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Culture of Animal Cell

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Abstract

Cell culture has become an indispensable technology in many branches of the life sciences. It provides the basis for studying the regulation of cell proliferation, differentiation and product formation in carefully controlled conditions, with processes and analytical tools which are scalable from the level of the single cell to in excess of 10 kg wet weight of cells. Cell culture has also provided the means to define almost the entire human genome, and to dissect the pathways of intracellular and intercellular signaling which ultimately regulate gene expression.

Roller bottle culture is considered the first scale-up step for anchorage-dependent cells from stationary flasks or bottles (Langdon SP et al 2004) this is achieved by using all the internal surface for cell growth, rather than just the bottom of a bottle. The added advantages are that: a smaller volume of medium and thus a higher product titre can be achieved; the cells are more efficiently oxygenated due to alternative exposure to medium and the gas phase; and dynamic systems usually generate higher unit cell densities than stationary systems.

Introduction

Cell culture has matured from a simple, microscope driven, observational science to a universally acknowledged technology with roots set as deep in industry as they are in academia. It stands among microelectronics, avionics, astrophysics and nuclear engineering, as one of the major bridges between fundamental research and industrial exploitation, and, in the current climate, perhaps the more commercial aspects will ensure its development for at least several more decades. The prospects for genetic therapy and tissue replacement are such that the questions are rapidly becoming ethical, as much as technical, as new opportunities arise for genetic manipulation, whole animal cloning and tissue transplantation. Several textbooks are already available to introduce the complete novice to the basic principles of preparation, sterilization, and cell propagation, so this book will concentrate on certain specialized aspects, many of which are essential for the complete understanding and correct application of the technology. Cultured cells, their derivation and characterization, and give guidance in those areas which require critical observation and control [1].

Material and Methods

Origin and Characterization

The list of different cell types which can be grown in culture is extensive, includes representatives of most major cell types and due largely to the improved availability of selective media and specialized cell cultures through commercial sources such as Clonetics.

The use of markers that are cell type specific has made it possible to determine the lineage from which many of these cultures were derived, although the position of the cells within the lineage is not always clear. During propagation, a precursor cell type will tend to predominate, rather than a differentiated cell. Consequently a cell line may appear to be heterogeneous, as some cultures, such as epidermal keratinocytes, can contain stem cells, precursor cells, and mature differentiated cells. There is constant renewal from the stem cells, proliferation and maturation in the precursor compartment, and terminal and irreversible differentiation in the mature compartment. Other cultures, such as dermal fibroblasts, contain a relatively uniform population of proliferating cells at low cell densities (about 10^4 cells/cm^2) and an equally uniform, more differentiated, non-proliferating population at high cell densities (10^5 cells/cm^2). This high density population of fibrocyte-like cells can re-enter the cell cycle if the cells are trypsinized or scraped (or 'wounded' by making a cut in the monolayer) to reduce the cell density or create a free edge. Most of the cells appear to be capable of proliferation, and there is little evidence of renewal from a stem cell compartment [2].

Culture heterogeneity also results from multiple lineages being present in the cell line. The only unifying factors are the selective conditions of the medium and substrate, and the predominance of the cell type (or types) which have the ability to survive and proliferate. This tends to select a common phenotype but, due to the interactive nature of growth control, may obscure the fact that the population contains several distinct phenotypes only detectable by cloning.

Cell Type

The cell type chosen will depend on the question being asked. For some processes, such as DNA synthesis, response to cytotoxins,

or apoptosis, the cell type may not matter, provided the cells are competent in other cases a specific process will require a particular cell type, for example surfactant synthesis in the lung will require a fresh isolate of type II pneumocytes or a cell line, such as NCI-H441, which still expresses surfactant proteins [3]. A reasonable first step would be to determine from the literature whether a cell line exists with the required properties.

Source of Tissue

Embryo or adult?

In general, cultures derived from embryonic tissues survive and proliferate better than those from the adult. This presumably reflects the lower level of specialization and higher proliferative potential in the embryo. Adult tissues usually have a lower growth fraction and a higher proportion of non-replicating specialized cells, often within a more structured and less readily disaggregated extracellular matrix. Initiation and propagation are more difficult, and the lifespan of the culture often shorter [4].

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

Examples of widely used embryonic cell lines are the various 3T3 lines (primitive mouse mesodermal cells) and WI-38, MRC-5, and other human fetal lung fibroblasts. Mesodermally-derived cells (fibroblasts, endothelium, myoblasts) are on the whole easier to culture than epithelium, neurons, or endocrine tissue. This selectivity may reflect the extensive use of fibroblast cultures during the early development of culture media and the response of mesodermallyderived cells to mitogenic factors present in serum. Selective media have now been designed for epithelial and other cell types, and with some of these it has been shown that serum is inhibitory to growth and may promote differentiation. Primary culture of epithelial tissues, such as skin, lung, and mammary gland, is routine in some laboratories, and prepared cultures are available commercially.

Selection of Medium

Regrettably, the choice of medium is still often empirical. What was used previously by others for the same cells, or what is currently being used in the laboratory for different cells, often dictates the choice of medium and serum. For continuous cell lines it may not matter as long as the conditions are consistent, but for specialized cell types, primary cultures, and growth in the absence of serum, the choice is more critical [5].

There are two major advantages of using more sophisticated media in the absence of serum: they may be selective for particular types of cell, and the isolation of purified products is easier in the absence of serum. Nevertheless in the presence or serum is still easier and often no more expensive, though less controlled. Two major determinants regulate the use of serum-free media:

1. Cost. Most people do not have the time, facilities, or inclination to make up their own media, and serum-free formulations, with their various additives, tend to be much more expensive than conventional media.
2. Requirements for serum-free Serum media are more cell type specific. Serum will cover many inadequacies revealed in its absence. Furthermore, because of their selectivity, a different medium may be required for each type of cell line. This problem may be particularly acute when culturing tumour cells where cell line variability may require modifications for cell lines from individual tumorus.

In the final analysis the choice is often still empirical: read the literature and determine which medium has been used previously. If several media have been used, as is often the case, test them all, with others added if desired. Measure the growth (*population doubling time* (PDT) *and saturation density*), cloning efficiency, and expression of specific properties (differentiation, transfection efficiency, cell products, etc.). The choice of medium may not be the same in each case, for example differentiation of lung epithelium will proceed in serum, but propagation is better without. If possible, include one or more serum-free media in the panel to be tested, supplemented with growth factors, hormones, and trace elements as required. Once a medium has been selected, try to keep this constant for as long as possible. Similarly, if serum is used, select a batch by testing samples from commercial suppliers and reserve enough to last six months to one year, before replacing it with another pre-tested batch. Testing procedures are as described above for media selection [6].

Gas Phase

The composition of the gas phase is determined by:

1. The type of medium (principally its sodium bicarbonate concentration);
2. Whether the culture vessel is open (Petri dishes, multiwell plates) or sealed (flasks, bottles); and
3. The amount of buffering required.

Several variables are in play, but one major rule predominates and three basic conditions can be described. The rule is that the bicarbonate concentration and carbon dioxide tension must be in equilibrium. It should be remembered that carbon dioxide/bicarbonate is essential to most cells, so a flask or dish cannot be vented without providing carbon dioxide in the atmosphere. Prepare medium to about pH 7.1-7.2 at room temperature, incubate a sample with the correct carbon dioxide tension for at least 0.5 h in a shallow dish, and check that the pH stabilizes at pH 7.4. Adjust with sterile I M HCI or I M NaOH if necessary.

Oxygen tension is usually maintained at atmospheric pressure, but variations have been described, such as elevated for organ culture and reduced for cloning melanoma aad some haematopoietic cells.

Culture System

Originally tissue culture was regarded as the culture of whole fragments of explanted tissue with the assumption that histological integrity was at least partially maintained. Now *'tissue culture'* has become a generic term and includes organ culture, where a small fragment of tissue or whole embryonic organ is explanted to retain tissue architecture, and cell culture, where the tissue is dispersed mechanically or enzymatically, or the cells migrate from an explant, and the cells are propagated as a suspension or attached monolayer.

Cell cultures are usually devoid of structural organization, have lost their histotypic architecture and often the biochemical properties associated with it, and generally do not achieve a steady state unless special conditions are employed [7]. They can, however, be propagated, expanded, and divided into identical replicates. They

can be characterizes and a denned cell population preserved by freezing, and they can be purified by growth in selective media, physical cell separation, or cloning, to give a characterized cell strain with Considerable uniformity.

Organ culture will preserve cell interaction and retain histological and biochemical differentiation for longer than cell culture. After the initial trauma of explantation and some central necrosis, organ cultures can remain in a steady state for a period of several days to years. However, they cannot be propagated easily, show greater experimental variation between replicates, and tend to be more difficult to use for quantitative determinations.

Purified cell lines can be maintained at high cell density to create histotypic cultures and different cell populations can be combined in organotypic culture, simulating some of the properties of organ culture.

Substrate

The nature of the substrate is determined largely by the type of cell and the use to which it will be put. Polystyrene which has been treated to make it wettable and give it a net negative charge is now used almost universally. In special cases the plastic is pre-coated with fibronectin, collagen, gelatin, or poly-L-lysine. Glass may also be used, but must be washed carefully with a non-toxic detergent.

Cell Culture

Primary cultures

The first step in preparing a primary culture is sterile dissection followed by mechanical or enzymatic disaggregation. The tissue may simply be chopped to around 1 mm^3 and the pieces attached to a dish by their own adhesiveness, by scratching the dish, or by using clotted plasma. In these cases cells will grow out from the fragment and may be used directly or sub cultured. The fragment of tissue, or explant as it is called, may be transferred to a fresh dish or the outgrowth trypsinized to leave the explant and a new outgrowth generated [8].

When the cells from the outgrowth are trypsinized and reseeded into a fresh vessel they become a secondary culture, and the culture is now technically a cell line. Primary cultures can also be generated by disaggregating tissue in enzymes such as trypsin (0.25% crude or 0.Ol-0.05% pure) or collagenase (200-2000 U/ml, crude) and the cell suspension allowed to settle on to, adhere, and spread out on the substrate. This type of culture gives a higher yield of cells though it can be more selective, as only certain cells will survive dissociation. In practice, many successful primary cultures are generated using enzymes such as collagenase to reduce the tissue, particularly epithelium, to small clusters of cells which are then allowed to attach and grow out.

When primary cultures are initiated, all details of procedures should be carefully documented to form part of the provenance of any cell line that may arise and be found to be important. A sample of tissue, or DNA extracted from it, should be archived to be available for DNA fingerprinting or profiling for authentication of any cell lines that arise.

Culture Modes

1. ***Batch culture:*** cells grow from seed to a final density over 4-7 days and are then harvested (typical monoclonal antibody yield is 6 mg l-' per batch run).
2. **Fed-batch culture:** additional media, or medium components, are added to increase the culture volume and density of cells.
3. **Semi-continuous batch culture:** this is in effect a batch culture that is partially harvested (e.g. 70%), topped up with fresh medium, allowed to grow up and then harvested again. Typically, three or four harvests can be made, although usually with diminishing returns at each harvest.
4. **Continuous-flow (chemostat) culture:** the culture is completely homogeneous for long periods of time whilst cells are in a steady state. An extremely useful tool for physiological studies but not very economical for production (by definition, cells are never at maximum density)
5. **Continuous perfusion cuhure:** differs from chemostat in that all nutrients are kept in excess and cells are retained within the hioreactor. The most productive culture system gives typical yields of l0 mg l^{-1} day^{-1} for 50-100 days.

There is no definitive answer as to the best system to use: it depends upon the nature of the cell and the product, the quantity of product, downstream processing capability, licensing regulations, etc. However, a rough guide to relative costs for producing monoclonal antibodies by perfusion, continuous-flow and batch culture is the ratio l: 2:3:5.

Biological Factors

Key factors for the successful scale-up of cell culture include using the best available cell line, inoculating cells that are in prime

Procedure: Roller Bottle Culture of Animal Cells

Materials and equipment

- Standard tissue culture media and reagents
- Roller bottles (e.g. 23 x 12-cm plastic bottle with surface area of 1400 cm^2 from, for example, Becton Dickinson, Sterilin).
- Roller bottle apparatus with speed control between 5 and 60 rph.

1. Add approximately 1 ml of complete culture medium at 37°C per 5-cm^2 culture area.
2. Add 5% CO_2 in air to headspace.
3. Add 1-2 x 10^5 cells cm^{-2}.
4. Seal bottle and place in roller culture apparatus.
5. Rotate bottle at 12-24 rph for the initial attachment phase (2-8 h, depending upon cell type). This faster speed is to get an even distribution of cells but should be reduced for cells with low attachment efficiency.
6. Reduce revolution rate to 5-10 per hour when culture is growing.
7. Cell growth can be monitored initially under an inverted microscope and later in the culture period by visual inspection.
8. A medium change can be carried out after 4-5 days if the pH becomes acid and/or maximum cell densities are required. This can also be carried out to change to a production medium, or when infecting cells, and a lower medium volume can be added to get a higher product concentration.
9. Harvest cells when confluent (5-6 days) by removing the medium and trypsinizing in the conventional way. After adding trypsin, roll the bottles at speeds of 20-60 rph until cells detach. Cell yields will be similar to or up to two-fold higher than in stationary cultures, and multilayering (non-diploid cell lines) will occur.

Results

Table: Comparison of culture systems indicating volumetric size, unit and system cell yields

Scale (1)	*System*	*Area (cm^2)* (batch size)	*Cells per batch* (cells 1^{-1})	
	Roller	1750 (x 100)	2×10^{10}	(2×10^{8})
	Multitray	24,000(x 10)	3×10^{10}	(3×10^{9})
	Cell Cube	85,000 (x 4)	4×10^{10}	(9×10^{9})
1	Hollow fibre	20,000	10^{10}	(10^{11})
20	Microporous microcarrier	2.5×10^{6}	10^{12}	(5×10^{10})
100	Glass sphere	10^{6}	10^{11}	(10^{9})
200	Stacked plate	2×10^{5}	10^{11}	(10^{9})
500	Microcarrier (with spin-filter)	2×10^{10}	10^{13}	(5×10^{10})
2000	Airlift		4×10^{12}	(2×10^{9})
4000	Microcarrier	5×10^{6}	8×10^{12}	(2×10^{9})
10,000	Stirred tank		2×10^{13}	(2×10^{9})

Condition and ensuring that nutritional, physiological and physicochemical conditions are optimized.

Successful roller bottle culture depends a great deal on solving various logistic problems. Larger roller bottles (1500 cm^2) are unwieldy to use and cannot he manipulated in many tissue culture cabinets. Special Class II cabinets are suitable; otherwise use the smaller size bottles. Also, do not underestimate the time involved in harvesting cells from roller bottles. If a large number of bottles are being used, trypsinize four at a time and get the cells in fresh medium with serum before beginning the next batch. Cells deteriorate very rapidly in trypsin, and also when being held at high concentrations in medium, so do not store whilst harvesting multiple batches of roller bottles, but put them in a new culture as soon as possible. Unless your laboratory is geared up for this type of work (multiple tissue culture cabinets and staff), limit the number of roller bottles to 8-16 at a time. It is very difficult to remove completely all the cells from a roller bottle without repeated washing (increases contamination risk, standing time of cells and centrifugation volume) and it is wise to assume that a 10-20% loss may occur. This is particularly important when judging how many bottles to set up as an inoculum for a larger culture.

Cells vary in their cultural characteristics. This is very noticeable when comparing clones from the same hybridization or transfection experiment. Differences in expression of the desired product are well recognized, hence the standard screening produced for high producers. However, there is also clonal variability in serum requirements, plating efficiency, growth rate, merabolism (e.g. glycolytic rate) and ability to grow in (or adapt to) suspension culture. It is thus beneficial to select a clone that has the desired characteristics for the scaled-up process that is to be used.

The quicker that cells can be harvested and re-inoculated into a new culture, the healthier they tend to be and the higher the chance of establishing a successful culture. Prolonged exposure to proteolytic enzymes (e.g. trypsin), standing in concentrated suspensions for long periods before inoculation and over-robust mixing (or pouring) whilst in the fragile post-trypsinized state must be avoided. Selecting cells in the late logarithmic phase and rapid processing into the new culture at an adequate inoculum level should allow an optimum initiation of the new culture with a short lag phase and maximum cell density. Always inoculate cells into stabilized culture conditions, because shifts in temperature, pH and oxygen levels during initiation can be damaging.

As one scales up, culture conditions become more demanding on the cells and the logistics of preparing large-cell inocula and maintaining sterility become more difficult. Therefore, do not be over-ambitious in the scale-up steps.

In this overview an explanation has been given for the wide and complex range of cell culture bioreactor systems that are available. This is to put some perspective on the ones that are described in more detail in the following chapters. The selection of a suitable system follows the adage '*horses for courses*' , i.e. a process is selected that fulfils the particular criteria for the cell, product, scale and quantity required, plus the resources in facilities, manpower and experience that are available [9].

There are some additional general factors to be taken into consideration. First, upstream processing is only part of the total process, which starts at the initiation of a cell line and finishes with a purified and packaged product, and therefore should not be treated in isolation. Secondly, increasing unit productivity does not only depend upon designing a bigger, better and more efficient bioreactor. Significant increases in productivity have been, and will be, achieved by designing better media, genetically constructing more efficient and robust cell lines and more critical monitoring (by biosensors) and control (by computer) of the process.

Roller bottle culture is considered the first scale-up step for anchorage-dependent cells from achieved try using all the internal surface for cell growth, rather than just the bottom of a bottle. The added advantages are that: a smaller volume of medium and thus a higher product titre can be achieved; the cells are more efficiently oxygenated due to alternative exposure to medium and the gas phase; and dynamic systems usually generate higher unit cell densities than stationary systems [10-17].

References

1. Lincoin CK, Michael G Gabridge (1998) Cell culture contamination: sources, consequences, prevention, and elimination. Sources consequences, prevention, and elimination. Methods in Cell Biology 57: 49-65.
2. Markovic O, Nenad Markovic (1998) Cell cross-contamination in cell cultures the silent and neglected danger in Vitro Cellular and Developmental Biology. Animal 34: 1-8.
3. Masters J R (2004) Human Cell Cross-contamination Since 1983. In Vitro Cellular & Dev Bio-Animal 4010-A.
4. Bubenk J (2000) Cross-Contamination of Cell lines in Culture. Folia Bioligica 46: 163-164.
5. Mirjalili A, Parmoor E, Moradi Bidhendi S, Sarkari B (2005) Microbial contamination of cell cultures. A 2-year study Biologicals 33: 81-85.
6. Vierck JL, Dodson MV (2000) Interpretation of cell culture phenomena. Methods in Cell Science 22: 79-81.
7. Hartung T, Coecke S, Balls M, Bowe G, Davis J, et al. (2002)

Good cell culture practice. ECVAM good cell culture practice task force report 1, ATLA 30: 407-414.
8. Hay RJ, Cleland M M, Durkin S, Reid, YA (2000) Cell Line Presentation and Authentication in "Animal Cell Culture". JRW Masters (ed). J Wiley. Inc. Oxford University Press. New York City
9. O' Brien S J (2001) Cell culture forensics, proceedings of the National Academy of Science USA 98: 7656-7658.
10. Nardone R M (2006) Eradication of Cross – Contaminated Cell lines: A call for action. Cell Biol Toxicol 23: 367-72.
11. Ausubel F M, Roger Brent, Robert E Kingston, David D Moore, J G Seidman, et al. (1991) Current protocols in molecular biology New York: Wiley Interscience
12. Buehring, Eby EA, Eby MJ (2004) Cell Line cross-contamination: how aware are mammalian cell culturists of the problems and how to monitor it? In vitro cellular and developmental Biology. Animal 40: 211-215.
13. Drexler H G (1997) DSMZ Catalog of Human and Animal Cell Lines. 6th edition.
14. Freshney RI (1993) Culture of Animal Cells, A Manual of Basic Technique, 3rd ed. New York Wiley-Liss.
15. Langdon SP (2004) Cell Culture Contamination: an overview Methods in Molecular Medicine 88: 309-317.
16. Langeler EG, van Uffelen CJ, Blankenstein MA, van Steenbrugge GJ, Mulder E (1993) Effect of culture conditions on androgen sensitivity of the human prostate cancer cell line LnCaP. Prostate 23: 213-223.
17. Spector D, Goldman RR, Leinwand LA (1998) Cells: a Laboratory Manual. Cold Spring Harbor Laboratory Press