Overview of Cell and Tissue Culture Techniques

The techniques for tissue and cell culture evolved from ex vivo studies of whole organs or tissue fragments that were kept in vitro for various length of time. To prolong their structural and functional integrity in vitro, the balanced salt solutions used in acute experiments were replaced, first by complex biological media (e.g., plasma, serum, or tissue extracts), then by synthetic media containing various proportions of biological fluids, and more recently by chemically defined media. At the same time, a variety of culture techniques have evolved, taking into account both the goal of a particular experimental approach and the particular growth requirements of a given cell or tissue. Culture preparations vary greatly in complexity, ranging from single isolated cells to three-dimensional histotypic cell structures. Besides cultures obtained directly from animal tissues (primary cultures), permanent cultures of continuously dividing cells have been established. Therefore, before utilizing cell cultures, there is a need to select among the multitude of established in vitro systems. In general, it appears that with the increasing complexity of the culture system, the relevance to the in vivo situation increases, while it becomes more difficult to control the cellular and molecular variables. It is unlikely that there will ever be a single in vitro approach adequate for all experimental needs. Therefore, to select a particular approach, both the advantages and disadvantages of each in vitro approach must be considered. The aim of the present unit is to highlight these issues.

CULTURE TYPES

Primary Cultures

Primary cultures are prepared with cells or tissues taken directly from the intact organism. They can be maintained in vitro for only a limited period of time, ranging from several days to months, depending upon the cell type and the culture conditions. Often, primary cultures are derived from immature cells or tissues, being allowed to differentiate and mature in vitro. In some cases, cultures of differentiated cells are prepared from tissue taken from a mature organism. It is often more difficult to obtain viable cells from differentiated tissues because of the relatively harsh dissociation methods that must be utilized. On the other hand, it is difficult to obtain cultures of a single defined cell type with immature undifferentiated tissue. The availability of multipotent cells, such as the hematopoietic precursor cells from bone marrow (Metcalf, 1984; Celis, 1998), embryonic stem cells prepared from preimplantation embryos (Conn, 1990; Celis, 1998), or stem cells from immature or adult brain (Celis, 1998) makes it possible to conduct lineage-specific cell differentiation using appropriate signaling factors. In general, primary cells undergo only a finite number of cell divisions (and may not proliferate at all in culture) and have a limited life span. With the exception of hemopoietic cells and certain transformed cells (see discussion of Suspension Cultures under Culture Techniques), most animal cells are adherent (they are therefore called "anchorage-dependent"). For growth and maintenance in vitro, these cells must attach either to a specifically treated artificial surface or to the surface of other cells. Furthermore, for the subdivision and replating of adherent cell populations ("subculturing" or "passaging"), specific techniques are used for cell detachment.

Continuous (Permanent) Cell Cultures

Continuous cultures are composed of cells that may proliferate indefinitely. Continuously dividing cells may arise spontaneously in the process of continuous subculturing of normal cells that proliferate in vitro (e.g., fibroblasts or astrocytes). Such a cell lineage is termed a *cell strain*. However, the majority of "immortal" cells belong to the class of transformed cells, and an established clone of such cells is termed a *cell line*. Transformed cells have undergone a stable heritable change and they are tumorigenic ("malignant")—i.e., they are able to form tumors in an appropriate recipient animal such as nude mice (Jakoby and Pastan, 1979). Historically, most cell lines were derived from tumors that occurred either spontaneously or after chemical or viral induction either in vivo or in vitro. Some of these cell lines exhibit a more or less undifferentiated phenotype, whereas others show certain, but never all, characteristics of the corresponding differentiated cell. Many of the currently available neural cell lines belong to the latter category, such as clones of the mouse C1300 neuroblastoma, the

Contributed by Paul Honegger
Current Protocols in Pharmacology (1999) 12.1.1-12.1.12

rat C6 glioma, the rat PC12 pheochromocytoma, and the rat RN22 schwannoma. A variety of strategies have been used in the search for new cell lines exhibiting specific differentiated phenotypes. An early approach was somatic cell hybridization (e.g., Jakoby and Pastan, 1979). By fusing transformed cells with somatic cells, immortal hybrid cells (hybridomas) were obtained that express characteristics of the normal parent cell. Cell fusion is promoted by chemicals, such as polyethylene glycol, or by viruses, such as β-propiolactone-inactivated Sendai virus. For isolation of the hybridomas, selection media containing an inhibitor of de novo nucleotide synthesis, such as aminopterin, methotrexate, or azaserine, in addition to the required purine and/or pyrimidine salvage precursor(s), such as hypoxanthine and/or thymidine, are used. For example, the fusion of normal B lymphocytes with transformed lymphocytes (myeloma cells) is a routine procedure for generating immortalized hybrids that produce the monoclonal antibody encoded by the original B lymphocyte. Also, many neuroblastoma and glioma cell hybrids are derived this way. Furthermore, techniques have been developed to prepare permanent cell lines by the transfection of immortalizing oncogenes (see discussion of Cell Immortalization under Culture Preparation and Maintenance). However, transfection procedures are generally harsh and require a relatively large number of cells. Therefore, virus-mediated transformation has been found to be a milder and more efficient method for this purpose (Cepko, 1989). Immortalization of cells is attained by retroviral gene transfer—e.g., using oncogene-containing retrovirus vectors. However, with both transfection and viral infection, heterogeneous cell populations are produced, exhibiting different sites of gene integration. To overcome this problem, a method was developed to generate genetically homogeneous immortalized cell lines from transgenic mice. An advantage of cell lines is that they can be propagated indefinitely, providing large quantities of a relatively homogenous cell population. Problems encountered with tumorigenic and hybridoma cell lines include their phenotypic and functional deviation from the normal parent cell(s), and their abnormal and unstable karyotype resulting in frequent intra- and interclonal heterogeneity. Nevertheless, cell lines are invaluable for many in vitro approaches, and are useful complementary systems for studying normal cells.

CULTURE TECHNIQUES

A number of excellent books detail the basic methods for tissue and cell culture, including medium preparation, sterilization, cell handling, aseptic working techniques, and quality control (e.g., Davis, 1994; Freshney, 1992; Jakoby and Pastan, 1979; Pollard and Walker, 1990; Boulton et al., 1992). Furthermore, there is a large body of literature providing detailed protocols of culture techniques for a great variety of cell types, including neural cells (Bottenstein and Sato, 1985; Conn, 1990; Banker and Goslin, 1991; Fedoroff and Richardson, 1997), epithelial cells (Shaw, 1996), liver cells (Brill et al., 1994), renal cells (Handler and Kreisberg, 1991), heart muscle and endothelial cells (Piper, 1990; Deli and Joo, 1996; Celis, 1998), keratinocytes (Daniels et al., 1996; Celis, 1998), and hemopoietic cells (Metcalf, 1984; Celis, 1998). Because of the wide variety of culture systems, it is sometimes difficult to select the most appropriate for study. The purpose of this unit is to provide guidance in the search for an appropriate in vitro system by detailing basic aspects of culture methodologies.

Suspension Cultures

Suspension cultures are generally used for anchorage-independent cells, such as hemopoietic cells and some transformed cell lines, and for free-floating cells or cell formations (e.g., isolated cells, aggregates, or tissue fragments). Anchorage-independent cells are grown in either a semisolid medium, such as agarose, or in fluid suspension culture under continuous agitation (stirring or shaking). Short-term suspension cultures of bulk, isolated brain cells have also been described (Verity, 1995). In general, suspension cultures are easy to maintain and are ideal for scale-up. For the latter, culture vessels of various sizes are available, ranging from small spinner flasks to industrial-size fermenters (e.g., Jakoby and Pastan, 1979).

Attached ("Monolayer") Cell Culture

Monolayer cell culture is the most widely used technique, with numerous protocol variants of this approach practiced in different laboratories (Sensenbrenner, 1977; Conn, 1990; Jakoby and Pastan, 1979). In these cultures, the cells grow attached to the surface of the culture vessel, although specific treatment of the culture vessel surface (e.g., with polylysine or polyornithine) may be necessary for better cell

attachment and/or attainment of more physiological growth conditions. The growth conditions are also influenced by the cell density. Techniques using different cell densities range from single-cell microcultures to high-density mass cell cultures. The yield in monolayer cultures is limited by the available surface to which the cells may attach. Therefore, for scaling up, cells may be grown in roller bottles (cylindrical bottles partially filled with medium and rotated around their horizontal axis at 40 to 50 rpm to provide continuous medium supply), in capillary perfusion systems (in which medium is circulated through tightly packed artificial capillaries), or attached to particulate microcarriers that can be maintained in suspension culture (Conn, 1990). With a high-cell-density culture, essential metabolites may be rapidly depleted, causing density-dependent growth inhibition and eventually cell death. One advantage of monolayer cultures is their ready accessibility for direct microscopic examination, as well as for morphological, immunocytochemical, and electrophysiological studies. On the other hand, it is relatively difficult to sample monolayer cultures for ultrastructural or biochemical analyses without destroying the culture. Furthermore, with these cultures, the cells are attached to an artificial substrate rather than to the natural extracellular matrix, limiting direct cell-cell interactions.

Three-Dimensional (Aggregate) Cell Cultures

Aggregate cultures are prepared from dissociated cells allowed to reaggregate under controlled conditions and continuous gyratory agitation to form regular, spherical cell structures. Such cultures permit a maximum of cell-cell interactions and thus the development of a natural cell matrix and histotypic cell formations. Aggregate cultures prepared from fetal cells show a particularly high capacity for cellular reorganization and maturation. Although it has been shown by Moscona, who invented this particular cell culture technique (Moscona, 1965), that immature cells of any tissue are able to reaggregate and mature into histotypic structures, this method is most suitable for immature neural cells (Conn, 1990; Fedoroff and Richardson, 1997). One advantage of aggregate cultures is that they provide large numbers of highly reproducible replicates, from which aliquots are readily sampled for multidisciplinary studies. On the other hand, aggregate cultures do not permit the direct microscopic observation of cells during culture. Furthermore, with three-dimensional cultures, oxygen and nutritional gradients are critical, limiting the size of the individual aggregate to a diameter less than 400 μm. Moreover, in certain preparations, such as aggregate cultures of fetal liver cells, an additional diffusion barrier is formed by a peripheral epitheloid cell layer. This problem is not encountered with aggregate cultures of fetal brain cells, which can be maintained for months at a highly differentiated stage. Tumor cells or explants grown in sponge-gel matrices form three-dimensional structures (called spheroids) and exhibit tissue-specific drug responses (Celis, 1998).

Explant Cultures

Explant cultures are prepared either from intact organs, such as dorsal root ganglia, sympathetic ganglia, parasympathetic ciliary ganglia, or from small tissue fragments that do not exceed 1 mm in any dimension. This approach is particularly useful for neural tissues which, due to their structural and functional complexity, are most difficult to reconstitute in vitro. Explants may be maintained in stationary culture on coverglasses or coverslips (Crain, 1976; Bornstein, 1995; Fedoroff and Richardson, 1997), or they may be placed on porous and transparent membranes (e.g., Millipore Millicell-CM; Falcon Cyclopore; Whatman Anodisc) that remain in contact with liquid medium in such a way that a thin film of liquid surrounds the tissue (Stoppini et al., 1991). Often, the attachment of explants is enhanced by substrate coating using collagen, laminin, or polylysine. As a further variant, brain slices embedded in a plasma clot on glass coverslips are maintained in a roller tube, which allows for alternating exposure of the tissue to air and liquid medium (Gähwiler, 1981). Explants (or microexplants from minced tissue) may also be maintained in nonadherent organ culture by using continuous gyratory agitation to keep the tissue in suspension. Stationary as well as free-floating explants preserve their cytoarchitectonic and gross anatomic cellular organization to a large extent, although structural and functional abnormalities relative to the in vivo situation occur. Roller tube slice cultures decrease in thickness to one to three cell layers within a few days of culture, but generally retain their histotypic organization. This preparation is particularly well suited for direct microscopic observation and electrophysiological studies. As a rule, explant cultures are the best approximation of the in vivo situation with respect to the organ-specific cellular organization and matu-

ration. For example, cerebellar explants develop normal architectural arrangements of cortical laminae and deep nuclei, and hippocampal explants form functional synaptic networks in culture, as well as normal dendritic arborizations of pyramidal and granule cells. Explant cultures also offer a unique system for the study of adjacent brain regions in coculture, such that they are able to form interacting afferent and efferent projections under in vitro conditions (Fedoroff and Richardson, 1997). The main disadvantages of explant cultures are the small amount of tissue available and the relatively low number and limited reproducibility of replicate cultures, making it difficult to use them for biochemical and molecular biology studies.

CULTURE PREPARATION AND MAINTENANCE

The technical requirements for culture preparation and maintenance vary greatly. Primary hemopoietic cells, and many permanent cell lines, are particularly easy to obtain and cultivate. A large number of cell lines originating from various tissues and species, including human tumor cell lines (Hay et al., 1994) have been characterized. Many can be purchased from commercial suppliers, including the American Type Culture Collection (ATCC) and the European Collection of Animal Cell Cultures of the Centre for Applied Microbiology and Research (ECACC/CAMR). In some cases, cell cultures have to be established from the original tissues, requiring more elaborate protocols for culture preparation and maintenance.

Tissue Dispersion

Tissue dispersion is required for preparing most types of primary cell cultures (Jakoby and Pastan, 1979). Suspensions of dissociated cells can be obtained mechanically (by cutting, mincing, shearing, or sieving), chemically (by omission of divalent cations with or without the addition of chelating agents), or enzymatically (by proteolytic separation of cells using enzymes such as trypsin, papain, dispase, collagenase, pronase, hyaluronidase, DNase, or a mixture of several of these enzymes). In some instances, a combination of dissociation techniques is used. The choice of a particular protocol depends upon the nature, developmental stage, amount of tissue, and the intended use of the cultures. In all cases, it is important to keep the tissue moist and to minimize physical and chemical trauma to the tissue and isolated cells.

Certain immature tissues, such as those from brain or liver, may be dissociated into single cells by mechanical means only. This may be accomplished by simple trituration using large-bore and/or fire-polished (constricted) glass pipets, and/or by passage through fine nylon filter mesh or stainless steel screen of defined pore size. For mechanical dissociation, the tissue is maintained in Ca^{2+}/Mg^{2+}-free balanced salt solution. In combination with proteolytic enzymes, Ca^{2+} chelators such as EDTA are used to disrupt intercellular and cell-matrix junctions. However, some proteolytic enzymes, such as collagenase, require Ca^{2+}, and DNase requires Mg^{2+}. To limit enzymatic digestion, specific protease inhibitors or serum are used. After dissociation, the resulting cell suspension is examined to assess cell yield (by counting the number of cells obtained from the original mass of tissue using a hemocytometer or an electronic cell counter) and viability (by exclusion of dyes such as trypan blue).

Cell Separation and Purification

Cell separation and purification may be necessary to prepare cultures of defined cellular composition, or to eliminate contaminating cells (e.g., erythrocytes or fibroblasts) and debris. Cells may be separated either immediately after dissociation, or after culture initiation. Dissociated cells with differences in size and/or buoyant density are separated by centrifugal elutriation (Celis, 1998) or by differential centrifugation (Pretlow and Pretlow, 1983). For density gradient centrifugation, solutions of Percoll, Ficoll, and/or sucrose can be used. Alternatively, cell separation or purification may be achieved with antibodies that recognize cell-specific surface markers. Thus, isolated cells may be separated using techniques such as fluorescence-activated cell sorting (FACS), selective immunoadsorption (immunopanning), or immunomagnetic separation. Alternatively, unwanted cells may be selectively destroyed by complement-mediated lysis. Cells may also be separated by exploiting differences in their attachment to artificial substrata using glass or plastic surfaces with or without special coating. For example, fibroblasts and macrophages attach to culture surfaces more rapidly than other cells. Oligodendrocytes derived from adult brains adhere relatively poorly to tissue culture plastic as compared to astrocytes or microglia, whereas microglial cells exhibit stronger adherence to plastic as compared to astrocytes (Fedoroff and Richardson, 1997). Cell separation by differential adhesivity may

be possible also in more advanced cultures. For example, highly purified glial subtypes can be isolated from confluent cultures of mixed glial cells using a shaking procedure (Conn, 1990; Fedoroff and Richardson, 1997). During cultivation, the enrichment of a specific cell type can be obtained using a selective growth medium, taking advantage of known cell type–specific nutritional requirements. For example, pure astroglial cultures can be obtained by use of a selective medium containing sorbitol instead of glucose, which eliminates neurons, oligodendrocytes, and microglia from mixed brain cell cultures (Fedoroff and Richardson, 1997; Wiesinger et al., 1991). Microglia can be removed chemically using defined medium containing 5 mM L-leucine methyl ester (Thiele and Lipsky, 1985). On the other hand, cultures of highly enriched brain microglia can be obtained by enhancing microglial proliferation with colony-stimulating factor (CSF-1), while eliminating the co-cultured astrocytes by starvation (Fedoroff and Richardson, 1997). Substituting lactate for glucose in the growth medium selects for oligodendrocyte precursors against differentiated oligodendrocytes, astrocytes, and microglial cells (Fedoroff and Richardson, 1997), and substituting D- for L-valine minimizes the proliferation of fibroblasts, since only cells of ectodermal origin express amino acid isomerase necessary to metabolize D-amino acids (Gilbert and Migeon, 1975). Cell type-specific toxins may be of use in some cases for selective cell elimination; for example, excitotoxins, such as kainic acid, may be used to selectively kill neurons. Overgrowing cell populations (e.g., fibroblasts or astrocytes) are often eliminated by applying antimitotic agents, such as cytosine arabinoside or 5-fluoro-2-deoxyuridine. However, if concentrations used are too high, they may also be toxic also to some nondividing cell types (Banker and Goslin, 1991).

Cell Immortalization

Immortal cell lines are generated from primary cells by integrating immortalizing oncogenes into their genome. With this approach, many immortalized cell lines have been derived, particularly from neural precursor cell populations (Gage et al., 1995). The most commonly used immortalizing oncogenes are either members of the *myc* oncogene family, or a temperature-sensitive (ts) mutant of the simian virus 40 (SV40) large T antigen. Transfection of DNA is accomplished using various enhancers such as DEAE-dextran, calcium phosphate, cationic liposomes, or electroporation. Easily transfectable cells are, for example, mouse L cells, as well as BHK, COS, and Vero cell lines. Retroviral transduction by infection with genetically modified viruses (using retroviruses such as Rous sarcoma virus) is generally better suited to generate stably integrated genes in cells of diverse origin (Cepko, 1989; Markowitz et al., 1988). However, this requires that the recipient cells be mitotically active. Nondividing cells, such as neurons, may be transfected through nontoxic mutants of other viruses such as adenovirus or herpes virus (Fedoroff and Richardson, 1997). Transfection of cells with the *myc* oncogene usually produces continuously dividing cells exhibiting different intermediate stages of differentiation. Transfection of the SV40 tsA58 strain large T antigen generates conditionally immortal cell lines which continuously proliferate at the permissive temperature (33°C), but are capable of differentiation at the nonpermissive temperature (39.5°C). Conditionally immortalized cell lines that are genetically homogeneous can be derived from transgenic mice harboring coding sequences of the tsA58 SV40 large T antigen. This strategy has proven to be applicable to various cell types, including fibroblasts, thymic stromal cells, and astrocytes (Baba et al., 1997; Jat et al., 1991).

Cloning

Cloning procedures establish a new culture from the progeny of a single parent cell (e.g., Jakoby and Pastan, 1979; Fedoroff and Richardson, 1997). By definition, a cell clone is a cell population derived from a single ancestral cell. For cloning by dilution plating, a cell suspension is sufficiently diluted to permit either the distribution of single cells to separate dishes, or the formation of isolated colonies of cells in the same dish. Alternatively, cells can be cloned by the physical isolation of single cells, for example by using the glass-shard method (Richelson, 1976). Often, isolated cells require particular media conditions for survival and/or growth. The conditioning of the medium by the cell ("autoconditioning") is facilitated by using small volumes of medium and small culture dishes (e.g., microwell plates). Growth conditions may be further improved by the addition of specific survival and/or growth-stimulating factors, conditioned medium from higher density cultures, or by the use of "feeder layer" supports—i.e., monolayer cells cultivated to confluency and then irradiated—which, in vivo, are normally in association with

the cells to be cloned, such as stromal cell feeder layers for cloned epithelial cells. The attachment of anchorage-dependent cells to the feeder layer is facilitated by the addition of polymerized collagen or fibrin. For cloning by isolated cell colonies, the cloning and culture procedure must be repeated several times to assure purity.

Passaging

Subculturing or passaging of cells is often necessary to reduce the density of growing cell cultures and is accomplished by transferring them to new culture vessels (e.g., Fedoroff and Richardson, 1997). To subdivide cultures of anchorage-dependent cells, the cells must first be detached from their substrate. Methods for the detachment and dispersal of cultured cells include mechanical shearing (shaking or scraping with a silicone rubber spatula), use of divalent-cation chelators such as EDTA, and proteolytic enzymes, such as trypsin, collagenase, or pronase. Often, a combination of these methods is optimal. Usually, a balanced salt solution is used for subculturing, and care is exercised to avoid any harsh physical manipulation and to minimize the duration of the procedure.

CRITICAL PARAMETERS

Culture medium

The specific nutritional requirements for individual cell types are only partially known, and, in many cases, the addition of poorly defined biological preparations, such as serum or tissue extracts, is still needed for successful culturing. Nevertheless, chemically defined media (Bottenstein and Sato, 1985) are suitable for a variety of culture systems, and with further progress in the identification of factors promoting cell survival, growth, and maturation, defined culture media will eventually become available for most in vitro systems. Medium components required by all or several culture systems are listed in Table 12.1.1 and include essential ions and trace elements, essential amino acids, and often additional amino acids, such as Cys, Gln, and Tyr which are synthesized in specialized cells, and in some cases also Gly, Pro, and others. Also included are essential fatty acids, metabolic substrates, and often additional metabolites such as pyruvate, choline, inositol, nucleosides, and putrescine. Various vitamins and cofactors are typically necessary along with hormones and other specific factors stimulating survival, growth, and/or differentiation (either with broad or narrow specificity). Transferrin is an absolute requirement for many cell types, and other proteins such as albumin may have beneficial effects in serum-free culture conditions. Antibiotics are often used for the elimination or suppression of contaminating microorganisms (Jakoby and Pastan, 1979). While a combination of penicillin and streptomycin is frequently used, an alternative is provided by gentamicin, which prevents the growth of mycoplasma as well as gram-positive and gram-negative bacteria. Most antibiotics, but particularly the fungicidal or fungistatic antibiotics such as amphotericin B and nystatin, are relatively toxic to most cell types and therefore not recommended for continuous application. Finally, phenol red is often used as a visual indicator of the pH.

Osmolality and pH

The optimum range of osmotic pressures for a particular cell type is relatively narrow. Optimal clonal growth in established cell lines is often found at 300 ± 25 mosmol/kg (Jakoby and Pastan, 1979), although certain primary cell cultures and cell lines may require up to 340 mosmol/kg. Cells should be maintained at relatively constant osmolality, particularly in the absence of serum and macromolecular medium components. Changes in osmolality due to evaporation should be avoided.

The optimal pH for cellular growth is usually 7.2 to 7.4. However, in high-density culture, cells may generate sufficient acidic metabolites to cause a considerable shift to lower pH values. This may require more frequent replenishment of the medium, or use of a buffer with a pK on the acidic side of the operational pH to increase the buffering capacity. Individual cell types may exhibit specific pH preferences (e.g., astrocytes appear to prefer slightly alkaline media, whereas neurons survive better under slightly acidic culture conditions). Also, the pH optimum of a given cell type may be different for growth and for other (differentiated) biological functions. For buffering, the CO_2/bicarbonate system, which is similar to the natural buffering system in blood, is most frequently used. In some cases, organic buffers such as HEPES (up to 25 mM) are used alone or together with inorganic buffer systems, such as phosphate. Some commercially available HEPES may be cytotoxic and, in the presence of DMSO, HEPES may enter cells and be cytotoxic.

Table 12.1.1 Media Components for Cell Culture

Components	Examples
Essential ions	Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, $H_2PO_4^-$, HCO_3^-
Trace elements	Fe^{3+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Mn^{2+}, Ni^{2+}, Sn^{2+}, $Mo_7O_{24}^{6-}$, SeO_3^{2-}, SiO_3^{2-}
Essential amino acids	Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val
Essential fatty acids	Linoleic acid, linolenic acid
Metabolic substrates	D-Glucose; in some cases also D-galactose
Vitamins and cofactors	Biotin, cobalamine, folate, nicotinamide, pantothenate, pyridoxal, thiamine, riboflavin, α-tocopherol, retinol, ascorbate, lipoic acid
Hormones	Insulin, hydrocortisone, triiodothyronine; in some cases also progesterone, estradiol
Specific factors stimulating survival, growth, and/or differentiation	With broad specificity, e.g., EGF, FGF, IGF, LIF, and PDGF; or with narrow specificity, e.g., interleukins, chemokines, and neurotrophins

Gas Phase

A mixture of air and CO_2 is used most frequently as the gas phase in conventional incubators (e.g., Jakoby and Pastan, 1979). This provides the oxygen necessary for aerobic cell metabolism as well as the CO_2 needed for maintenance of the proper pH in the growth medium when using the CO_2/bicarbonate buffering system. Although the amount of oxygen in air (~18%) is adequate for many culture systems, O_2 regulation may be necessary in certain cases. Ideally, for a given culture type, the pO_2 optimum has to be determined using a CO_2/O_2 incubator, but in practice this is rarely done. The partial pressure of oxygen in normal body fluids is significantly less than that of air, and some cells may function better at comparable (reduced) oxygen levels. Other cell types, such as neurons, exhibit a relatively high oxygen requirement. However, high partial pressure of oxygen may increase the risk of oxygen radical formation and cell damage. The presence of selenium, a component of the enzyme glutathione peroxidase, and/or α-tocopherol (a strong antioxidant) in the culture medium can decrease the detrimental effects of high oxygen concentrations. Using the CO_2/bicarbonate buffer system, it is necessary to adjust the bicarbonate concentration to the CO_2 tension in the incubator. Thus, the requirement for $NaHCO_3$ is 2.2 g/liter with 5% CO_2 and 3.7 g/liter with 10% CO_2. In addition to their roles in pH buffering, CO_2 and bicarbonate are involved in a number of biosynthetic reactions, so that at low cell density an extracellular source must be provided. High humidity (>98%) in the incubator atmosphere is necessary when working with open culture systems to minimize the evaporation of culture medium.

Specific Culture Conditions

Cell adhesion

In vivo, many cells are in contact with an extracellular matrix (ECM) formed by specific proteins and polysaccharides. Furthermore, within each tissue, cells usually form tight contacts with neighboring cells through specialized cellular junctions. It is thought that the ECM plays an important role in morphogenesis, cellular development, and function. Depending on the developmental stage, a particular cell type may encounter different types of ECM, which may greatly influence the cellular responsiveness to hormones and growth factors and thus the cellular functions. Therefore, dispersed cell cultures of anchorage-dependent cells often depend on attachment factors and/or ECM components for optimal cell adhesion and function (e.g., Ronnett, 1995). Some types of cells can adhere to and grow on borosilicate glass or on polystyrene plastic chemically treated to decrease hydrophobicity by creating an overall negatively charged surface comparable to glass. Others may require the presence of positively charged surfaces that can be obtained, for example, by coating with polymers such as polylysine or polyornithine. Again, others may need more specific attachment to ECM components such as collagen subtypes, fibronectin, or laminin. Once attached, some cells may eventually synthesize their own ECM molecules, whereas in other cases it may be advantageous to use culture dishes with perma-

nent ECM coating. However, in vivo, the ECM composition is tissue-specific and development-dependent.

Cell density

In general, cell density has a profound effect on the survival, growth and/or function of cultured cells. Excessively high or low cell densities may have detrimental effects. For example, primary neurons in monolayer culture require a minimal cell density 10^4 cells/cm^2, with maximal density at ~1.5×10^6 cells/cm^2. Also, the seeding requirement for oligodendrocytes is relatively stringent, whereas astrocytes and microglia survive at relatively low density (Fedoroff and Richardson, 1997). A minimum number of cells per culture vessel is required for medium conditioning, which occurs due to the accumulation of metabolic intermediates (e.g., amino acids, CO_2, pyruvate, and lactate), detoxifying molecules, and endogenous macromolecular factors stimulating cell survival, growth, and development. On the other hand, if the cell density is too high, adverse effects will result from the depletion of essential metabolites, the accumulation of waste products, and drastic shifts in pH. With the exception of some transformed cell lines, most cells cease multiplication at high population density—a phenomenon termed density-dependent inhibition. The use of suspension cultures in industrial quantities may require fermenters built for continuous feeding, with feedback loops for automatic control of nutrient supply and pH. Three-dimensional cell cultures and explant cultures naturally provide optimal (tissue-like) cell densities and cell-cell interactions. However, the size of the cellular formations must be limited to 300 to 400 μm diameter to ensure a sufficient supply of oxygen, since the critical oxygen diffusion distance is ~150 to 200 μm. Anoxic necrosis can occur in the center of multicellular structures in stationary culture. This may be prevented by continuous agitation, which reduces glucose and oxygen gradients between medium and cells.

TROUBLESHOOTING

Problems with cell or tissue cultures are readily apparent when increased cell death, slow growth, extreme pH shifts, or turbid medium is observed. Sometimes, problems are detected only by careful analysis of the cultures. However, identification of the problem may be difficult because of the large number of variables that need to be considered.

Contamination

Contamination of cultures by bacteria or fungi is usually easy to detect by direct inspection. In contrast, infections by mycoplasma and viruses, or cross-contamination with another cell type, can be detected only by the use of specialized techniques. Most problems with infections stem from improper culture techniques or contaminated culture equipment such as culture hoods, culture vessels, pipets, filtration units, and incubators. Mycoplasma and viruses are able to pass through the 0.2-μm filters used for the sterilization of liquids, and therefore are most frequently introduced by medium supplementation with biological preparations such as serum or tissue extracts. Mycoplasma may also be accidentally introduced directly by the operator. Insidious infections by mycoplasma, viruses, or foreign cells are most problematic when using cell lines. Therefore, a number of precautions should be taken in advance. First, cell lines should be obtained only from certified sources and stock cultures should be prepared from early passages and kept frozen in liquid nitrogen. Secondly, cultures should be handled individually to avoid cellular cross-contamination and tested regularly for mycoplasma and other possible contaminants. The continuous use of antibiotics is not recommended, particularly with cell lines, since they may hide latent infections. On the other hand, antibiotics are usually required for the initiation of primary cultures and may be used in acute cell culture experiments.

Besides the contamination of cultures due to improper handling, there is also the potential risk of exposing workers to pathogens residing in cultures. Therefore, it is important to apply the rules of good laboratory practice, to use the required safety containment facilities, and to follow the official safety guidelines. Particular measures are needed when working with tumorigenic or transformed cells, with pathogenic viruses, and human cell cultures (e.g., Jakoby and Pastan, 1979).

Toxic Chemicals

Toxic chemicals in the growth medium may seriously affect cell growth and viability. The most frequent source of toxic chemicals is the water used for culture preparation. Therefore, only highly purified water should be used, e.g., water which has been passed through a Milli-Q or Nanopure water purification system with added organic- and pyrogen-removal cartridges. Toxic compounds may also be introduced by the recycling of cultureware. If glass-

ware is used, all organic residues from detergent washes must be removed prior to use for culturing. Toxic chemicals may also be introduced through impure medium components. In this case, cultures maintained in serum-free media are more susceptible to toxic contaminants because of the lack of macromolecules that may bind and inactivate such chemicals. Water and biological preparations may contain also endotoxins (lipopolysaccharides from the membranes of gram-negative bacteria). Serum also contains complement factors that may damage cultured cells. Complement can be inactivated by incubating the serum in a water bath at 56°C for 30 min prior to use (also see *APPENDIX 2A*).

Alterations in materials or methods are frequent causes of culture problems, though they are often difficult to identify. Media or media components may slowly degrade during storage. Different batches of media components, particularly serum, may vary in their composition. Cell lines may alter their characteristics with repeated subculturing, and methods may vary between individual co-workers or may change slightly over the years. It is therefore important to maintain strict rules for working with cell cultures. Commercially available materials should always be purchased from the same supplier to avoid variations. The shelf life of all products must be strictly respected, and batches of products (e.g., serum) that are known to be highly variable should be tested prior to use. When working with cell lines, stock cultures should be prepared and stored with great care. Cell cultures should be regularly subjected to quality controls, evaluating their viability, growth characteristics, and phenotypes.

ANTICIPATED RESULTS

Cell and tissue culture offers the possibility of studying biological processes at the cellular, subcellular, and molecular levels, in a system less complex than the whole organism. These techniques increase the accessibility of cells and their environment for manipulation and investigation. On the other hand, complementary studies are usually needed to verify the physiological relevance of observations in vitro. Tissue and cell culture have found numerous applications in research, clinical diagnosis, toxicology, and industrial biotechnology and gene targeting.

Culture Characterization

Growth characteristics and phenotype expression of cultures are the most common criteria for culture characterization (e.g., Ronnett, 1995; Jakoby and Pastan, 1979). Routinely, growth is estimated by measuring the rate of incorporation of radiolabeled thymidine, by the tetrazolium salt (MTT) assay, and/or by direct cell count. To determine cell type-specific differentiation, various criteria or endpoints are used, including morphological, immunocytochemical, biochemical, genetic, and electrophysiological properties. Dispersed cell cultures are particularly accessible for direct observation under a phase-contrast inverted stage microscope, making it possible to monitor gross morphological changes over time in culture. More critical morphological analysis by high-resolution light microscopy and transmission electron microscopy is feasible with most of the conventional culture preparations, although embedding and sectioning of the cultures is required (Fedoroff and Richardson, 1997). For immunocytochemical analyses, numerous monoclonal and polyclonal antibodies are available to probe for cell type–specific antigens (Fedoroff and Richardson, 1997), including cell surface components, cytoskeletal proteins, and specific intracellular enzymes, proteins, or metabolites. Biochemical assays can be used to probe for cell type–specific protein expression, specific enzyme activities, receptor-binding properties, characteristics of plasma membrane channels and transporters, or synthesis of specific metabolites. Also, specific gene expression is studied at the transcriptional level. In certain culture systems, the specificity of certain phenotypic markers may be limited because they are either coexpressed in different cell types, or are aberrantly expressed. The latter condition has been observed in transformed cells and in some cell types grown in isolation. Therefore, for the phenotypic characterization or identification of a given cell type, a different set of criteria is usually needed.

Functional Studies

Any of the immunocytochemical, biochemical, or molecular criteria for characterizing a culture may also be useful for the study of normal cell functions. The type of cell culture suitable for functional studies depends largely on the degree of functional integrity needed relative to the in vivo situation. For example, simple cell lines (e.g., CHO cells) are particularly suitable for the pharmacological study of channels or receptor complexes after the genes have been stably transfected into these cells. On the other hand, to study signal

transduction or other regulatory mechanisms involved in cell function, a culture system closely mimicking the in vivo tissue would appear to be the most appropriate. Electrophysiological studies in neural tissues also require neuronal interactions comparable to the in vivo situation. Therefore, the complex explant cultures, which retain the greatest degree of original histotypic organization, are best suited for this purpose. Furthermore, cell culture systems of different complexities are indispensable in many research areas—for example in developmental studies (including cell lineage studies), for the analysis of functional variations of cytosolic free calcium concentrations, for the elucidation of cell-matrix interactions, for the identification and study of trophic factors, and for the investigation of pathogenic mechanisms such as microglial reactivity, neurotoxicity, oxygen radical damage, senescence, and mechanisms of cell death.

Screening Procedures

Relatively simple cell culture systems can be used for bioassays and for drug screening using defined cellular targets. Various cell lines are employed for the detection and quantification of bioactive substances such as cytokines, neuroactive agents, or toxins. Three-dimensional histocultures of tumor explants provide test systems to evaluate the efficacy and pharmacokinetics of chemotherapy drugs. Culture systems are used to replace or complement animal models. Genetically engineered cells are used successfully for high-throughput screening.

Cell-Derived Products

Cell culture systems of different complexity can also be used for the synthesis of biological products. Various cell lines synthesize and release cytokines and other trophic molecules that can be used for research or as media additives for other culture systems. The hybridoma technology is employed for monoclonal antibody production. Cultured human epidermal keratocytes serve as autografts for burn victims. Cell cultures may also be used for virus propagation and purification, and as host cell lines for the infection of primary cells with viral vectors. Transfection of various types of eukaryotic cells is also used for the production of certain eukaryotic proteins. Embryonic stem cells are most suitable for gene targeting, and are employed in transgenic technology. Furthermore, genetically engineered cell lines that produce biologically active factors, such as specific neurotrophic factors or hormones, can be encapsulated in a porous polymer fiber and implanted in a host tissue (Tseng et al., 1997).

TIME CONSIDERATIONS

Cell and tissue culture techniques have the reputation of being particularly laborious and time-consuming. However, with the steady increase of culture applications, and increasing demand, standardized methods have been developed which greatly reduce the time necessary for culture preparation and maintenance. Also, most of the components and equipment needed for cell culture are now commercially available, and the use of disposable plastic materials reduces the need for specialized washing and sterilizing procedures. Nevertheless, in some cases, special equipment is still needed (e.g., for aggregate cell cultures), and some techniques still are particularly tedious and labor-intensive, such as some explant-culture techniques. In contrast, the preparation and growth of most primary cell cultures, as well as work with cell lines, have become common laboratory practice. When cell lines are used, it is easiest to regularly purchase fresh certified cells instead of storing them. Using exclusively freshly obtained or disposable materials—including cells, media, culture dishes, and pipets—saves time and avoids the need for specialized equipment for media preparation, cleaning of glassware, sterilization, and quality control. This approach is particularly advantageous for small laboratories.

The practice of standard culture methods is best acquired in a laboratory where culturing is routinely performed. Also, in the beginning, it is best to use only proven protocols from well-established laboratories. Once the basic conditions are working, new protocol variants may be attempted. Much time is saved if a particular culture technique is mastered in a laboratory where it is used. Many published protocols lack small but critical technical details, and therefore do not allow reliable reproduction. In any case, sterile techniques and standard good laboratory practice are essential for successful work. Besides the basic cell culture equipment, such as a laminar flow hood and a CO_2 incubator, a special facility may be needed to meet the safety standards for working with pathogenic viruses, human cell cultures, or transformed cell lines.

LITERATURE CITED

Baba, H., Nakahira, K., Morita, N., Tanaka, F., Akita, H., and Ikenaka, K. 1997. GFAP gene expression during development of astrocytes. *Dev. Neurosci.* 19:49-57.

Banker, G. and Goslin, K. 1991. Culturing Nerve Cells. MIT Press, Cambridge, Mass.

Bornstein, M.B. 1995. Organotypic cultures of mammalian nerve tissues: A model system for neurotoxicological investigations. *In* Neurotoxicology. Approaches and Methods (L.W. Chang and W. Slikker, eds.) pp. 573-579. Academic Press, San Diego.

Bottenstein, J.E. and Sato, G. 1985. Cell Culture in the Neurosciences. Plenum, New York.

Boulton, A.A, Baker, G.B., and Walz, W. 1992. Practical Cell Culture Techniques. Humana Press, Totowa, N.J.

Brill, S., Holst, P.A., Zvibel, I., Fiorino, A.S., Sigal, S.H., Somasundaran, U., and Reid, L.M. 1994. Extracellular matrix regulation of growth and gene expression in liver cell lineages and hepatomas. *In* The Liver: Biology and Pathobiology, 3rd ed. (W.B. Jakoby, D.A. Schachter, and D.A. Shafritz, eds.) pp. 869-897. Raven Press, New York.

Celis, J.E. (ed.) 1998. Cell Biology: A Laboratory Handbook, vol. 1, 2nd ed. Academic Press, San Diego.

Cepko, C.L. 1989. Immortalization of neural cells via retrovirus-mediated oncogene transduction. *Annu. Rev. Neurosci.* 12:47-67.

Conn, P.M. (ed.) 1990. Cell Culture. *In* Methods in Neurosciences, Vol. 2. Academic Press, San Diego.

Crain, S.M. 1976. Neurophysiologic Studies in Tissue Culture. Raven Press, New York.

Daniels, J.T., Kearney, J.N., and Ingham, E. 1996. Human keratinocyte isolation and cell culture: A survey of current practices in the UK. *Burns* 22:35-39.

Davis, J.M. 1994. Basic Cell Culture: A Practical Approach. IRL Press Oxford.

Deli, M.A., and Joo, F. 1996. Cultured vascular endothelial cells of the brain. *Keio J. Med.* 45:183-198.

Fedoroff, S. and Richardson, A. (eds.) 1997. Protocols for Neural Cell Culture (2nd ed.). Humana Press, Totowa, N.J.

Freshney, R.I. 1992. Animal Cell Culture. A Practical Approach (2nd ed.). IRL Press, Oxford.

Gähwiler, B.H. 1981. Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Methods* 4:329-342.

Gage, F.H., Ray, J., and Fisher, L.J. 1995. Isolation, characterization, and use of stem cells from the CNS. *Annu. Rev. Neurosci.* 18:159-192.

Gilbert, S.F. and Migeon, B.R. 1975. D-valine as a selective agent for normal human and rodent epithelial cells in culture. *Cell* 5:11-17.

Handler, J.S. and Kreisberg, J.I. 1991. Biology of renal cells in culture. *In* The Kidney, 4th ed. (B.M. Brenner and F.C. Rector, eds.) pp. 110-131. W.B. Saunders, Philadelphia.

Hay, R.J., Park, J.-G., and Gazdar, A. 1994. Atlas of Human Tumor Cell Lines. Academic Press, San Diego.

Jakoby, W.B. and Pastan, I.H. (eds.) 1979. Cell Culture. *Methods Enzymol.* vol. 58.

Jat, P.S., Noble, M.D., Ataliotis, P., Tanaka, Y., Yannoutsos, N., Larsen, L., and Kioussis, D. 1991. Direct derivation of conditionally immortal cell lines from an *H2-K^b*-tsA58 transgenic mouse. *Proc. Natl. Acad. Sci. U.S.A.* 88:5096-5100.

Markowitz, D., Gaff, S., and Bank, A.A. 1988. A safe packaging line for gene transfer: Separating viral genes on two different plasmids. *J. Virol.* 62:1220-1224.

Metcalf, D. 1984. Clonal culture of hemopoietic cells: Techniques and applications. Elsevier/North-Holland, Amsterdam.

Moscona, A.A. 1965. Recombination of dissociated cells and the development of cell aggregates. *In* Cells and Tissues in Culture, Vol. 1 (E. Willmer, ed.) pp. 489-529. Academic Press, New York.

Piper, H.M. 1990. Cell Culture Techniques in Heart and Vessel Research. Springer, Berlin.

Pollard, J.W., and Walker, J.M. (eds.) 1990. Animal Cell Culture. *In* Methods in Molecular Biology, vol. 5. Humana Press, Totowa, N.J.

Pretlow, T.G. and Pretlow, T.P. 1983. Cell Separation: Methods and Selected Applications, vol. II. Academic Press, London.

Richelson, E. 1976. The culture of established clones for neurobiologic investigations. *In* Metabolic Compartmentation and Neurotransmission. (S. Berl, D.D. Clarke, and D. Schneider, eds.) Plenum, New York.

Ronnett, G.V. 1995. Human neuronal cell lines as in vitro models. *In* Neurotoxicology. Approaches and Methods (L.W. Chang and W. Slikker, eds.) pp. 581-593. Academic Press, San Diego.

Sensenbrenner, M. 1977. Dissociated brain cells in primary cultures. *In* Cell, Tissue, and Organ Cultures in Neurobiology (S. Fedoroff and L. Hertz, eds.) pp.191-213. Academic Press, New York.

Shaw, A.J. 1996. Epithelial Cell Culture: A Practical Approach. IRL Press, Oxford.

Stoppini, L., Buchs, P.-A., and Muller, D. 1991. A simple method for organotypic cultures of nervous tissue. *J. Neurosci. Methods* 37:173-182.

Thiele, D.L. and Lipsky, P.E. 1985. Modulation of human natural killer cell function by L-leucine methyl ester: Monocyte-dependent depletion from human peripheral blood mononuclear cells. *J. Immunol.* 134:786-793.

Tseng, J.L. Baetge, E.E., Zurn, A.D., and Aebischer, P. 1997. GNDF reduces drug-induced rotational behavior after medial forebrain bundle transection by a mechanism not involving striatal dopamine. *J. Neurosci.* 17:325-333.

Verity, M.A. 1995. Cell suspension techniques in neurotoxicology. *In* Neurotoxicology. Approaches and Methods (L.W. Chang, and W. Slikker, Jr., eds.) pp. 573-579. Academic Press, San Diego.

Wiesinger, H., Schuricht, B., and Hamprecht, B. 1991. Replacement of glucose by sorbitol in growth medium causes selection of astroglial cells from heterogeneous primary cultures derived from newborn mouse brain. *Brain Res.* 550:69-76.

Contributed by Paul Honegger
Universite de Lausanne
Lausanne, Switzerland