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Cell culture is the art of growing cells in vitro. Although cells are probably influenced by the presence of each other, they have not become organized into a tissue. Cell culture allows one to look at cells in their entirety from the outside - that is, as a whole - before examining the intricacy of their component parts. In this chapter, we will provide the tools for using cell culture as an adjunct to the cell biology and molecular biology methods that break down the traditional borders between the disciplines of biology and virology.

As with any chapter on the methodology of cell culture, one must first determine the cell type of choice needed for a particular study. There are over 3200 characterized cell lines today, derived from over 75 species, including hybridomas and plant cultures. Several national and international cell banks, including the American Type Culture Collection (Rockville, Maryland, U.S.A.) and the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, U.K.) are excellent resources for cells and specific cell information. Detailed information on media composition, plaque cultures, microcultures, and specialized techniques are also well documented (Freshney 1983; Schmidt 1989).

Once the cell type that exhibits some unique characteristic(s) has been selected, make certain that the life expectancy of these cells in vitro is sufficient to complete the project. It is preferable to use a cell line which can be propagated for at least 15–20 passages or subcultures beyond the seed stock while retaining its special characteristics.
Cell cultures

Primary cells

Primary cells are freshly isolated cells that are derived directly from the tissue of origin. The tissue source for the majority of primary cell cultures is either laboratory animals or human pathology specimens. Research institutions should have in place a review and approval process for procuring laboratory animals and human tissue. When a project utilizing laboratory animals, and particularly human tissue, is being developed, this approval process must be followed explicitly.

Healthy young or embryonic animals are preferable to adults. The younger the animal, the greater the potential for success in culturing the cells. Because there are numerous cell types, there are numerous methods of culturing these cells. The following is a general protocol for processing primary monkey kidney cells; it may need modification for use with other species or organs.

Removing the organ

1. The special skills of a veterinarian, or highly trained technician under the supervision of a veterinarian, are required for the successful removal of the monkey kidney. The kidney must be removed with a minimum of trauma to the animal and to the organ. The animal handlers and any technician involved in this process must be trained and certified by the supervising veterinarian.
2. To prevent drying and loss of viable cells, the kidney is placed in a flask containing an isotonic solution (balanced salt solution). Antibiotics are generally added to the medium to help prevent contamination.

Processing the organ

1. With sterile forceps, transfer the kidney to a beaker, and carefully remove the capsule with the forceps and surgical scissors. With surgical scissors, mince the kidney into small pieces (approximately 3 mm in diameter), add 100 ml of 0.22% trypsin-0.02% versene, and pour the contents of the beaker into a 250 ml indented Erlenmeyer flask (Melnick), which contains a magnetic stir bar.
2. Place the beaker on a magnetic mixer. Adjust the stirring speed to prevent foaming and stir for 2 min. Discard the first supernate-suspension as a wash, then add fresh enzyme and pour off every 20 min, repeating the interval harvest until the cortex tissue is exhausted. Collect the cell suspension by filtering through two layers of sterile gauze into 250 ml centrifuge bottles, and add 25-50 ml of cold growth medium.
3. Centrifuge the cell suspension immediately after each harvest at 65 g for 20 min at 4°C. Aspirate the trypsin-versene solution from the cells.
4. Add 50 ml of growth media to each bottle of packed cells and swirl vigorously until an even suspension is obtained.
5. After completing the cell harvest, filter the entire suspension through two layers of sterile gauze.
6. Determine the cell concentration (see page 13). Crystal violet is the dye of choice for primary cells, however, since cell fragments will also stain, count only the whole cells.
7. Seed the primary cells at $3 \times 10^5$ ml$^{-1}$.
Continuous cell lines

Cell lines are continuously growing subcultures of the primary cells. Most cell lines may be propagated in an unaltered form for a limited number of cell generations, beyond which they either die out or give rise to continuous cell lines. The ability of a cell line to grow continuously probably reflects its capacity for genetic variation, which allows subsequent selection to occur (Freshney 1983).

Euploid cells

Normal human fibroblasts remain predominantly euploid (containing exact multiples of the normal chromosomal number) throughout their culture life-span, rarely give rise to continuous cell lines and at crisis (usually around 50 generations) will stop dividing (Hayflick and Moorhead 1961).

Aneuploid cells

Mouse fibroblasts and cell cultures from a variety of human and animal tumors, on the other hand, often become aneuploid (chromosome number is not an exact multiple of the normal number) in culture and give rise to continuous cultures with fairly high frequency. Continuous cell lines are usually aneuploid and have a chromosome complement between the diploid and tetraploid values. Nonfibroblast normal cells rarely give rise to continuous cell lines.

The alteration in a culture giving rise to a continuous cell line is commonly called 'in vitro transformation' and may occur spontaneously or be chemically or virally induced. The term ‘transformation’ has been applied to the process of formation of a continuous cell line partly because the culture undergoes morphological and kinetic alterations, but also because the formation of a continuous cell line is often accompanied by an increase in tumorigenicity (Freshney 1983). The condition that most predisposes primary cells to develop a continuous cell line is probably inherent genetic variation, so it is not surprising to find genetic instability perpetuated in continuous cell lines.

Insect cells

The most commonly utilized insect lines are the SF9 (ovary, fall armyworm, *Spodoptera frugiperda* (ATCC # CRL 1710) and mosquito cell lines such as *Aedes aegypti* (ATCC # CRL 125), and *Aedes albopictus* (ATCC # CRL 126).

The SF9 cell line culture medium consists of Graces’ medium plus 3.3 g of lactalbumin hydrolysate and 3.3 g of yeastolate per liter with 10% heat-inactivated fetal bovine serum. This cell line is highly susceptible to infection with Baculoviruses and can be used to study recombinant genes in Baculovirus expression vectors and for other needs (Vaughn et al 1977; Smith et al 1985).

Mosquito lines are suitable for the replication of many mosquito-borne viruses (Singh 1968; Singh 1971; Yunker and Cory 1969; Buckley 1969). Mitsuhashi and Maramorosch Insect Medium supplemented with 5–20% fetal bovine serum is commonly used for the growth and maintenance of mosquito cell lines. Subcultures are prepared by scraping (see page 7) or by vigorous pipetting. A subcultivation ratio of 1:4 to 1:10 is recommended.

Propagation of cell cultures

All procedures involving cell lines should be performed in a Class II biological safety cabinet under strict aseptic conditions to minimize the risk of contaminating the cells with bacteria, yeasts, and mycoplasmas. All pipetting should be done using an automatic pipet-aid or manual propipetter, since absolutely no mouth pipetting should be allowed in the laboratory. Laboratory personnel should wear lab coats and surgical gloves while performing cell culture manipulations. All cell lines should
be treated as potentially hazardous. Continuous cell lines are assumed to be free of infectious viruses, but in fact may harbor latent viruses that have the potential to infect the laboratory worker. Transformed cells may spontaneously produce a potentially oncogenic virus (Barkley 1979). Therefore, all cell lines should be treated as potentially hazardous, and all laboratory workers should become familiar with safe laboratory practices. Institution directors and laboratory supervisors should provide safety training and should enforce safety regulations. Laboratory workers should also share responsibility for their own safety.

Media and buffers

Most media in use today are chemically defined, but are supplemented with 5–20% serum. The medium used for any particular cells will be determined by the cell type and the procedures to be performed with these cells. Cells purchased from cell repositories will be accompanied by an instruction sheet which recommends the appropriate medium, subculture procedures, correct seeding concentrations, feeding schedule, and any safety concerns.

Generally, cells will grow well at pH 7.0–7.4. Phenol red is commonly used as an indicator in the culture medium. It is red at pH 7.4, becoming orange at pH 7.0 and yellow at pH 6.5, and in the alkaline direction, it becomes bluish at pH 7.6 and purple at pH 7.8 (Freshney 1983).

Culture media require buffering under two sets of conditions: (1) use of open dishes, in which exposure to oxygen causes the pH to rise, and (2) when CO₂ and lactic acid are overproduced in transformed cell lines at high cell concentrations, when the pH will fall. A buffer may be incorporated in the medium to stabilize the pH, but in open dishes, exogenous CO₂ may still be required by cell lines, particularly at low cell concentrations, to prevent the total loss of dissolved CO₂ and bicarbonate from the medium. In an overproduction scenario, it is usually preferable to leave the cap loose or to use a CO₂-permeable cap to promote the release of CO₂. Flasks with gas-permeable caps are available from Costar (Cambridge, MA), Corning (Corning, NY), and others. (Use of trade names is for identification purposes only and does not imply endorsement by the Public Health Service or U.S. Department of Health and Human Services.)

The CO₂ level is extremely important; a 1% increase can result in cell death in some cases. Therefore, even with a state-of-the-art incubator with digital read-outs, the CO₂ level must be monitored, since it can drift undetected by the read-out. In our laboratory, a Bacharach Fyrite Gas Analyzer (Bacharach, Inc., Pittsburgh, PA) is used once a week to monitor the CO₂ incubators.

The need for endotoxin-free water

Pyrogen-free water should be used to make any medium components made in-house. Small, inexpensive reagent grade water systems are available commercially (Culligan, Marietta, GA; Millipore, Bedford, MA). These units will produce Type I reagent grade water as described by NCCLS Document C3–A2: Preparation and Testing of Reagent Water in the Clinical Laboratory. We recommend that a reputable manufacturer of pure water equipment be contacted for guidance on the purchase of an appropriate system for laboratory applications. The manufacturer’s recommendations will primarily depend on their analysis of the quality of the source water. The manufacturer will provide maintenance and sanitization on a contract basis or provide written procedures for laboratory maintenance.

Type I reagent grade water must be used as soon as it is produced because when it is stored, its resistivity will decrease, metals and/or organic contaminants will be leached from the storage container, and bacterial contamination will occur (NCCLS Document C3–A2, 1991). Due to this degradation, purchased
bottled water is not an acceptable alternative to an in-house pure water system.

**Sterilization**

Most reagents or media can be sterilized either by autoclaving if they are heat-stable (water, salt solutions, amino acid hydrolysates) or by membrane filtration if they are heat-labile. Filters appropriate for the sterilization of cell culture media are available from many sources such as Costar, Corning, Nalge, and Gelman. The type of filter membrane is important and care should be taken when selecting a filter system. Choose from cellulose acetate membranes for applications involving low protein binding. Cellulose nitrate membranes are appropriate for general purpose filtration in the cell culture laboratory. A membrane pore size of 0.22 μm should be used for sterilization. Thick cotton pads or membranes of larger pore size should be used for prefiltering.

**Propagating cells by scraping**

Removing anchorage dependent (adherent) cells from the cell culture flask requires a chemical or physical method. The enzymes used to detach cell monolayers may be toxic to some cells; therefore, disposable cell scrapers (Cat. # 3010, Costar Corp., Cambridge, MA) may be used. Cell scraping is a fairly simple method for cell harvesting. The growth medium is removed from the culture flask, and the cell sheet is then detached by physically scraping the cells from the flask surface. To disperse the cells, a growth medium is added to the flask and the medium is pipetted over the flask surface to obtain a single cell suspension. The cells are then counted and appropriately diluted (see page 13) for passage to new flasks or other types of cell culture containers.

Some laboratories depend on 'split ratios' rather than on cell counts to passage cells. These laboratories determine the optimum ratio at which a confluent flask may be divided to yield viable confluent monolayers within the time frame necessary for their projects. This method is an option to be considered; however, dye exclusion cell counts are more reliable for consistent results because these counts provide the number of 'viable' cells, and the quantity of living cells that are actually being subcultured can be determined without guesswork.

**Propagating cells by enzyme treatment**

**Advantages and disadvantages**

For chemical detachment of adherent cells from tissue culture flasks, certain enzymes (trypsin, pronase and collagenase) and chelating agents (versene or ethylene-diamine-tetra-acetic acid (EDTA)) are routinely used. Utilizing a combination of trypsin and versene is a convenient and efficient method of detaching the majority of adherent cell lines with a minimum of cell damage; this method is detailed below. Note that some enzymes remove receptors or other important cell surface molecules; therefore, the effects of these enzymes on the characteristics of the cell line chosen must be determined.

**Subculture protocol**

1. Observe the cell monolayer under a microscope to determine the general condition of the cells and any obvious contamination. If the cells are healthy and appear to be free of contamination, proceed with trypsinization.
2. Decant or aspirate the spent medium and wash the cell layer with PBS that has been warmed to 37°C; the washing removes any remaining serum, which would inhibit the enzyme activity of the trypsin/versene.
3. Decant the wash solution and add trypsin/versene (0.05% trypsin, 0.53 mM EDTA; Cat. # 25300-047, GIBCO BRL, Grand Island, NY) to the
flask, approximately 3 ml per T-75 or 6 ml per T-150 flank. Place the flask at room temperature with the cell surface down until the cells detach. If trypsinizing a roller bottle, the bottle may be rotated at the work station during the wash, but should be placed on the roller drum while waiting for the cells to detach. Occasionally, a cell line will be difficult to remove from the flask; incubating at 37°C for a few minutes or gently tapping the flask against the heel of your hand will accelerate the process. Some cell lines such as MDCK may require two washes of trypsin–versene to accelerate detachment.

4. When cells detach, add growth medium to the flask and wash all flask surfaces by repeatedly pipetting up and down to suspend as many cells as possible in the medium and to break up any existing cell clumps.

5. Perform a cell count (see page 13) and seed new flasks or other vessels accordingly. Incubate the newly seeded vessels at 37°C.

Subculture protocol

The subculture of suspension cells is a simple and fast procedure.

1. Allow the suspension to settle, and aspirate most of the spent medium before doing a cell count. Proceed with subculture by transferring the number of cells determined necessary to establish the new culture to the appropriate vessel containing fresh growth medium. If the cells do not settle well, centrifugation at a low speed may be necessary.

2. Suspension cells may also be cultured by dilution. A 1:10 dilution is usually sufficient to establish a new culture; one part cell suspension transferred to nine parts fresh growth medium will accomplish the subculture rapidly and efficiently. This procedure is less precise, however, and viability has not been assessed.

The pH of suspension cultures is critical. Observe cultures closely for the color changes described previously (page 6) and initiate appropriate procedures if the pH changes drastically. Culture vessels with vented caps are helpful in maintaining the pH in suspension cultures as well as in adherent cultures that require a CO₂ atmosphere.

Suspension cultures

Usefulness

Some cells will not adhere to a culture vessel but can be propagated in suspension. Many mouse and human leukemias and ascites tumors grow in suspension. These cells are incubated on shakers or on roller drums or allowed to settle to the bottom of a large culture flask such as a roller bottle. Alternatively, a mechanical method is available which forces normally adherent cells to grow in suspension. This is usually performed by using a spinner apparatus and medium formulated for suspension culture. Cells are available that are easily adapted to spinner culture, such as HeLa S3 (ATCC # CCL 2.2).

Lymphocyte cultures

Fresh mononuclear cells can be isolated from whole blood by Ficoll-Hypaque gradient centrifugation (Boyum 1968) and cultured in suspension. These cells have multiple uses, including the following: after stimulation using
lectins, such as phytohemagglutinin (PHA), they can be cocultivated with blood specimens to determine whether human immunodeficiency virus (HIV) is present. Other uses for these cells for diagnosis, evaluation of immunity, detection of disease or cellular networking are too numerous to discuss in this chapter.

Procedures for the isolation of particular subpopulations of cells (T cells, B cells, and macrophages) from peripheral blood or lymphoid tissues may be found in a National Institutes of Health publication (Coligan et al 1991).

**Maintenance of adherent and suspension cells**

**The need for routine medium changes**

Once a culture has been passaged, routine medium changes (feeding) (i.e. to replenish constituents in the medium, such as amino acids, vitamins, and glucose, which are essential for metabolism) are necessary to allow the cells to proliferate. The initial cell concentration and the metabolism rate will determine any feeding schedule instituted.

**The need for subculturing**

Close observation of color changes in the medium, which indicate an increase or decrease in pH level and the rate of metabolism, is necessary to determine if cells require feeding or subculturing. Epithelial cells such as HeLa and HEp2 metabolize rapidly and can be subcultured twice a week. Fibroblast cells such as MRC5 and WI-38 metabolize slowly and would probably be ready for subculture only once a week and would require feeding at least once during that week. Suspension cultures must be subcultured or the volume of medium increased as the cell population reaches $2 \times 10^6$ cells ml$^{-1}$, because, in general, the viability of suspension cells drops rapidly at higher concentrations.

Seeding concentrations of a rapidly proliferating cell line such as HeLa may be lowered to reduce subculturing to once a week. A seeding concentration as low as 20,000–30,000 cells cm$^{-2}$ of surface area will result in a healthy monolayer. If cell viability begins to decrease, then the twice-a-week subculture schedule should be resumed.

A ‘maintenance’ medium, which is the regular medium with only 1 or 2% serum rather than 10–20%, may be used to hold some cell lines at a single cell layer for an extended period, even for 2–3 weeks if necessary. This holding or maintenance medium tends not to stimulate mitosis in most untransformed cells, which usually accompanies a medium change. This holding medium can also be used to maintain cell lines with a finite life-span without using up the limited number of cell generations available to them. Transformed cells, on the other hand, are unsuitable for this procedure because they may either continue to divide successfully or the culture may deteriorate.

**Cell synchronization**

The growth of cells in cultures can be divided into three stages: (1) a lag period, immediately after the inoculum; (2) a growth phase, during which the cell number increases rapidly and usually in an exponential fashion; and (3) a plateau or stationary phase, during which the cell number remains constant. In exponentially growing populations, the cells are distributed asynchronously throughout the cell cycle in its four phases – G$_1$, S, G$_2$ and M (Ashihara and Baserga 1979).

The M (mitosis) phase is the cell's reproductive cycle. The much longer phase between one cell division and the next is known as interphase, consisting of the G$_1$, S, and G$_2$ phases. The G$_1$ phase (G = gap) is the interval between the end of mitosis and the beginning of DNA synthesis or S phase. The G$_2$ phase is the interval between DNA synthesis and the beginning of mitosis.
Any study of cellular events at specific times during the cell cycle is facilitated by culture synchronization, such that cells can be induced to proceed through the cell cycle together. Many ways to synchronize cultured cells have been described (Table 1.1); the method chosen depends on which cells are used and which events are to be studied. Synchrony of human diploid cells is short-lived, usually much less than the mean cell cycle time, and never complete. Human non-diploid established lines, such as HeLa cells, may respond to any of the methods generally applicable to mammalian cells, and synchrony may be more successful and lasting (Priest 1971).

Ideally, synchronized populations of cells in culture should meet the following criteria: (1) they should be perfectly synchronized at a specific point in the cell cycle; (2) the procedure used for synchronizing cells should have little or no effect on the metabolic processes of the cell; and (3) especially for the biochemist, the method should allow the harvesting of synchronized cells in large quantities (Ashihara and Baserga 1979).

Because mammalian cell populations have an intrinsic variability in cell doubling times, it is impossible to achieve perfect synchrony, i.e. all the cells passing through every point in the mitotic cycle simultaneously. The best that can be achieved is for all the cells to be at one single point in the cycle at a given time; thereafter they will lose synchronization as they progress through the mitotic cycle. Continued synchronization is only likely to be maintained by repeated treatment of the cell population, but the lack of synchronization at some time interval after a single treatment does not necessarily imply that the original synchronization procedure was ineffective (Nias and Fox 1971).

An example of synchronization is described by Held et al. (1989). Their procedure is a simple, nontoxic protocol based on the selection of a rapidly attaching subpopulation of trypsinized cells. By limiting the time of attachment of trypsinized cells and the subsequent removal of unattached cells, a G₁ population of cells is isolated.
Quality assurance and contamination

Bacteria, yeasts, fungi

Many types of organisms may be found as contaminants of cell cultures. Some of them, such as bacteria, yeasts, and fungi may be detected by turbidity and pH change of the culture medium or, more definitively, by examining the cultures by microscope. Detailed protocols for the detection of most bacteria and fungi that would be expected to survive in cell cultures may be found elsewhere (ATCC 1985). These protocols should also be incorporated into the quality assurance of a cell culture media production laboratory to preclude the contamination of cells by the growth media.

Mycoplasmas and viruses

Organisms such as mycoplasmas and viruses usually do not cause turbidity or pH change and may or may not produce cytopathological effects (CPE) in the cells. They are thus difficult to detect and may be passaged with the cells indefinitely without detection, unless specific testing is performed. Primary rhesus monkey kidney cells (MK), primary African green monkey kidney cells (AGMK), and primary bovine kidney cells (PBEK) are particularly notorious for adventitious virus contamination (Crandell et al 1978; Hsiung 1969; Schmidt 1989). Commercial testing services are available for mycoplasmas and viruses. Test kits are commercially available for mycoplasma, such as the MycoTect test (GIBCO #189-5672). Two of the most common methods for mycoplasma detection in the cell culture laboratory are culture and fluorescent staining. These methods may be incorporated into the quality assurance procedures of a laboratory relatively easily. The DNA specific fluorescent stain (Chen 1977) may be used as the presumptive test, and the culture method (Hayflick 1973) as the confirmatory test.

Other cell lines

Cross-contamination between cell lines has occurred often, the most problematic being the intrusion of HeLa cells into many other lines. Therefore, it is extremely important to take the following precautions. Never work with more than one cell line at a time. Do not use a bottle of medium, trypsin/versene etc on more than one cell line. Allow a 'resting' period of approximately 30 min between cell lines in a biological safety cabinet, and decontaminate cabinet surfaces before introducing another cell line to the work area.

Determining species of origin

Species of origin can be determined for cell lines by a variety of immunological tests, by isoenzymology and/or by cytogenetics (ATCC 1985). This testing requires expertise that is not available in most cell culture laboratories; however, testing services are available for these procedures (e.g. Childrens’ Hospital, Detroit, MI).

If a laboratory routinely receives cell lines from other research institutions, an area should be established to isolate these cell lines until the quality assurance testing can be performed on them and they are declared to be free of contamination and the correct species is identified. This may seem extreme, but our laboratory has on several occasions
received cells that were contaminated with mycoplasma or with another cell line. Also, quality testing information from the sending laboratory should be reviewed by the receiving laboratory before culturing the cells.

We cannot emphasize strongly enough that these quality assurance tests must be performed _routinely_ in any laboratory in which cell cultures are used. Contaminants may cause irreversible changes in cell cultures, and they can completely confound the interpretation of a diagnostic test. Therefore, results from diagnostic tests or any research in which cells were used would be suspect unless these quality assurance tests were performed with satisfactory results.
Quantifying by cell counts

Purpose and choice of method

Cells are counted in order to seed stock cultures with a known number of viable cells, to determine cell propagation rates, and to determine viability in a cell culture. Cells are usually counted in the presence of a vital stain, a dye which is only incorporated into a cell if it is no longer alive. Hence dyes such as trypan blue, which stain only the dead cells, can be used to determine cell viability. Since the cell membrane of living cells prevents the cells from taking up the dye, procedures in which these vital stains are used are known as dye exclusion tests.

Trypan blue vital stain procedure

A cell suspension sample which has been combined with a dye is placed in the counting chamber of a hemocytometer, and the cells are counted with a low-power microscope. The count is then mathematically converted to the number of cells per milliliter. The stained (dead) cells and the unstained (living) cells are counted separately to determine the percentage that are viable. Trypan blue and erythrosine B are the most common dyes used for viability dye exclusion tests. Other dyes, such as methylene blue, acridine orange, eosin, nigrosin, and safranin, have been used as ‘exclusion’ dyes but are no longer preferred (Tolnai 1975). Dyes such as crystal violet stain both living and dead cells and are best used to clarify morphology (Bird and Forrester, 1981).

A working solution of trypan blue (0.4% in physiological saline) that has been filtered through membrane (and can be stored at room temperature) may be purchased through several suppliers, including GIBCO BRL (Cat. # 15250-012).

1. Gather the cell suspension sample, trypan blue dye, counting chamber (Improved Neubauer 0.1 mm deep, conversion factor = 10,000), coverslip, and hand tally counter (the counting chamber, coverslips, and tally counter may be purchased through a scientific supply catalog such as Fisher or Thomas).

2. Prepare the chamber and coverslip. Clean the counting chamber and coverslip with 70% alcohol. Dry both thoroughly, being careful not to scratch either. Moisten the edges of the coverslip slightly and apply to the supporting edges of the counting chamber.

3. Mix the cell sample thoroughly and add 1 ml of dye to 1 ml of the cell sample. Allow mixture to stand for at least 5 min, but not more than 15 min, before performing the cell count.

4. Using a Pasteur pipette, gently fill one side of the counting chamber. For an accurate count, the chamber should not be underfilled or overfilled.

5. Examine the cells through a microscope at about 100× magnification by first focusing on the grid of the chamber and then on the cells themselves. Count the cells in the four large corner squares. Each corner square is made up of sixteen small squares. Count cells within each small square, and to avoid counting some cells twice, count overlapping cells using the line method (i.e. count
the left and top lines of each small square or the right and bottom lines, but not both). Count each cell in a clump of cells.

6. Determine the number of viable cells per milliliter in the original suspension, using the following formula:

\[
\text{[(no. of cells counted) ÷ (no. of squares counted)] × [counting chamber conversion factor] × [dilution factor]} = \text{cells per ml}
\]

(For counting chamber conversion factor see package insert.)

Example: 1 ml of dye added to a ml of cell suspension = 1:2 dilution, therefore the dilution factor = 2. The conversion factor of the hemocytometer = 10,000. Therefore, 356 (cells) ÷ 4 (large sqs.) × 10,000 (conversion) × 2 (dilution) = 1.78 × 10^6 (cells/ml)

We recommend that at least 100 cells be counted for routine subcultures. It may be necessary to concentrate the cell suspension by centrifugation for more accurate results. If the cell suspension is heavily concentrated and results in too many cells to count, the sample may be diluted in growth medium before the dye is added.

Example: 1 ml cell suspension added to 4 ml of medium = 1:5 dilution. 1 ml of the 1:5 dilution added to 1 ml of dye = 1:2 dilution, and thus the dilution factor used in the formula is the product of both factors: 5 × 2 = 10 (the total dilution factor).

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**Viability determination**

For viability determination, use the cell counting procedure described above to count viable and nonviable cells.

Using the following formula, determine the percentage of viable cells.

\[
\frac{\text{Viable cells (Unstained)}}{\text{Viable Cells + Dead Cells (Stained)}} \times 100 = \% \text{ Viable cells}
\]

Example: 254 ÷ (254 + 5) × 100 = 98% Viable cells

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**Stock cell culture seeding**

The volume of the initial cell suspension needed to seed subsequent cultures is determined by the standard volume/concentration equation:

\[
\text{Initial Concentration} \times \text{Volume of Initial Concentrate} = \text{Final Concentration} \times \text{Final Volume}
\]

For example: To determine what volume of a cell suspension containing 5 × 10^6 cells ml^-1 should be diluted to 1 ml to yield 2 × 10^6 cells ml^-1

\[
5 \times 10^6 \text{ cells ml}^{-1} \times (X \text{ ml}) = 2 \times 10^6 \text{ cells ml}^{-1} \times (1 \text{ ml})
\]

\[
X = \frac{2 \times 10^6 \text{ cells ml}^{-1}}{5 \times 10^6 \text{ cells ml}^{-1}} = 0.4 \text{ ml}
\]

Therefore: 0.4 ml of original suspension diluted with 0.6 ml of medium will yield 1 ml of suspension at 2 × 10^6 cells ml^-1.
Cell preservation and storage

Cryopreservation procedure

Cells may be preserved by freezing or 'cryopreserved'. This provides a ready stock of cells if cells are lost due to contamination, malfunction of the incubator, natural senescence, or a genetic drift that may affect their ability to exhibit the expected response characteristics to a routine procedure or assay.

As most laboratories are not equipped with a controlled rate freezing apparatus, we find the following procedure yields successful cryopreservation results. First and foremost, only cells that are healthy, free from contamination, and in active logarithmic growth phase should be selected for preservation. In general, cells should be frozen at a minimum of $1 \times 10^6$ cells ml$^{-1}$; a routine number of $2-4 \times 10^6$ cells ml$^{-1}$ is used as the standard in our laboratory.

After enumeration by trypan blue cell counting technique (page 13), the cells are centrifuged at 80–100 g and resuspended in the preservation or freeze medium that has been warmed to room temperature. The preservation medium consists of the culture medium containing 5–20% serum plus 5% dimethylsulfoxide (DMSO) or glycerol, preferably DMSO (Lovelock and Bishop 1959; Freshney 1983; Schmidt 1989).

The cells are then dispensed into cryovials, 1 ml per vial. Cryovials, which are recommended for $-70\degree C$ or nitrogen storage, may be purchased from numerous commercial sources, such as the Nalgene Cryovial (Nalge Company, Rochester, NY).

The cell suspension should be frozen slowly at $1\degree C$ min$^{-1}$ (Leibo and Mazur 1971; Harris and Griffiths 1977). If a controlled rate freezing apparatus is not available, the cells may be cooled gradually by placing at $4\degree C$ for 1 h, then at $-20\degree C$ for at least 1 h and transferred to $-70\degree C$ or a liquid or gas phase nitrogen freezer. The much lower temperature ($-165\degree C$) obtained in nitrogen is preferable for long-term storage.

An inexpensive and extremely efficient controlled rate freezing apparatus is now available (9001 BTRL Cell Freezer, Biotech Research Laboratories, Inc., Rockville, MD). The 9001 BTRL Cell Freezer consists of a can with a foam grid support and vial grid that will hold 24 vials. The can is filled with methanol, ethanol, or isopropanol as a refrigerant to a level that will be slightly higher than the level of the cell suspension in the freezing vials. After the vials are placed in the vial grid, the 9001 freezer can is sealed and placed in a $-70\degree C$ to $-90\degree C$ freezer. The 9001 unit should be left undisturbed for at least 5 h, after which the vials can be transferred to a liquid nitrogen freezer.

Recovery of frozen cells

A vial of cells is thawed rapidly by agitating the vial in a water bath at $37\degree C$. The cells are then diluted, but not washed, with the appropriate culture medium and placed in a cell culture flask. The dilution should be determined by the concentration of viable cells present at...
the time of freezing. A vial containing $2 \times 10^6$ cells ml$^{-1}$ may be diluted successfully to 1:10 or 1:20.

During the initial growth period, the pH should be carefully controlled. Therefore, it is standard procedure in our laboratory to place these cultures in a 5% CO$_2$ atmosphere. The medium must be replaced after 24 h to remove all traces of DMSO or glycerol. The medium exchange on a suspension culture would necessitate centrifugation and resuspension in the new culture medium.

During all phases of this procedure, precautions should be taken to prevent inhalation of DMSO fumes and contact of DMSO with the skin. A face shield or safety glasses should be worn while removing the vial from the freezer and during the thawing process.
Transformation and transfection

Most vertebrate cell lines die after a finite number of divisions in cell culture, although rare 'immortal' variant cells can arise spontaneously in culture and can be maintained indefinitely as cell lines. DNA cloning and genetic engineering have given us techniques that enable us to isolate specific genes, redesign them, and insert them back into cells. This technology has revolutionized the study of living cells. Eukaryotic cell lines are being developed with indefinite replication ability to express at least some of the differentiated properties of their cell of origin, and to not necessarily cause tumors if injected into animals.

The procedures for generation of transformed cell lines are well documented (Freshney 1983). Genetic engineering procedures are too numerous and involved for this chapter; an excellent source of detailed procedures is Ausubel et al (1991).
Cytopathologic effects in cell cultures due to viruses

The general principles of collection, shipment, and processing of specimens, choice of viral isolation systems, and choice of diagnostic tests can be found in the next chapter and in other reference works (Fields et al 1990; Hierholzer 1993; Leland and French 1988; Mandell et al 1990; Schmidt and Emmons 1989). Information surrounding viral CPE is given here because cell culture is so vital to the laboratory diagnosis of viral infections.

Viral growth is usually evidenced by the CPE that occur in the infected cell monolayer. CPE are scanned under light microscopy at 40–100× and observed in greater detail at 200–400×. The exact nature of the CPE and the time required for it to appear in a particular cell type are often indicative of the virus group present there. Thus, careful observation of the monolayers is important so that the proper identification test can be expediently applied. CPE caused by different viruses in various cells are summarized below.

Types of CPE in standard cell cultures

Enlarged, round, refractile cells in clusters

Grape-like clusters of round, refractile, enlarged cells in cultures of HEK, HEP2, A549, NCI-H292, HeLa, and many other cells indicate adenovirus or herpes simplex virus. For adenovirus, the same CPE may develop, but more slowly, in fibroblast cells. At complete (4+) CPE, all cells become lysed and detached from the glass surface. Generally, both viruses grow in the same cells, but adenovirus grows more slowly (3–14 days) depending on serotype, and makes more discrete, irregularly-shaped cell clusters, whereas herpes type 1 grows very fast (1–2 days), makes fewer clusters, and may produce ballooned, multinucleate giant cells with granulated cytoplasm.

Multinucleate giant cell CPE

Measles virus produces classic giant cell CPE in AGMK, Vero, and HEK cells after 7–14 days of roller culture. This CPE is characterized by fusion of the cells, with the nuclei of the fused cells surrounding a granular area in the cytoplasm. Cytomegalovirus produces giant cells resembling elongated foci of refractile, swollen cells, but more slowly (12–30 days) and only in diploid fibroblast cells, also on a roller apparatus.

Syncytial CPE

The classic example of this CPE is respiratory syncytial virus (RSV), which produces patches of multicell syncytia in HEP2, HeLa, and NCI-H292 cells in 5–12 days in roller cultures. The ball-ed-up syncytia usually become detached from the glass surface and float freely in the medium. Some strains of RSV, particularly of type B, produce more cellular degeneration than syncytia both in HEP2 and NCI-H292 cells.

The influenza, parainfluenza, and mumps viruses cause a combination of syncytia, rounding, and degeneration in roller cultures of NCI-H292 and MK cells. In addition, chick embryo and MDCK cells for influenza virus isolation and NCI-H292 cells for parainfluenza virus isolation require a fortified medium containing trypsin for optimal sensitivity (Castells et al 1990; Frank et al 1979; Klenk et al 1975; Meguro et al 1979). The syncytia may be
accompanied by vacuolation with influenza, especially type B; by a granular degeneration with parainfluenza virus types 1–4; and is often noted as large syncytia with mumps virus. The CPE induced by these viruses may develop in 4–7 days, but the cultures must generally be blind-passaged and held an additional week to ensure viral growth. The cells rarely become detached.

Enlarged, round, glassy cells in small foci

Varicella-zoster (VZ) virus exhibits this CPE in 2–10 days; it is distinct from that caused by CMV, although both grow in diploid fibroblast cells in roller culture. VZ can produce large foci of multinucleate giant cells when the individual foci coalesce.

Cell fusion CPE with plaques

The cell fusion/plaque type of CPE is caused by vaccinia virus and certain other poxviruses growing in MK, Vero, NCI-H292, and diploid fibroblast cells. Plaques ranging from 1 mm to 6 mm in diameter, depending on the virus, are formed in 2–4 days, during which the infected cells fuse, form cytoplasmic bridging, and then disintegrate.

Shrunken cell degeneration CPE

The enteroviruses and rhinoviruses typify this CPE in NCI-H292, MK, RD, and diploid fibroblast cells, preferably in roller cultures. The CPE is often observed as tadpole shaped, shrunken cells with pycnotic nuclei, beginning in patches at the edges of the monolayer and progressing inward. CPE for polioviruses and some coxsackie B viruses is very rapid, becoming 4+ in 1–3 days with all cells detached from the glass. CPE for the remaining enteroviruses and the rhinoviruses generally requires 4–7 days or longer, is often accompanied by individual small, rounded, and sometimes refractile cells or by a degenerative appearance across the monolayer, and may not ever progress to 4+ or general cell lysis.

Granular, degenerative, slowly-lytic CPE

Reoviruses cause a nondescript, gradual degeneration with granulation of the cytoplasm in NCI-H292, MK, and HeLa cells under stationary or roller conditions after 5–14 days of culture. MK cultures must be rolled. Polyoma BK virus in HEK, NCI-H292, and diploid fibroblast cells, and the polyoma JC virus in primary human fetal glial cells cause a similar, slowly-developing CPE.

Certain arboviruses belonging to the Togaviridae, Flaviviridae, Filoviridae, Bunyaviridae, and Arenaviridae families can be isolated in primary hamster kidney, chick or duck embryo cells, or derivative cell lines such as BHK-21, Vero, and LLC-MK2, after 2–10 days of culture. The CPE may be nonexistent at first, but become a generalized degeneration in sub-passages or form plaques under agarose. Other arboviruses grow best in mosquito suspension cultures (see pages 5 & 8), whereas others can only be recovered in whole animal systems such as embryonated chicken eggs, suckling mice or hamsters, or mosquitoes (these systems not discussed here).

The gastroenteritis viruses also belong to diverse virus families and have unique requirements for culture. Rotaviruses, after treatment with trypsin, will replicate in BSC-1, MA104, and primary AGMK cells under roller conditions (Babiuk et al 1977). Caliciviruses and astroviruses may replicate in primary HEK under a fortified medium with trypsin. Enteric adenoviruses (see Chapter 2) and enteric coronaviruses (discussed below) round out this difficult group of viruses.
New or specialized culture systems

Organ cultures

Laboratory cultures of pieces of human embryonic lung, kidney, intestine, and other organs have proven essential to the discovery of many viruses, such as the respiratory coronaviruses and rhinoviruses. Organ cultures are tiny explants of whole tissue, so that many cell types are present in their natural form, and feeding medium has to be perfused through the explants to maintain viability. The human enteric coronaviruses—although their existence is still in dispute—may replicate in primary human fetal intestinal organ cultures containing trypsin. Once the tissue culture is inoculated with a suspected virus specimen, the presence of virus may be detected by cessation of ciliary movement (in lung and intestine) or by specific tests for the viral products accumulated in the medium (Caul and Clarke 1975; Caul and Egglestone 1977; McIntosh et al 1967; Tyrrell and Blamire 1967).

Microcultures

Early attempts to reduce the size of cell cultures and to manipulate cultures into 4-, 6-, 8- and 96-well formats were met with severe problems of external contamination, cross-contamination between wells, overoxygenation, and toxic plastics. All of these problems have now been overcome. Today, microcultures of most cell types are commonplace in the 4- to 8-well format in plastic plates with sealable lids (or snug lids for CO₂ incubators) for plaque assays. Microculture plates in the 96-well microtiter plate configuration, but with flat well bottoms, made of nontoxic plastic, and sterilized without toxic residues, are also a standard laboratory item today. Microcultures are especially useful for large volume neutralization tests, tissue culture EIA tests, monoclonal antibody testing, and most screening assays requiring cell cultures (Anderson et al 1985; Hierholzer and Bingham 1978; Hierholzer et al 1990, 1993; Schmidt and Emmons 1989). Macrocultures in standard glass tubes, however, are still preferred for primary virus isolation because of the ease of setting up stationary or roller cultures in ordinary incubators, of reading the monolayers for CPE, and of obtaining sufficient volume of virus culture to use in identification tests and for subpassaging and storage.

Shell vial cultures

These cultures utilize the Leighton tube concept, in which the cell monolayer is established on one side of a glass coverslip inside a glass tube. The tube is then inoculated as usual and is subjected to a low-speed centrifugation, which apparently distorts the cell surface and renders it more susceptible to viral attachment. After just 1–3 days of incubation, regardless of the presence of any CPE, the coverslip is brought out, washed briefly, and tested by IFA or immunoperoxidase tests for suspected viruses. Shell vials have been particularly useful as a rapid culture test for adenoviruses, myxoviruses, herpes simplex, and cytomegalovirus, to name a few (Espy et al 1987; Gleaves et al 1985; Leland and French 1988; Matthey et al 1992).

Other specialized systems

Suspension cultures of human peripheral blood lymphocytes are sensitive to the Epstein-Barr herpes virus, where it is detected by a transformation assay, and, under cocultivation procedures, to the HTLV and HIV retroviruses; these highly specialized techniques are described elsewhere (Fields et al 1990; Leland and French 1988; Schmidt and Emmons 1989; Warfield and Feorino 1992). Immortalized human microvascular endothelial cell cultures derived from foreskin are sensitive to a wide variety of viruses under special culture conditions, and potential for use as a
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model to study the biology of these important cells (Ades et al 1992).

Extracellular matrix systems have recently been introduced for the three-dimensional growth of cells, as opposed to monolayer cultures. The Matrigel Invasion Chamber (Collaborative Biomedical Products, Bedford, MA), for instance, can support the growth of many fastidious cell types, and is currently being explored for its use in viral culture and alterations in cell physiology following viral infection (Bissell et al 1990; Thompson et al 1991).

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