

Basics of animal cell culture

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INTRODUCTION

The culture of animal cells and tissues is a generally and widely used technique that involves isolation of cells, tissues and organs from animals and growing them in an *in vitro* or artificial environment. The term culture means to keep alive and grow in an appropriate medium that simulates the natural conditions. The list of different cell types which can now be grown in culture include connective tissues such as fibroblasts, skeletal, cardiac and smooth muscle, epithelial tissues, neural cells, endocrine cells and many different types of tumour cells). *In vitro* culture has been proven to be the most valuable method to study the functions and mechanism of operations of many cells. A particular group of cells can be cultured in large quantities to study their cellular activities, differentiations and proliferations. Cell culture is highly essential to biotechnology; the major areas of application of animal cell culture are; cancer research, vaccine manufacturing, recombinant protein production, drug selection and improvement, gene therapy, stem cell biology and *in vitro* fertilization technology. Growing tissues of living organisms outside the body are made possible in an appropriate culture medium, which contains a mixture of nutrients either in solid or

liquid form. Nutritional factors like serum, Ca^{2+} ions, hormones etc. can be added to the medium to aid growth, differentiation and proliferation of cells. Cells can be propagated, expanded and divided into identical replicates, which can be characterized and preserved by freezing. They can also be purified phenotypically by

growth in selective media. Historical landmarks in the development of cell culture are presented in Table 1. The objective of this review was to provide useful and basic information on animal cell culture for early career scientists.

PROCEDURE OF CELL CULTURE

Primary culture Freshly isolated cultures are known as primary cultures until they are passaged or subculture. They are usually heterogeneous, and have a low growth fraction, but they are more representative of the cell types in the tissue from which they were derived and in the expression of tissue specific properties. The first step in obtaining the primary culture is isolation of tissues from the whole part or organ, followed by disaggregation of cells from the tissues. This is done by addition of low trypsin to the tissue for proper disintegration and isolation of cells. Trypsin is added to the tissues in order to degrade extracellular proteases and glycosidase. The externally exposed proteins are digested by the action of trypsin for dissociation of cells of the tissues in order to harvest individual

cells. The cells obtained after trypsin digestion are incubated in the presence or absence of serum and culture in a medium.

Subculture culture A subculture is a new culture taken from a primary culture and grown separately in the culture medium. Subculture allows the expansion of the culture (it is now known as a cell line). The advantage of sub-culturing primary culture into a cell line is the provision of large amounts of consistent material suitable for prolonged use. The subculture does not need trypsinization. The medium required is based on the type of specialized cells in culture. This subculture may not need serum for continuous propagation.

Monolayer culture This is a type of culture in which the bottom of the culture plate is covered by a continuous or a single layer of cells in a culture medium. They do not require enzymatic or mechanical dissociation. Growth is limited by concentration of cells in the medium, which allows easy scale-up.

Suspension culture Suspension cultures are cells which can be grown within suspension of the medium. They are easier to passage as there is no need to detach them. They do not require enzymatic or mechanical dissociation.

Adherent culture Cells are dissociated enzymatically before they are cultured. They require periodic passaging, but allow easy visual inspection under inverted microscope. They are referred to as anchorage dependent cells. Seeding density and cell propagation The conditions which favour cell proliferation

are low cell density, low Ca^{2+} concentration (100 to 600 μM) and the presence of growth factors such as epidermal growth factor (EGF), fibroblast growth factors (FGF), and platelet derived growth factor (PDGF). High cell density ($>10^5$ cells/ cm^2) will favour cyto-stasis and differentiation. Different conditions are therefore required for propagation and differentiation, and hence an experimental protocol may require a growth phase to increase cell number, this is followed by a non-growth maturation phase, which allows replication of samples and an increased in the expression of differentiated functions. In general, cultures derived from embryonic tissues will survive and grow better than those of the adult. They presumably reflect the lower level of specialization and presence of replicating precursor or stem cells in the embryo Adult tissues will usually have a lower growth fraction and high proportion of nonreplicating specialized cells. Cells cultured from neoplasma, however, can express at least partial differentiation, e.g. B16 Mouse melanoma, while retaining capacity to divide The list of main equipment required for cell culture is provided in Table 3.

TYPE OF CULTURE SYSTEMS

Batch This can be defined as the usual type of culture in which cells are inoculated into a fixed volume of medium. As the culture grows, nutrients are consumed and metabolites are accumulated. The environment is therefore continually changing and this, in turn, enforces changes to cell metabolism, often

referred to as physiological differentiation. Batch culture is suitable for both monolayer and suspension cells. The media are added to the culture in three different ways: (1) By replacing a constant fraction of the culture with an equal volume of fresh medium. (2) By increasing continuously the volume at a constant rate, but withdrawing culture aliquots at intervals. (3) Perfusion: by the continuous addition of medium to the culture and the withdrawal of an equal volume of spent (cell-free) medium. Perfusion can be open, i.e. the complete removal of medium from the system or closed which is recirculation of the medium; usually, a secondary vessel is used to regenerate the medium by gassing and pH correction.

Continuous-flow culture This system gives true homeostatic conditions with no fluctuations of nutrients, metabolites or cell number. It depends on the medium entering the culture with a corresponding withdrawal of medium with cells. It is only suitable for suspension culture cells or monolayer cells growing on micro-carriers. The system has a chemostat attached to it. A fixed volume of culture in which medium is fed in, at a constant rate mixed with the cells and then leaves at the same rate).

GROWTH KINETICS IN CELL CULTURE

The standard growth kinetics of a culture cycle begins with a lag phase, proceeding through the logarithmic or exponential phase to a stationary phase, and finally to the decline and death of cells. The phases of cell growth in culture are discussed below:

The lag phase: This is the period of adaptation of cells to the new environment. New enzymes are synthesized, a slight increase in cell mass and volume occurs, but there is no increase in cell number. If this phase is prolonged, there could be low inoculum volume and poor inoculum condition (that is, high percentage of dead cells). The log or exponential phase: This is the period of balanced growth in which all cell components grow at the same rate. The cells have adjusted to their new environment and multiply rapidly (exponentially). Growth rate is independent of nutrient concentration, as nutrients are in excess. The composition of the biomass remains constant and the phase results in a straight line graph. Growth (increase in cell numbers or mass) can be defined in the following terms: Specific growth rate (μ) or proliferation rate (r): **The exponential growth**

rate is the first order of reaction. The rate of biomass is correlated with the specific growth rate (μ) and the biomass concentration or cell number, X. A measure of the rapidity of growth has dimension T⁻¹. $dx/dt = \mu \cdot X$ (1) Where, ΔX = increase in cell mass/number; Δt = time interval, and X = cell mass/number. Doubling time (t_d) that is the time for a population to double in number or mass: (2) Degree of multiplication (n) or number of doublings (that is, the number of times the inoculum has replicated) is defined as: (3) $n = 3.34 \times (\log X_o - \log X)$ (4) The proliferation rate, (r) or specific growth rate (μ) = $3.32 \times (\log NF - \log NI) / (T_2 - T_1)$ (Griffiths, 1972). Where, r = Proliferation rate, NF =

number of Final cell count, N_f = Number of Initial cell count, T_2 = Time at harvest, T_1 = Initial time. Population doubling time (PD): This is the number of times the inoculum has replicated within 24 hours. It is defined as: $(PD) = 24/(r)$, where r is proliferation rate. The deceleration phase: The exponential phase is followed by deceleration phase, which is the period of unbalanced growth. The growth decelerates due to either depletion of one or more essential nutrients. There is an accumulation of toxic by-products of growth period of unbalanced nutrients in the medium. Cells undergo internal restructuring to increase their chances of survival. The stationary phase: This phase starts when the net growth rate is zero. Cells may have an active metabolism to produce secondary metabolites. Secondary metabolites are non-growth-related which may be antibiotics like pigments. The decline and death phase is characterized by the living cell population decreasing with time, due to lack of nutrients and toxic metabolic byproducts. The rate of death is defined as K_d is the cell death constant. N = Cell number concentration (cell number /L).

. MEDIA REQUIREMENTS FOR CELL CULTURE Criteria for selecting culture media The choice of medium to be used for culture is dependent on the cell type specifics which significantly affects the success of cell culture experiments. The selection of the media also depends on the purpose of the culture and resources available in the laboratory. Different cell types have

highly specific growth requirements; therefore, the most suitable media for each cell type must be determined experimentally. In general, it's always good to start with MEM for adherent cells and RPMI-1640 for suspension. Media are the sources of nutrients for the cells in culture. They are rich in essential nutrients such as amino acids, glucose, ions, fructose, and hormones with or without serum. The varieties of artificial culture media available can be grouped into:

Serum containing media Serum is a complex mixture of many small and large biomolecules with different, physiologically balanced growth-promoting and growth-inhibiting activities. Serum performs major functions which are hormonal factors; stimulating cell growth and functions; attachment and spreading factors; transport proteins carrying hormones, minerals, lipids etc. Among other, the serum contains proteins which serve as carriers and protective agents for other molecules. Albumin is a protein in serum which performs a lot of functions. Albumin binds vitamins such as pyridoxal, fatty acids: such as oleic, linoleic, linolenic $td = In2 \mu = 0.693 \mu$ k N dt d arachidonic, myristic and palmitic acids, and ions such as copper. However, the use of serum is limited because of its complexity, expensive and a potential source of adventitious biological contaminants. Eliminating the use of serum in cell culture, it requires an understanding of what it does in in vitro systems. Examples of types of serum available for culture are Fetal Bovine Serum, New

Calf Serum, Donor Horse Serum, Porcine Serum etc.). Serum-free media The development of effective serum-free and serumreplacement medium formulations has become essential for the future growth of the biotechnology. The elimination of animal serum-borne components aims to advance standardization, consistency and to reduce risk of contamination by serum-borne adventitious agents in cell culture processes. Biological industries have developed serum-free formulations for all commercially significant cell and tissue cultures Examples are CHO Cell Culture Media, PER.C6 and 293 Media, Insect Cell Media, Immunology Media, Stem Cell Media, Hybridoma Media, Primary Cell Media. The two categories of serum free media are: (i) Chemically defined media: A chemically defined medium is one in which the exact chemical composition is known e.g. Expression media. (ii) Protein free media: The common examples are hybridoma serum free media, PFHM II is protein-free media.

NUTRIENT UTILIZATION IN CELL CULTURE Nutrient that is likely to be exhausted first is glutamine, and is enzymatically converted (by serum and cellular enzymes) to glutamic acid, leucine and isoleucine. L-Glutamine supports the growth of cells that have high energy demands and synthesize large amounts of proteins and nucleic acids. L-Glutamine is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently. Glucose and pyruvate are other nutrients needed by the cells in culture. When added to a culture at high concentrations, they

are stimulatory to maturation of cells and modulation of metabolism of the substrate Cysteine is also efficiently utilized by human diploid cells. It must be noted that nutrients become growth-limiting before they become exhausted. As the concentration of amino acid falls, the cells find it increasingly difficult to maintain sufficient intracellular pool levels.

Table 2. Commercial culture media and their compositions.

Culture medium Composition DMEM High Glucose - 4 mM L-glutamine 4500 mg glucose/L 1500 mg/L sodium bicarbonate. RPMI Modified to contain 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate McCoy High Glucose, L-glutamine, Bacto-peptone, Phenol Red, HEPES with or without Sodium Pyruvate. M 2 Magnesium Sulfate, Potassium Chloride, Potassium Phosphate, Sodium Bicarbonate, Sodium Chloride, Albumin, Bovine Fraction, D-Glucose, HEPES, Phenol, Pyruvic Acid, DL-Lactic Acid. Nutrient Mixture F-10 (Ham's F10) Sodium bicarbonate, without L-glutamine; supplement with 0.146 gm/L L-glutamine. Nutrient Mixture F-12(Ham's F-12) L-glutamine and Sodium bicarbonate Minimum Essential Medium Eagle Earle's salts, L-glutamine and Sodium bicarbonate, Sterile-filtered, Endotoxin tested.

List of main equipment for cell culture. Type of Equipment
Function

Bio Safety Cabinets It offers protection from contaminants during culture. **CO2 Incubators** Cells are grown in an atmosphere of 5%-10% CO₂. It keeps constant level of humidity **Microscopes** Inverted microscopes are used for this purpose. **Culture vessels** These consist of petri dishes, multi-well plates, microtitre plates, roller bottles, screw cap flasks T-25, T-75, T150. **Centrifuges** Cells are centrifuged at low temperature and low speed. **Freezer** For freezing and short term storage **Hemocytometer** To determine the cell counts before or after culture. **Water bath with shaker** For cell dissociation and trypsinization **Liquid N₂ Cylinder** For long-term cryopreservation **pH meter** To determine the pH of the medium.

This is exaggerated in monolayer cultures because as the cells become more tightly packed together, the surface area which is available for nutrient uptake becomes smaller. Glucose is often another limiting factor as it is destructively utilized by cell rather than adding high concentrations at the beginning, it is more beneficial to supplement after 2-3 days. In order to maintain a culture some additional feeding often has to be carried out either by complete, partial media changes or by perfusion. The efficiency of medium changes is probably due to the high extracellular concentration of nutrients it provides, thus stimulating a further replicative cycle. Many cell types are either totally dependent upon or can only perform optimally when certain growth factors are present. Cell aggregation is often a

problem in suspension cultures. Media lacking calcium and magnesium ions have been designed specifically for suspension cells because of the role of these ions in attachment. This problem has also been overcome by including very low levels of trypsin in medium. Other conditions for cell culture: effect of pH, temperature and oxygen on cell growth. In addition to nutrients, the pH of the growth medium is also important for cell growth rate and cell density. The optimal growth pH for most cells is near neutral. Cells can grow reasonably well over a range of pH 5.5 to 8.5. Extreme pH beyond this range will significantly decrease the cell growth rate and may sometimes even cause cell death. pH is another limiting factor for cell growth in addition to nutrition exhaustion and accumulation of toxic metabolites. The medium's pH is determined by medium compositions, buffers, cellular metabolites, and aeration conditions. Cells produce large quantities of acetic acid if the growth medium contains little or no oxygen causing the growth medium to reach pH 4 or lower. Acetic acid is the major metabolic inhibitor under anaerobic growth condition. With proper aeration, cells will be able to use many organic acids as carbon sources and the pH of the growth medium will be maintained at near neutral or basic ranges. Cells cannot grow well at temperatures higher than 42°C. They can tolerate lower temperatures with lower growth rate. Temperature range from 15 to 30°C is the most optimal range for most cells depending on the cell type and species of animals.

Cell growth stops when the medium is kept at 4°C or shifted above 37°C

CRYOPRESERVATION AND STORAGE Liquid Nitrogen is often used to preserve tissue culture cells, either in the liquid phase (-196°C) or in the vapour phase (-156°C). Freezing can be lethal to cells due to the effects of damage by ice crystals, alterations in the concentration of electrolytes, dehydration, and changes in pH. To minimize the effects of freezing, several precautions are taken. First, a cryoprotective agent which lowers the freezing point, such as glycerol or dimethylsulfoxide (DMSO) is added. A typical freezing medium is 90% serum and 10% DMSO. In addition, it is best to use healthy cells that are growing in log phase and to replace the medium 24 h before freezing. Also, the cells are slowly cooled from room temperature to -80°C to allow the water to move out of the cells before it freezes. The optimal rate of cooling is 1 to 3°C per minute. Some laboratories use isopropanol at room temperature and the freezing vials containing the cells are placed in the container and the container is placed in the -80°C freezer. The effect of the isopropanol is to allow the tubes to come to the temperature of the freezer slowly, at about 1°C per minute. To maximize recovery of the cells when thawing, the cells are warmed very quickly by placing the tube directly from the liquid nitrogen container into a 37°C water bath with moderate shaking. As soon as the last ice crystal is melted, the cells are immediately diluted into pre-warmed

medium. Cultures should be examined daily, by observing the morphology, the colour of the medium and the density of the cells. A tissue culture logbook should be maintained and it should contain: the name of the cell line, the medium components and any alterations to the standard medium, the dates on which the cells were split and/or fed, a calculation of the doubling time of the culture and any observations relative to the morphology (Jacob and Allison, 2009).

CELL LINE IDENTIFICATION Cell line identification is done to determine if there is presence or absence of cross-contamination. It also confirms the origin of species of the cell line. It detects the transformed cells and evaluates if there are genetic instabilities. The following techniques discussed below are used to prove the integrity of the cultured cells. **Morphology and STR analyses** Observation of morphology is the most direct technique to characterize cell lines. The study of size, shape and structure of cells can authenticate the type and origin of a particular cell line. Most cells can be divided into five basic categories based on their morphology. The structures are fibroblastic, epithelial-like, lymphoblast-like, endothelial and Neuronal (Figures 2, 3, 4, 5 and 6). Short tandem repeats (STR) profile of a reference sample and other known cell line STR profile can be compared. STR is repetitive sequence elements 3 to 7 base pairs of DNA long scattered throughout the human genome. By amplifying and analyzing these polymorphic loci,

comparing the resulting STR profile to that of a reference sample, the origin of biological samples such as cells or tissues can be identified and verified .

Chromosomal and Karyotyping analyses: This is done to detect the presence of genetic abnormalities within the cell. A karyotype is the number and appearance of chromosomes in the nucleus of eukaryotic cells. The chromosomes are depicted in a standard format known as Karyogram. The karyotyping is done to determine species identification

Isoenzyme analysis Isoenzyme analysis is based on the existence of enzymes with similar or identical specificity, but different molecular structure. It is used to study the pattern of migration of isoenzymes present in cell lysates following electrophoresis using agarose gels. Examples of isoenzymes available are; Aspartate aminotransferase, Glucose-6-phosphate dehydrogenase, Lactate dehydrogenase, Malate dehydrogenase).

ELISA The **enzyme-linked immuno-sorbent assay (ELISA)** is a common laboratory technique which is used to measure the concentration of a substance (usually antibodies or antigens) in solution. The basic ELISA, or enzyme immunoassay (EIA), is distinguished from other antibodybased assays because of its separation of specific and non-specific interactions which occur via serial binding to a solid surface. Usually a polystyrene multi-

well plate can be achieved due to the quantitative results that can be obtained (Braitbard et al., 2006). The steps of the ELISA result in a colored end product correlates to the amount of a substance present in the original sample. ELISAs were first developed in the early 1970s as a replacement for radio-immunoassays. They remain in wide use in their original format and in expanded format with modifications that allow multiple analyses per well, highly sensitive readouts, and direct cell-based output

Biochemical tests A large number of **cell lines** and strains can be shown to derive from a particular tissue or tumour by the presence of specific synthetic abilities or metabolic pathways. The human trophoblastic are cell lines isolated from a malignant gestational choriocarcinoma of a foetal placenta. Interestingly, the line has been shown to secrete a spectrum of placental hormones, including human chorionic gonadotrophin, placental lactogenic, oestrogen, oestradiol, oestriol and progesterone in culture. Biochemical tests are the tests used for the identification of cells based on the differences in the biochemical activities of different cell functions. These differences in carbohydrate metabolism, protein metabolism, fat metabolism, production of certain enzymes, hormones and ability to utilize a particular compound, etc. help to identify them by their biochemical tests). Tests for microbial contamination These tests are suitable for detection of most microorganisms that would be

expected to survive as contaminants in cell lines or culture fluids. The common examples of contaminants that could affect culture are bacteria, fungi, mycoplasma and viruses. The following tests should be carried out to avoid any microbial contaminant: microbial environmental aseptic monitoring, container integrity testing, pre-sterilization bio-burden testing, media filtering before use and sterility testing (Tests for intra-species cross-contamination)

A quiet number of cell lines are being developed; there is a high risk of intra-species cross-contaminants in the laboratory. The problem is common, especially in laboratories where many different cell lines of human and murine origins are being developed. Tests for polymorphic isoenzymes, surface marker antigens and unique karyology are all important tools to detect cellular cross-contamination within a given species. The other methods for identification of cell lines include Giemsa binding, tissue-specific antigen, cell type specific marker etc.

CONCLUSION Animal cell culture is important to all fields of bioscience; especially from medicine to agriculture. It is an important tool to study cell specific functions, physiology and biochemical components. The major advantage of cell culture is its consistency and reproducibility of results that can be obtained from using clonal cells. They serve as building blocks and stem cells for bioscience research and biological repairs. Priority should be given to developing good cell culture laboratories especially in developing countries where they are

not yet fully established. Conflict of Interests The authors have not declared any conflict of interests.

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