasma efficiently and quickly without causing any adverse effects to the cells. One such product is Mynox®, a biological agent that integrates into the membrane of mycoplasma, compromising its integrity and eventually initiating its disintegration. This process apparently occurs within an hour of applying Mynox® and may have the added advantage that it is not an antibiotic and as a result will not lead to the development of resistant strains. It is safe to cultures and eliminated once the medium has been replaced. Moreover, this reagent is highly sensitive, detecting as little as 1–5 fg of mycoplasma DNA, which corresponds to two to five mycoplasma per sample and is effective against many of the common mycoplasma contaminations encountered in cell culture.

2.5 TYPES OF ANIMAL CELL, CHARACTERISTICS AND MAINTENANCE IN CULTURE

The cell types used in cell culture fall into two categories generally referred to as either a primary culture or a cell line.

2.5.1 Primary cell cultures

Primary cultures are cells derived directly from tissues following enzymatic dissociation or from tissue fragments referred to as explants. These are usually the cells of

preference, since it is argued that primary cultures retain their characteristics and reflect the true activity of the cell type *in vivo*. The disadvantage in using primary cultures, however, is that their isolation can be labour-intensive and may produce a heterogeneous population of cells. Moreover, primary cultures have a relatively limited lifespan and can be used over only a limited period of time in culture.

Primary cultures can be obtained from many different tissues and the source of tissue used generally defines the cell type isolated. For instance, cells isolated from the endothelium of blood vessels are referred to as endothelial cells whilst those isolated from the medial layer of the blood vessels and other similar tissues are smooth muscle cells. Although both can be obtained from the same vessels, endothelial cells are different in morphology and function, generally growing as a single monolayer characterised by a cobble-stoned morphology. Smooth muscle cells on the other hand are elongated, with spindle-like projections at either end, and grow in layers even when maintained in culture. In addition to these cell types there are several other widely used primary cultures derived from a diverse range of tissues, including fibroblasts from connective tissue, lymphocytes from blood, neurons from nervous tissues and hepatocytes from liver tissue.

2.5.2 Continuous cell lines

Cell lines consist of a single cell type that has gained the ability for infinite growth. This usually occurs after transformation of cells by one of several means that include treatment with carcinogens or exposure to viruses such as the monkey simian virus 40 (SV40), Epstein–Barr virus (EBV) or Abelson murine leukaemia virus (A-MuLV) amongst others. These treatments cause the cells to lose their ability to regulate growth. As a result, transformed cells grow continuously and, unlike primary culture, have an infinite lifespan (become 'immortalised'). The drawback to this is that transformed cells generally lose some of their original *in vivo* characteristics. For instance, certain established cell lines do not express particular tissue-specific genes. One good example of this is the inability of liver cell lines to produce clotting factors. Continuous cell lines, however, have several advantages over primary cultures, not least because they are immortalised. In addition, they require less serum for growth, have a shorter doubling time and can grow without necessarily needing to attach or adhere to the surface of the flask.

Many different cell lines are currently available from various cell banks, which makes it easier to obtain these cells without having to generate them. One of the largest organisations that supplies cell lines is the European Collection of Animal Cell Cultures (ECACC) based in Salisbury, UK. A selection of the different cell lines supplied by this organisation is listed in Table 2.1.

2.5.3 Cell culture media and growth requirements for animal cells

The cell culture medium used for animal cell growth is a complex mixture of nutrients (amino acids, a carbohydrate such as glucose, and vitamins), inorganic salts (e.g. containing magnesium, sodium, potassium, calcium, phosphate, chloride, sulphate,

Table 2.1 **Examples of cell lines supplied by commercial sources**

Cell line	Morphology	Species	Tissue origin
BAE-1	Endothelial	Bovine	Aorta
BHK-21	Fibroblast	Syrian hamster	Kidney
CHO	Fibroblast	Chinese hamster	Ovary
COS-1/7	Fibroblast	African green monkey	Kidney
HeLa	Epithelial	Human	Cervix
HEK-293	Epithelial	Human	Kidney
HT-29	Epithelial	Human	Colon
MRC-5	Fibroblast	Human	Lung
NCI-H660	Epithelial	Human	Lung
NIH/3T3	Fibroblast	Mouse	Embryo
THP-1	Monocytic	Human	Blood
V-79	Fibroblast	Chinese hamster	Lung
HEP1	Hepatocytes	Human	Liver

and bicarbonate ions) and broad-spectrum antibiotics. In certain situations it may be essential to include a fungicide such as amphotericin B, although this may not always be necessary. For convenience and ease of monitoring the status of the medium, the pH indicator phenol red may also be included. This will change from red at pH 7.2–7.4 to yellow or fuchsia as the pH becomes either acidic or alkaline, respectively.

The other key basic ingredient in the cell culture medium is serum, usually bovine or fetal calf. This is used to provide a buffer for the culture medium, but, more importantly, enhances cell attachment and provides additional nutrients and hormone-like growth factors that promote healthy growth of cells. An attempt to culture cells in the absence of serum does not usually result in successful or healthy cultures, even though cells can produce growth factors of their own. However, despite these benefits, the use of serum is increasingly being questioned not least because of many of the other unknowns that can be introduced, including infectious agents such as viruses and mycoplasma. The recent resurgence of 'mad cow disease' (bovine spongiform encephalitis) has introduced an additional drawback, posing a particular risk for the cell culturist, and has increased the need for alternative products. In this regard, several cell culture reagent manufacturers have now developed serum-free medium supplemented with various components including albumin, transferrin, insulin, growth factors and other essential elements required for optimal cell growth. This is proving very useful, particularly for the pharmaceutical and biotechnology companies involved in the manufacture of drugs or biological products for human and animal consumption.

2.5.4 Preparation of animal cell culture medium

Preparation of the culture medium is perhaps taken for granted as a simple straightforward procedure that is often not given due care and attention. As a result, most infections in cell culture laboratories originate from infected media. Following the simple yet effective procedures outlined in Section 2.4.1 should prevent or minimise the risk of infecting the media when they are being prepared.

Preparation of the medium itself should also be carried out inside the culture cabinet and usually involves adding a required amount of serum together with antibiotics to a fixed volume of medium. The amount of serum used will depend on the cell type but usually varies between 10% and 20%. The most common antibiotics used are penicillin and streptomycin, which inhibit a wide spectrum of Gram-positive and Gram-negative bacteria. Penicillin acts by inhibiting the last step in bacterial cell wall synthesis whilst streptomycin blocks protein synthesis.

Once prepared, the mixture, which is referred to as complete growth medium, should be kept at 4 °C until used. To minimise wastage and risk of contamination it is advisable to make just the required volume of medium and use this within a short period of time. As an added precaution it is also advisable always to check the clarity of the medium before use. Any infected medium, which will appear cloudy or turbid, should be discarded immediately. In addition to checking the clarity, a close eye should also be kept on the colour of the medium, which should be red at physiological pH owing to the presence of phenol red. Media that looks acidic (yellow) or alkaline (fuchsia) should be discarded, as these extremes will affect the viability and thus growth of the cells.

2.5.5 Subculture of cells

Subculturing is the process by which cells are harvested, diluted in fresh growth medium and replaced in a new culture flask to promote further growth. This process, also known as **passaging**, is essential if the cells are to be maintained in a healthy and viable state, otherwise they may die after a certain period in continuous culture. The reason for this is that adherent cells grow in a continuous layer that eventually occupies the whole surface of the culture dish and at this point they are said to be **confluent**. Once confluent, the cells stop dividing and go into a **resting state** where they stop growing (**senesce**) and eventually die. Thus, to keep cells viable and facilitate efficient transformation, they must be subcultured before they reach full contact inhibition. Ideally, cells should be harvested just before they reach a confluent state.

Cells can be harvested and subcultured using one of several techniques. The precise method used is dependent to a large extent on whether the cells are adherent or in suspension.

Subculture of adherent cells

Adherent cells can be harvested either mechanically, using a rubber spatula (also referred to as a ‘rubber policeman’) or enzymatically using proteolytic enzymes. Cells in suspension are simply diluted in fresh medium by taking a given volume of cell suspension and adding an equal volume of medium.

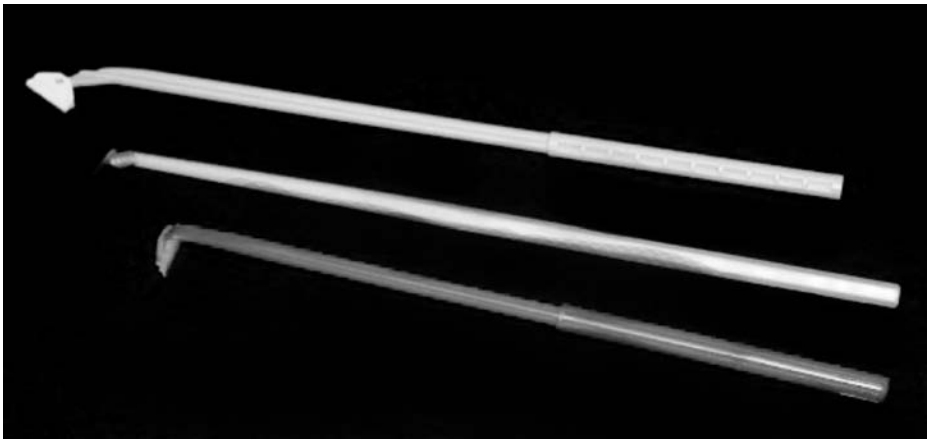

Fig. 2.5 Cell scrapers.

Harvesting of cells mechanically

This method is simple and easy. It involves gently scraping cells from the growth surface into the culture medium using a rubber spatula that has a rigid polystyrene handle with a soft polyethylene scraping blade (Fig. 2.5). This method is not suitable for all cell types as the scraping may result in membrane damage and significant cell death. Before adopting this approach it is important to carry out some test runs where cell viability and growth are monitored in a small sample of cells following harvesting.

Harvesting of cells using proteolytic enzymes

Several different proteolytic enzymes can be exploited including trypsin, a proteolytic enzyme that destroys proteinaceous connections between cells and between cells and the surface of the flask in which they grow. As a result, harvesting of cells using this enzyme results in the release of single cells, which is ideal for subculturing as each cell will then divide and grow, thus enhancing the propagation of the cultures.

Trypsin is commonly used in combination with EDTA, which enhances the action of the enzyme. EDTA alone can also be effective in detaching adherent cells as it chelates the Ca^{2+} required by some adhesion molecules that facilitate cell–cell or cell–matrix interactions. Although EDTA alone is much gentler on the cells than trypsin, some cell types may adhere strongly to the plastic, requiring trypsin to detach.

The standard procedure for detaching adherent cells using trypsin and EDTA involves making a working solution of 0.1% trypsin plus 0.02% EDTA in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline. The growth medium is aspirated from confluent cultures and washed at least twice with a serum-free medium such as Ca^{2+} or Mg^{2+}-free PBS to remove traces of serum that may inactivate the trypsin. The trypsin–EDTA solution (approximately 1 cm^3 per 25 cm^2 of surface area) is then added to the cell monolayer and swirled around for a few seconds. Excess trypsin–EDTA is aspirated, leaving just enough to form a thin film over the monolayer. The flask is then incubated at 37 °C in a cell culture incubator for 2–5 min but monitored under an inverted light microscope at intervals to detect when the cells

are beginning to round up and detach. This is to ensure that the cells are not overexposed to trypsin, as this may result in extensive damage to the cell surface, eventually resulting in cell death. It is important therefore that the proteolysis reaction is quickly terminated by the addition of complete medium containing serum that will inactivate the trypsin. The suspension of cells is collected into a sterile centrifuge tube and spun at 1000 r.p.m. for 10 min to pellet the cells, which are then resuspended in a known volume of fresh complete culture medium to give a required density of cells per cubic centimetre volume.

As with all tissue culture procedures, aseptic techniques should be adopted at all times. This means that all the above procedures should be carried out in a tissue culture cabinet under sterile conditions. Other precautions worth noting include the handling of the trypsin stock. This should be stored frozen at −20 °C and, when needed, placed in a water bath just to the point where it thaws. Any additional time in the 37 °C water bath will inactivate the enzymatic activity of the trypsin. The working solution should be kept at 4 °C once made and can be stored for up to 3 months.

Subculture of cells in suspension

For cells in suspension it is important initially to examine an aliquot of cells under a microscope to establish whether cultures are growing as single cells or clumps. If cultures are growing as single cells, an aliquot is counted as described in Section 2.5.6 below and then reseeded at the desired seeding density in a new flask by simply diluting the cell suspension with fresh medium, provided the original medium in which the cells were growing is not spent. However, if the medium is spent and appears acidic, then the cells must be centrifuged at 1000 r.p.m. for 10 min, resuspended in fresh medium and transferred into a new flask. Cells that grow in clumps should first be centrifuged and resuspended in fresh medium as single cells using a glass Pasteur or fine-bore pipette.

2.5.6 Cell quantification

It is essential that when cells are subcultured they are seeded at the appropriate seeding density that will facilitate optimum growth. If cells are seeded at a lower seeding density they may take longer to reach confluency and some may expire before getting to this point. On the other hand, if seeded at a high density, cells will reach confluency too quickly, resulting in irreproducible experimental results. This is because trypsin can digest surface proteins, including receptors for drugs, and these will need time (sometimes several days) to renew. Failure to allow these proteins to be regenerated on the cell surface may therefore result in variable responses to drugs specific for such receptors.

Several techniques are now available for quantification of cells and of these the most common method involves the use of a haemocytometer. This has the added advantage of being simple and cheap to use. The haemocytometer itself is a thickened glass slide that has a small chamber of grids cut into the glass. The chamber has a fixed volume and is etched into nine large squares, of which the large corner squares

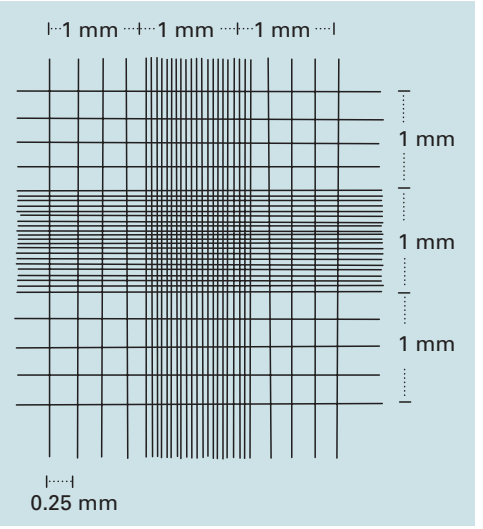

Fig. 2.6 Haemocytometer.

contain 16 small squares each; each large square measures 1 mm × 1 mm and is 0.1 mm deep (see Fig. 2.6).

Thus, with a coverslip in place, each square represents a volume of 0.1 mm^3 (1.0 mm^2 area × 0.1 mm depth) or 10^{-4} cm^3. Knowing this, the cell concentration (and the total number of cells) can therefore be determined and expressed per cubic centimetre. The general procedure involves loading approximately 10 µl of a cell suspension into a clean haemocytometer chamber and counting the cells within the four corner squares with the aid of a microscope set at 20× magnification. The count is mathematically converted to the number of cells per cm^3 of suspension.

To ensure accuracy, the coverslip must be firmly in place and this can be achieved by moistening a coverslip with exhaled breath and gently sliding it over the haemocytometer chamber, pressing firmly until **Newton's refraction rings** (usually rainbow-like) appear under the coverslip. The total number of cells in each of the four 1-mm^3 corner squares should be counted, with the proviso that only cells touching the top or left borders but not those touching the bottom and right borders are counted. Moreover, cells outside the large squares, even if they are within the field of view, should not be counted. When present, clumps should be counted as one cell. Ideally ~100 cells should be counted to ensure a high degree of accuracy in counting. If the total cell count is less than 100 or if more than 10% of the cells counted appear to be clustered, then the original cell suspension should be thoroughly mixed and the counting procedure repeated. Similarly, if the total cell count is greater than 400, the suspension should be diluted further to get counts of between 100 and 400 cells.

Since some cells may not survive the trypsinisation procedure it is usually advisable to add an equal volume of the dye trypan blue to a small aliquot of the cell suspension before counting. This dye is excluded by viable cells but taken up by dead cells. Thus, when viewed under the microscope, viable cells will appear as bright translucent

structures while dead cells will stain blue (see Section 2.5.12). The number of dead cells can therefore be excluded from the total cell count, ensuring that the seeding density accurately reflects viable cells.

Calculating cell number

Cell number is usually expressed per cm^3 and is determined by multiplying the average of the number of cells counted by a conversion factor which is constant for the haemocytometer. The conversion factor is estimated at 1000, based on the fact that each large square counted represents a total volume of $10^{-4}\,cm^3$.

Thus:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor}$$

If the cells were diluted before counting then the dilution factor should also be taken into account.

Therefore:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor} \times \text{dilution factor}$$

To get the total number of cells harvested the number of cells determined per cm^3 should be multiplied by the original volume of fluid from which the cell sample was removed,

i.e.:

$$\text{total cells} = \text{cells cm}^{-3} \times \text{total volume of cell suspension}$$

Example 1 CALCULATION OF CELL NUMBER

Question Calculate the total number of cells suspended in a final volume of 5 ml, taking into account that the cells were diluted 1 : 2 before counting and the number of cells counted with the haemocytometer was 400.

Answer

$$\text{Cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{large squares counted}} \times \text{conversion factor}$$

$$= \frac{400}{4} \times 1000$$

$$= 100\,000 \text{ cells cm}^{-3}$$

Because there is a dilution factor of 2, the correct number of cells cm^{-3} is given as:

$100\,000 \times 2 = 200\,000$ cells cm^{-3}

Thus in a final volume of 5 cm^3 the total number of cells present is:

$200\,000 \times 5 = 1\,000\,000$ cells

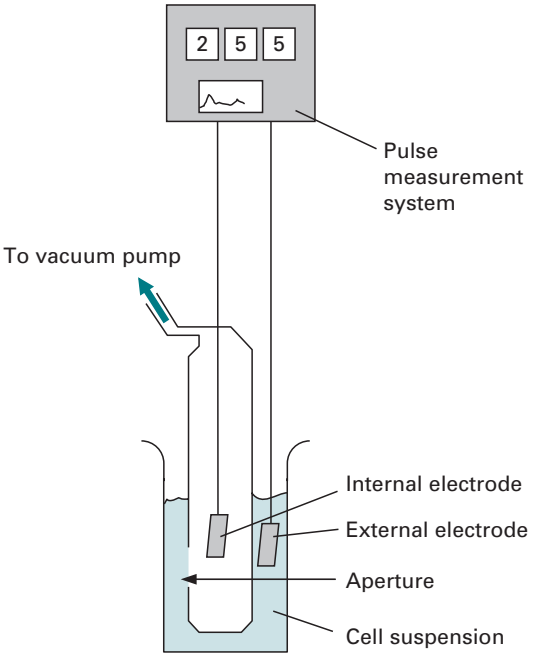

Fig. 2.7 Coulter counter. Cells entering the aperture create a pulse of resistance between the internal and external electrodes that is recorded on the oscilloscope.

Alternative methods for determination of cell number

Several other methods are available for quantifying cells in culture, including direct measurement using an **electronic Coulter counter**. This is an automated method of counting and measuring the size of microscopic particles. The instrument itself consists of a glass probe with an electrode that is connected to an oscilloscope (Fig. 2.7). The probe has a small aperture of fixed diameter near its bottom end. When immersed in a solution of cell suspension, cells are flushed through the aperture causing a brief increase in resistance owing to a partial interruption of current flow. This will result in spikes being recorded on the oscilloscope and each spike is counted as a cell. One disadvantage of this method, however, is that it does not distinguish between viable and dead cells.

Indirectly, cells can be counted by determining total cell protein and using a protein versus cell number standard curve to determine cell number in test samples. However, protein content per cell can vary during culture and may not give a true reflection of cell number. Alternatively, the DNA content of cells may be used as an indicator of cell number, since the DNA content of diploid cells is usually constant. However, the DNA content of cells may change during the cell cycle and therefore not give an accurate estimate of cell number.

2.5.7 Seeding cells onto culture plates

Once counted, cells should then be seeded at a density that promotes optimal cell growth. It is essential therefore that when cells are subcultured they are seeded at the

appropriate seeding density. If cells are seeded at a lower density they may take longer to reach confluency and some may die before getting to this point. On the other hand, if seeded at too high a density cells will reach confluency too quickly, resulting in irreproducible experimental results as already discussed above (see Section 2.5.6). The seeding density will vary depending on the cell type and on the surface area of the culture flask into which the cells will be placed. These factors should therefore be taken into account when deciding on the seeding density of any given cell type and the purpose of the experiments carried out.

2.5.8 Maintenance of cells in culture

It is important that after seeding, flasks are clearly labelled with the date, cell type and the number of times the cells have been subcultured or passaged. Moreover, a strict regime of feeding and subculturing should be established that permits cells to be fed at regular intervals without allowing the medium to be depleted of nutrients or the cells to overgrow or become super confluent. This can be achieved by following a standard but routine procedure for maintaining cells in a viable state under optimum growth conditions. In addition, cultures should be examined daily under an inverted microscope, looking particularly for changes in morphology and cell density. Cell shape can be an important guide when determining the status of growing cultures. Round or floating cells in subconfluent cultures are not usually a good sign and may indicate distressed or dying cells. The presence of abnormally large cells can also be useful in determining the well-being of the cells, since the number of such cells increases as a culture ages or becomes less viable. Extremes in pH should be avoided by regularly replacing spent medium with fresh medium. This may be carried out on alternate days until the cultures are approximately 90% confluent, at which point the cells are either used for experimentation or trypsinised and subcultured following the procedures outlined in Section 2.5.5.

The volume of medium added to the cultures will depend on the confluency of the cells and the surface area of the flasks in which the cells are grown. As a guide, cells which are under 25% confluent may be cultured in approximately 1 cm^3 of medium per 5 cm^2 and those between 25% and 40% or $\geqq$ 45% confluency should be supplemented with 1.5 cm^3 or 2 cm^3 culture medium per 5 cm^2, respectively. When changing the medium it is advisable to pipette the latter on to the sides or the opposite surface of the flask from where the cells are attached. This is to avoid making direct contact with the monolayers as this will damage or dislodge the cells.

2.5.9 Growth kinetics of animal cells in culture

When maintained under optimum culture conditions, cells follow a characteristic growth pattern (Fig. 2.8), exhibiting an initial lag phase in which there is enhanced cellular activity but no apparent increase in cell growth. The duration of this phase is dependent on several factors including the viability of the cells, the density at which the cells are plated and the media component.

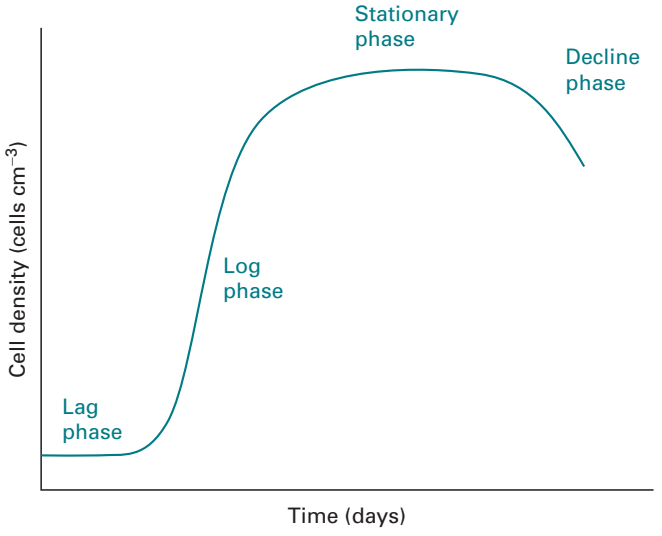

Fig. 2.8 Growth curve showing the phases of cell growth in culture.

The lag phase is followed by a **log phase** in which there is an exponential increase in cell number with high metabolic activity. These cells eventually reach a **stationary phase** where there is no further increase in growth due to depletion of nutrients in the medium, accumulation of toxic metabolic waste or a limitation in available growth space. If left unattended, cells in the stationary phase will eventually begin to die, resulting in the **decline phase** on the growth curve.

2.5.10 Cryopreservation of cells

Cells can be preserved for later use by freezing stocks in liquid nitrogen. This process is referred to as **cryopreservation** and is an efficient way of sustaining stocks. Indeed, it is advisable that, when good cultures are available, aliquots of cells should be stored in the frozen state. This provides a renewable source of cells that could be used in future without necessarily having to culture new batches from tissues. Freezing can, however, result in several lethal changes within the cells, including formation of ice crystals and changes in the concentration of electrolytes and in pH. To minimise these risks a cryoprotective agent such as DMSO is usually added to the cells prior to freezing in order to lower the freezing point and prevent ice crystals from forming inside the cells. In addition, the freezing process is carried out in stages, allowing the cells initially to cool down slowly from room temperature to $-80\,^{\circ}C$ at a rate of $1–3\,^{\circ}C\ min^{-1}$. This initial stage can be carried out using a freezing chamber or alternatively a cryo freezing container ('Mr Frosty') filled with isopropanol, which provides the critical, repeatable $-1\,^{\circ}C\,min^{-1}$ cooling rate required for successful cell cryopreservation. When this process is complete, the cryogenic vials, which are polypropylene tubes that can withstand temperatures as low as $-190\,^{\circ}C$, are removed and immediately placed in a liquid nitrogen storage tank where they can remain for an indefinite period or until required.

The actual cryogenic procedure is itself relatively straightforward. It involves harvesting cells as described in Section 2.5.5 and resuspending them in 1 cm^3 of freezing medium, which is basically culture medium containing 40% serum. The cell suspension is counted and appropriately diluted to give a final cell count of between 10^6 and 10^7 cells cm^{-3}. A 0.9-cm^3 aliquot is transferred into a cryogenic vial labelled with the cell type, passage number and date harvested. This is then made up to 1 cm^3 by adding 100 mm^3 of DMSO to give a final concentration of 10%. The cells should then be mixed gently by rotating or inverting the vial and placed in a 'Mr Frosty' cryo freezing container. The container and cells are placed in a −80 °C freezer and allowed to freeze overnight. The frozen vials may then be transferred into a liquid nitrogen storage container. At this stage cells can be stored frozen until required for use.

All procedures should be carried out under sterile conditions to avoid contaminating cultures as this will appear once the frozen stocks are recultured. As an added precaution it is advisable to replace the growth medium in the 24-h period prior to harvesting cells for freezing. Moreover, cells used for freezing should be in the log phase of growth and not too confluent in case they may already be in growth arrest.

2.5.11 Resuscitation of frozen cells

When required, frozen stocks of cells may be revived by removing the cryogenic vial from storage in liquid nitrogen and placing in a water bath at 37 °C for 1–2 min or until the ice crystals melt. It is important that the vials are not allowed to warm up to 37 °C as this may cause the cells to rapidly die. The thawed cell suspension may then be transferred into a centrifuge tube, to which fresh medium is added and centrifuged at 1000 r.p.m. for 10 min. The supernatant should be discarded to remove the DMSO used in the freezing process and the cell pellet resuspended in 1 cm^3 of fresh medium, ensuring that clumps are dispersed into single cells or much smaller clusters using a glass Pasteur pipette. The required amount of fresh pre-warmed growth medium is placed in a culture flask and the cells pipetted into the flask, which is then placed in a cell culture incubator and the cells allowed to adhere and grow.

Practical hints and tips in resuscitation of frozen cells

It is important to handle resuscitated cells delicately after thawing as these may be fairly fragile and could degenerate quite readily if not treated correctly. In addition, it is important to dilute the freezing medium immediately after thawing to reduce the concentration of DMSO or freezing agent to which the cells are exposed.

2.5.12 Determination of cell viability

Determination of cell viability is extremely important, since the survival and growth of the cells may depend on the density at which they are seeded. The degree of viability is most commonly determined by differentiating living from dead cells using the dye exclusion method. Basically, living cells exclude certain dyes that are readily taken up by dead cells. As a result, dead cells stain the colour of the dye used whilst living cells remain refractile owing to the inability of the dye to penetrate into the

cytoplasm. One of the most commonly used dyes in such assays is trypan blue. This is incubated at a concentration of 0.4% with cells in suspension and applied to a haemocytometer. The haemocytometer is then viewed under an inverted microscope set at 100 × magnification and the cells counted as described in Section 2.5.6, keeping separate counts for viable and non-viable cells.

The total number of cells is calculated using the following equation as described previously:

$$\text{cells cm}^{-3} = \frac{\text{number of cells counted}}{\text{number of squares counted}} \times \text{conversion factor} \times \text{dilution factor}$$

and the percentage of viable cells determined using the following formula:

$$\% \text{ viability} = \frac{\text{number of unstained cells counted}}{\text{total number of cells counted}} \times 100$$

To avoid underestimating cell viability it is important that the cells are not exposed to the dye for more than 5 min before counting. This is because uptake of trypan blue is time sensitive and the dye may be taken up by viable cells during prolonged incubation periods. Additionally, trypan blue has a high affinity for serum proteins and as such may produce a high background staining. The cells should therefore be free from serum, which can be achieved by washing the cells with PBS before counting.

2.6 STEM CELL CULTURE

Stem cells are unspecialised cells which have the ability to undergo self-renewal, replicating many times over prolonged periods, thereby generating new unspecialised cells. More importantly, stem cells have the potential to give rise to specialised cells with specific functions by the process of **differentiation**. Because of this property, stem cells are now being developed and exploited for cell-based therapies in various disease states. It has therefore become essential to be able to isolate, maintain and grow these cells in culture. This is however an emerging field where protocols to be used routinely are still being developed. This section of the chapter will focus on techniques that are now becoming routine for stem cell culture, focussing essentially on human embryonic stem cells (hESCs). The latter are cells derived from the inner cell mass of the **blastocyst** which is a hollow microscopic ball made up of an outer layer of cells (the **trophoblast**), a fluid-filled cavity (the **blastocoel**) and the cluster of inner cell mass.

Culturing of hESCs can be carried out in a standard cell culture laboratory using equipment already described earlier in the chapter. As with normal cell culture, the important criteria are that good aseptic techniques are adopted together with good laboratory practice. Unlike normal specialised cells, however, culture of hESCs requires certain conditions specifically aimed at maintaining these cells in a viable undifferentiated state. Historically, hESCs, and indeed other stem cells, have been cultured on what are referred to as **feeders** which act to sustain growth and maintain cells in the undifferentiated state without allowing them to lose their **pluripotency**

(i.e. ability to differentiate, when needed, into specialised cell types of the three germ layers). The most common feeder cultures used are fibroblasts derived from embryos. The methodology for this together with other techniques for successful maintenance and propagation of hESCs are described below. Other protocols such as freezing and resuscitation of frozen cells are similar to those already described and the reader is therefore referred to the relevant sections above.

2.6.1 Preparation of embryonic fibroblasts

Typically, fibroblasts are isolated under sterile conditions in a tissue culture cabinet from embryos obtained from mice at 13.5 days of gestation. Each embryo is minced into very fine pieces using sterile scissors and incubated in a cell culture incubator at 37 °C with trypsin/EDTA (0.25% (w/v)/5 mM) for 20 minutes. The mixture is then pipetted vigorously using a fine-bore pipette until it develops a sludgy consistency. This process is repeated, returning the digest into the incubator if necessary, until the embryos have been virtually digested. The trypsin is subsequently neutralised with culture medium containing 10% serum ensuring that the volume of medium is at least twice that of the trypsin used. The minced tissue is plated onto a tissue culture flask and incubated overnight at 37 °C in a tissue culture incubator. The medium is subsequently removed after 24 h and the cell monolayer washed to remove any tissue debris and non-adherent cells. Adherent cells are cultured to 80–90% confluency before being passaged using trypsin as described in Section 2.5.5. If needed, the trypsinised cells could be propagated, otherwise they should be frozen as described in Section 2.5.10 and used as stock. If the latter is preferred, ensure that cells are frozen at no higher than passage three.

Practical hints and tips in using fibroblast feeders

Mouse fibroblasts should be used as feeders for stem cell culture between passages three and five. This is to ensure that fibroblasts support the growth of undifferentiated cells. After passage five the cells may begin to senesce and could also potentially fail to maintain stem cells in the undifferentiated state. Each batch of feeders prepared should be tested for their ability to support cells in an undifferentiated state.

2.6.2 Inactivation of fibroblast cells for use as feeders

Fibroblasts isolated should be inactivated before they can be used as feeders in order to prevent their proliferation and expansion during culture. This can be achieved using one of two protocols which include either irradiation or treatment with the antibiotic DNA cross-linker mitomycin C. With the former, cells in suspension are exposed to 80 Gy of irradiation using a caesium-source gamma irradiator. This is the dose of irradiation normally used for mouse fibroblasts; however, the radiation dose and exposure time may vary between batches of fibroblasts. As a result, a dose

curve should be performed to determine the effective irradiation that is sufficient to stop cell division without cellular toxicity. Once irradiated, cells are spun at 1000 r.p.m. before resuspending the pellet using the appropriate medium and at the appropriate density for freezing or plating on gelatin-coated plates.

With the mitomycin procedure, cells are normally incubated with the compound at a concentration of 10 $\mu g\,cm^{-3}$ for 2–3 h at 37 °C in a cell culture incubator. After this, the mitomycin solution is aspirated and the cells washed several times with phosphate buffered saline or serum-free culture medium to ensure that there are no trace amounts of mitomycin that could affect the stem cells. The cells are then trypsinised, neutralised with serum containing medium, centrifuged and re-plated onto gelatin-coated dishes at the appropriate cell density.

Practical hints and tips with feeders

Of the two methods, exposure of cells to a gamma irradiation is the much preferred methodology because this gives a more consistent and reliable inactivation of cells. More importantly, mitomycin can be harmful and toxic, with embryonic cells showing particular sensitivity to this compound. Use of mitomycin-inactivated fibroblasts should therefore generally be avoided if irradiated feeders can be obtained. If frozen stocks are required of inactivated feeders, these can be prepared as described in Section 2.5.10. It is, however, important to ensure that stocks are not kept frozen for periods exceeding 4 months to avoid degeneration of cells. In addition, once plated, feeders should be used for stem cell culture within 24 h or no longer than 5 days after plating.

2.6.3 Plating of feeder cells

As with standard cell culture, fibroblast feeders are plated on tissue culture grade plastics but usually in the presence of a substrate such as gelatin, to provide the extracellular matrix component needed for cell attachment of the inactivated fibroblasts. In brief, the plates or flasks are incubated for 1 h at room temperature or overnight at 4 °C with the appropriate volume of 0.1% sterile gelatin. Excess gelatin is subsequently removed and the feeder cells plated at the approriate density for each cell line, e.g. 3.5×10^5 cells per 25-cm^2 flask. Feeders should be ready for use after 5–6 h but are best left to establish overnight for better results.

Practical hints and tips in plating feeders

It is important to ensure that the seeding density is optimal for each cell line otherwise feeders may fail to maintain the hESCs in the undifferentiated state. If frozen stocks of feeders are used for plating, these should be resuscitated, resuspended in fresh growth medium and plated on gelatin-coated plates as described in Section 2.5.11. Again the density of post-thaw feeders required to support the cells in an undifferentiated state should be established for each batch of frozen feeders since there is cell loss during the freeze–thaw process.

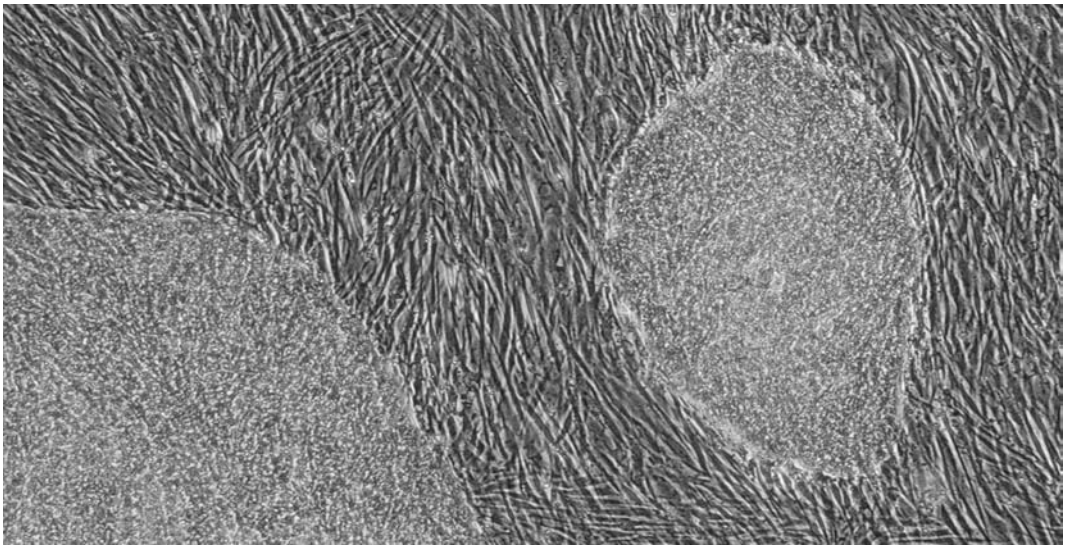

Fig. 2.9 Undifferentiated hESCs on mouse feeder cells.

2.6.4 Culture of human embryonic stem cells

Once the feeders are ready, hESCs can be plated directly by depositing the suspension of hESC onto the feeder layer. The dishes are placed in a cell culture incubator and the cells allowed to attach and establish over a 24-h period. Any non-adherent cells are removed during the first culture medium change. The cells are monitored and fed on a daily basis until the colonies are ready to be passaged. Depending on the conditions of growth, this can usually take up to 6 days.

As with the feeders, frozen stocks of hESCs should be resuscitated and diluted in fresh growth medium as described in Section 2.5.11.

Practical hints and tips in hESC culture

It is important to ensure that the colonies do not grow too large and to the point where adjacent colonies touch each other as this will initiate their differentiation. Similarly, the seeding density should be high enough to sustain growth otherwise sparsely plated colonies will grow very slowly and may never establish fully.

Colonies should be plated on healthy feeders that are not more than 4 days old. More importantly, only tightly packed colonies containing cells with the typical hESC morphology should be passaged (see Fig. 2.9). Any colony that has a less defined border (see Fig. 2.10) at the periphery, with loose cells spreading out or cells with atypical morphology, should not be passaged because these characteristics are evidence of cell differentiation. Should cells differentiate, these should be excised or aspirated before passaging the undifferentiated cells. Alternatively, if the majority of the colonies appear differentiated and no colonies display the characteristic morphology of undifferentiated cells, then it is advisable to discard the cultures and start with a new batch of undifferentiated hESCs.

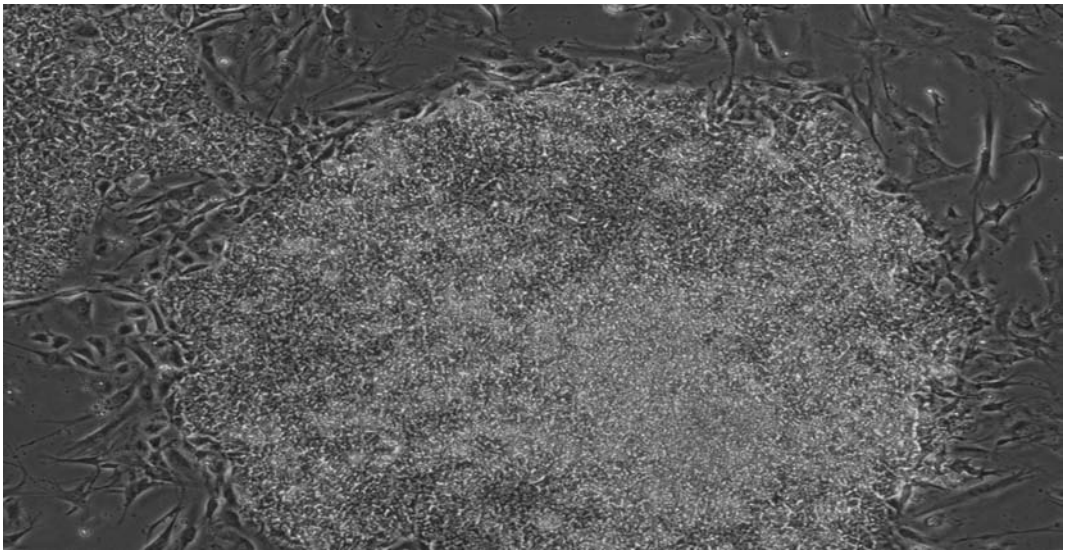

Fig. 2.10 Partially differentiated hESCs on mouse feeder cells.

2.6.5 Enzymatic subculture of hESCs

As with standard cell culture, hESCs can be passaged using enzymes but in this case an enzyme that does not disperse clusters of cells into single cells is preferred. This is because hESCs need to grow in colonies since single cells may not adhere to the feeders and may differentiate easily.

One of the most commonly used enzymes for subculturing hESCs is **collagenase**. When employed, hESC colonies are washed with phosphate-buffered saline and then incubated for 8–10 min with collagenase IV made up in serum-free medium at a concentration of 1 mg cm^{-3}. Curled up colonies can then be dislodged with gentle pipetting using a 5-ml pipette to break large clumps. Alternatively, colonies can be fragmented using glass beads. These are then washed with culture medium to remove the enzyme which may otherwise impair the attachment and growth of the cells, thus reducing the plating efficiency. hESCs can be washed by allowing the colonies to sediment slowly over 5–10 mins, leaving any residual feeder cells in the supernatant which are removed by aspiration. The colonies are subsequently resuspended in growth medium and are usually plated at a ratio of between 1 : 3 and 1 : 6. Alternatively, fragmented colonies could be frozen as described in Section 2.5.10 and stored for later use.

2.6.6 Mechanical subculture of hESCs

An alternative to the enzymatic method of subculturing hESCs is to manually cut colonies into appropriate size fragments using a fine-bore needle or a specially designed cutter such as the STEMPRO® EZPassage™ disposable stem cell passaging tool from Invitrogen. To do this, the dish of hESCs is placed under a dissecting

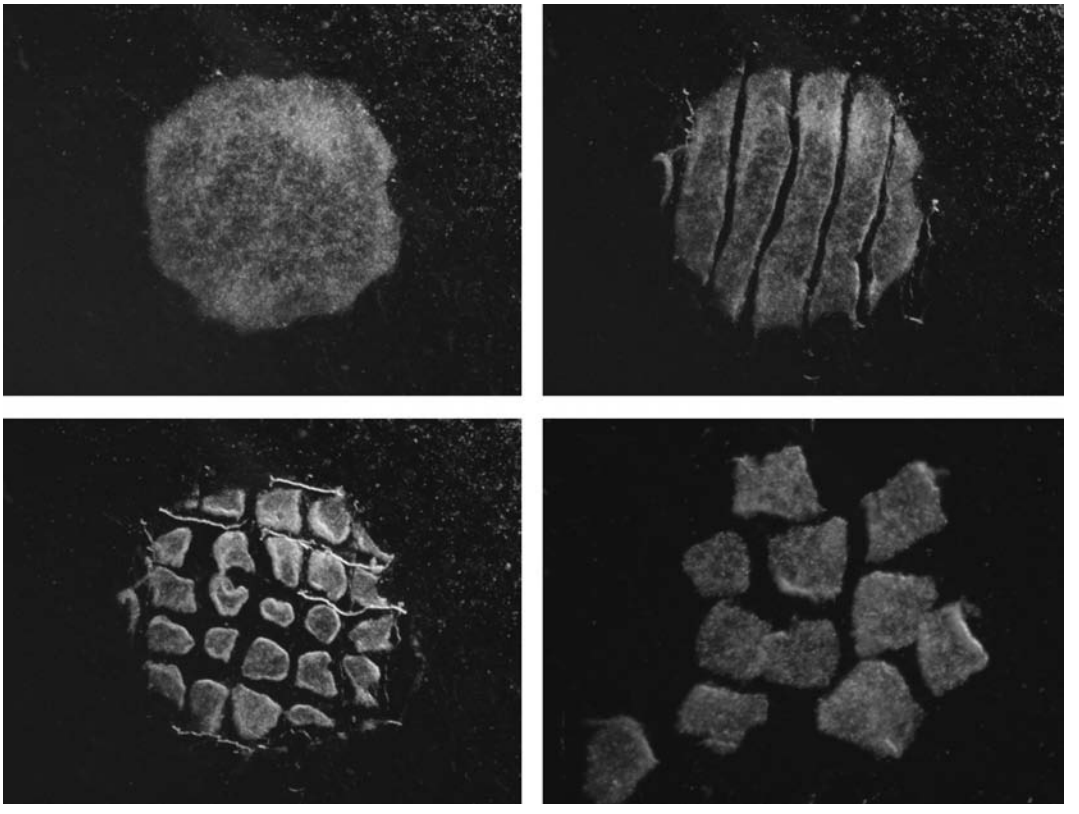

Fig. 2.11 Mechanically harvested hESCs.

microscope in a tissue culture hood. Undifferentiated colonies are identified by their morphology and then cut into grids (see Fig. 2.11) by scoring across and perpendicular to the first cut. Using a 1-ml pipette or pastette, the cut segments are transferred to dishes containing fresh feeders and culture medium. The colony fragments are placed evenly across the feeders (see Fig. 2.12) to avoid the colonies clumping together and attaching to the dish as one mass of cells. The dishes are then carefully transferred to a tissue culture incubator and left undisturbed for 1 day before replacing the spent medium with fresh. Established colonies are then fed every day until subcultured.

2.6.7 Feeder-free culture of hESCs

Although culture of hESCs on feeders has been extensively used, there have been concerns over this procedure when stem cells are being considered for clinical use in humans. One of the main drawbacks of using feeders is the concern over potential transmission of animal pathogens to humans and the possibility of expression of immunogenic antigens. Feeders are also inconvenient, expensive, and time-consuming to generate and inactivate. As a result of these limitations, there has been a drive towards developing a feeder-free culture system using feeder-conditioned media or media supplemented with different growth factors and other signalling molecules essential

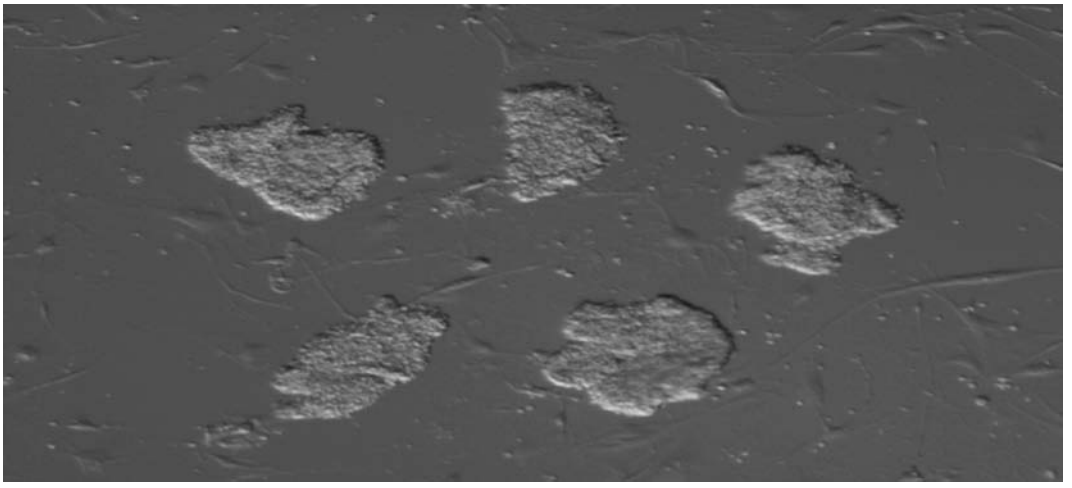

Fig. 2.12 Plating of hESCs onto feeder layer.

for sustaining growth. The conditioned medium can be generated by incubating normal growth medium with feeder cells for 24 h before use.

Feeder-free culture of hESCs is often carried out on tissue culture plastics coated with **Matrigel**, a substrate derived from mouse tumour and rich in **extracellular matrix** proteins such as **laminin**, **collagen** and **hepran sulphate proteoglycan**. It is also rich in growth factors such as basic fibroblast growth factor (bFGF) which can help to sustain and promote stem cell growth whilst maintaining them in an undifferentiated state.

Practically, dishes are coated with 5% Matrigel made up in culture medium. Just prior to use, the Matrigel is removed and replaced with culture medium before plating cells. The hESCs, subcultured from feeders or obtained from frozen stocks, are resuspended in conditioned medium supplemented often with bFGF at a concentration of 4 ng ml^{-1} before seeding. Alternatively, normal growth medium could be used but this will require a much higher concentration usually around 100 ng ml^{-1} bFGF. Once established, hESCs are fed every day with fresh growth medium. Colonies on Matrigel tend to show a different morphology to those on feeders; they tend to be larger and less packed initially than when cultured on feeders.

Practical hints and tips in using Matrigel

All work with Matrigel, other than plating of the hESCs, should be carried out at 4 °C. Thus, when coating tissue culture plastics with Matrigel, all the plates and pipette tips should be kept on ice and used cold to prevent the Matrigel solidifying. Stock Matrigel is usually in the solid form and should be placed on ice or in the fridge at 4 °C overnight until it liquefies. Once liquefied, the Matrigel should be diluted in ice-cold culture medium at a final concentration of 5%. Each plate should have a smooth even layer of Matrigel and if this is not the case, the plates should be incubated at 4 °C until the Matrigel liquefies and settles as a uniform layer. Once coated, Matrigel plates should be used within 7 days of preparation.

2.7 BACTERIAL CELL CULTURE

As with animal cells, pure bacterial cultures (cultures that contain only one species of organism) are cultivated routinely and maintained indefinitely using standard sterile techniques that are now well defined. However, since bacterial cells exhibit a much wider degree of diversity in terms of both their nutritional and environmental requirements, conditions for their cultivation are diverse and the precise requirements highly dependent on the species being cultivated. Outlined below are general procedures and precautions adopted in bacterial cell culture.

2.7.1 Safety considerations for bacterial cell culture

Culture of microbial cells, like that involving cells of animal origin, requires care and sterile techniques, not least of all to prevent accidental contamination of pure cultures with other organisms. More importantly, utmost care should be given towards protecting the operator, especially from potentially harmful organisms. Aseptic techniques and safety conditions described for animal cell culture should be adopted at all times. Additionally, instruments used during the culturing procedures should be sterilised before and after use by heating in a Bunsen burner flame. Moreover, to avoid spread of bacteria, areas of work must be decontaminated after use using germicidal sprays and/or ultraviolet radiation. This is to prevent airborne bacteria from spreading rapidly. In line with these precautions, all materials used in microbial cell culture work must be disposed of appropriately; for instance, autoclaving of all plastics and tissue culture waste before disposal is usually essential.

2.7.2 Nutritional requirements of bacteria

The growth of bacteria requires much simpler conditions than those described for animal cells. However, due to their diversity, the composition of the medium used may be variable and largely determined by the nutritional classification of the organisms to be cultured. These generally fall into two main categories classified as either **autotrophs** (self-feeding organisms that synthesise food in the form of sugars using light energy from the sun) or **heterotrophs** (non-self-feeding organisms that derive chemical energy by breaking down organic molecules consumed). These in turn are subgrouped into **chemo**- or **photoautotrophs** or heterotrophs. Both chemo- and photoautotrophs rely on carbon dioxide as a source of carbon but derive energy from completely different sources, with the chemoautotrophs utilising inorganic substances whilst the photoautotrophs use light. Chemoheterotrophs and photoheterotrophs both use organic compounds as the main source of carbon with the photoheterotrophs using light for energy and the chemo subgroup getting their energy from the metabolism of organic substances.

2.7.3 Culture media for bacterial cell culture

Several different types of medium are used to culture bacteria and these can be categorised as either complex or defined. The former usually consist of natural

substances, including meat and yeast extract, and as a result are less well defined, since their precise composition is largely unknown. Such media are, however, rich in nutrients and therefore generally suitable for culturing fastidious organisms that require a mixture of nutrients for growth. Defined media, by contrast, are relatively simple. These are usually designed to the specific needs of the bacterial species to be cultivated and as a result are made up of known components put together in the required amounts. This flexibility is usually exploited to select or eliminate certain species by taking advantage of their distinguishing nutritional requirements. For instance, bile salts may be included in media when selective cultivation of enteric bacteria (rod-shaped Gram-negative bacteria such as *Salmonella* or *Shigella*) is required, since growth of most other Gram-positive and Gram-negative bacteria will be inhibited.

2.7.4 Culture procedures for bacterial cells

Bacteria can be cultured in the laboratory using either liquid or solid media. Liquid media are normally dispensed into flasks and inoculated with an aliquot of the organism to be grown. This is then agitated continuously on a shaker that rotates in an orbital manner, mixing and ensuring that cultures are kept in suspension. For such cultures, sufficient space should be allowed above the medium to facilitate adequate diffusion of oxygen into the solution. Thus, as a rule of thumb, the volume of medium added to the flasks should not exceed more than 20% of the total volume of the flask. This is particularly important for aerobic bacteria and less so for anaerobic microorganisms.

In large-scale culture, **fermenters** or **bioreactors** equipped with stirring devices for improved mixing and gas exchange may be used. The device (Fig. 2.13) is usually fitted with probes that monitor changes in pH, oxygen concentration and temperature. In addition most systems are surrounded by a water jacket with fast-flowing cold water to reduce the heat generated during fermentation. Outlets are also included to release CO_2 and other gases produced by cell metabolism.

When fermenters are used, precautions should be taken to reduce potential contamination with airborne microorganisms when air is bubbled through the cultures. Sterilisation of the air may therefore be necessary and can be achieved by introducing a filter (pore size of approximately 0.2 mm) at the point of entry of the air flow into the chamber.

Solid medium is usually prepared by solidifying the selected medium with 1–2% of the seaweed extract agar, which, although organic, is not degraded by most microbes thereby providing an inert gelling medium on which bacteria can grow. Solid agar media are widely used to separate mixed cultures and form the basis for isolation of pure cultures of bacteria. This is achieved by streaking diluted cultures of bacteria onto the surface of an agar plate by using a sterile inoculating loop. Cells streaked across the plate will eventually grow into a colony, each colony being the product of a single cell and thus of a single species.

Once isolated, cells can be cultivated either in **batch** or **continuous cultures**. Of these, batch cultures are the most commonly used for routine liquid growth and entail

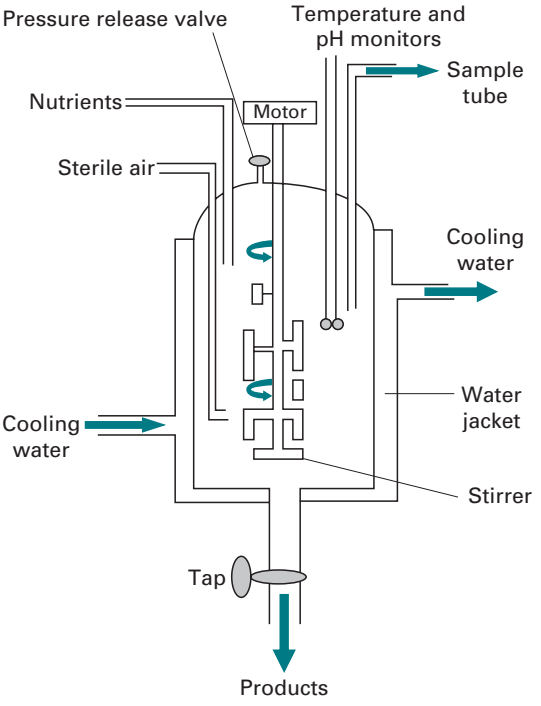

Fig. 2.13 Schematic representation of a fermenter.

inoculating an aliquot of cells into a sterile flask containing a finite amount of medium. Such systems are referred to as closed, since nutrient supply is limited to that provided at the start of culture. Under these conditions, growth will continue until the medium is depleted of nutrients or there is an excessive build-up of toxic waste products generated by the microbes. Thus, in this system, the cellular composition and physiological status of the cells will vary throughout the growth cycle.

In continuous cultures (also referred to as **open systems**) the medium is refreshed regularly to replace that spent by the cells. The objective of this system is to maintain the cells in the exponential growth phase by enabling nutrients, biomass and waste products to be controlled through varying the dilution rate of the cultures. Continuous cultures, although more complex to set up, offer certain advantages over batch cultures in that they facilitate growth under steady-state conditions in which there is tight coupling between cell division and biosynthesis. As a result, the physiological status of the cultures is more clearly defined, with very little variation in the cellular composition of the cells during the growth cycle. The main concern with the open system is the high risk of contamination associated with the dilution of the cultures. However, applying strict aseptic techniques during feeding or harvesting cells may help to reduce the risk of such contaminations. In addition, the whole system can be automated by connecting the culture vessels to their reservoirs through solenoid valves that can be triggered to open when required. This minimises direct contact with the operator or outside environment and thus reduces the risk of contamination.

2.7.5 Determination of growth of bacterial cultures

Several methods are available for determining the growth of bacterial cells in culture, including directly counting cells using a haemocytometer as described (Section 2.5.6). This is, however, suitable only for cells in suspension. When cells are grown on solid agar plates, colony counting can be used instead to estimate growth. This method assumes that each colony is derived from a single cell, which may not always be the case, since errors in dilution and/or streaking may result in clumps rather than single cells producing colonies. In addition, suboptimal culture conditions may cause poor growth, thus leading to an underestimation of the true cell count. When cells are grown in suspension, changes in the turbidity of the growth medium could be determined using a spectrophotometer and the absorbance value converted to cell number using a standard curve of absorbance versus cell number. This should be constructed for each cell type by taking the readings of a series of known numbers of cells in suspension (see also Section 12.4.1).

2.8 POTENTIAL USE OF CELL CULTURES

Cell cultures of various sorts from animal and microbes are becoming increasingly exploited not only by scientists for studying the activity of cells in isolation, but also by various biotechnology and pharmaceutical companies for the production of valuable biological products including viral vaccines (e.g. polio vaccine), antibodies (e.g. OKT3 used in suppressing immunological organ rejection in transplant surgery) and various recombinant proteins. The application of recombinant DNA techniques has led to an ever-expanding list of improved products, both from mammalian and bacterial cells, for therapeutic use in humans. These products include the commercial production of factor VIII for haemophilia, insulin for diabetes, interferon-α and β for anticancer chemotherapy and erythropoietin for anaemia. Bacterial cultures have also been widely used for other industrial purposes including the large-scale production of cell proteins, growth regulators, organic acids, alcohols, solvents, sterols, surfactants, vitamins, amino acids and many more products. In addition, degradation of waste products particularly those from the agricultural and food industries is another important industrial application of microbial cells. They are also exploited in the bioconversion of waste to useful end products, and in toxicological studies where some of these organisms are rapidly replacing animals in preliminary toxicological testing of xenobiotics. The advent of stem cell culture now provides the possibility of treating diseases using cell-based therapy. This would be particularly important in regenerating diseased or damaged tissues by transplanting stem cells programmed to differentiate into a specific cell type specialised in carrying out a specific function.

ACKNOWLEDGEMENTS

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2.9 SUGGESTIONS FOR FURTHER READING

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