Animal cell culture

a practical approach

Edited by
R I Freshney

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Preface

In recent years the use of animal cell culture has undergone a major expansion from being a purely experimental procedure to become an accepted technological component of many aspects of biological research and commercial exploitation. New developments have arisen to improve both versatility and standardisation and it is the aim of this book to make these developments readily accessible to scientists and technologists in the field. These developments include major advances that have been made in serum-free culture and in the scaling up of animal cell culture to industrial and semi-industrial levels. The importance of cell line characterisation, standardisation and banking are now widely recognised and information is presented to allow the reader both to perform their own banking and to take advantage of central repositories and data banks.

Analytical and preparative fractionation of cell populations is dealt with in two chapters on Flow Cytometry and Centrifugal Elutriation, and analysis at the cellular and subcellullar level is described in a chapter on *in situ* hybridisation. The importance of cell interaction and retention of histological structure is stressed in a chapter on organ culture, where most of the standard techniques are described. Finally, a chapter on viability and cytotoxicity measurements has been included to cover the requirements of *in vitro* toxicology and anti-neoplastic drug screening, as well as routine monitoring of culture viability.

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Abbreviations

ATCC	American Type Culture Collection
BSS	balanced salt solution
CAM	chorioallantoic membrane
CSIB	cell source information banks
dCTP	deoxycytidine triphosphate
DGDW	double glass distilled water
dUTP	deoxyuridine triphosphate
EDTA	ethylenediamine tetraacetic acid
HBSS	Hanks' balanced salt solution
HEK cells	human embryonic kidney cells
HLA	human leukocyte antigens
IEF	isoelectric focusing
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5 phenyl tetrazolium chloride
LED	light emitting diode
MEM	minimal essential medium
ODR	oxidation-reduction (redox) potential
OTR	oxygen transfer rate
PA	prostate antigen
PBMEM	phosphate-buffered Eagle's MEM
PBS	phosphate-buffered saline
PMT	photomultiplier tubes
RBCs	red blood cells
SSC	standard saline citrate

Contents

CHAPTER 1

Introduction: Principles of Sterile Technique and Cell Propagation

R.I.FRESHNEY

1. INTRODUCTION

The culture of animal cells and tissues is now a widely used technique in many different disciplines from the basic sciences of cell and molecular biology to the rapidly evolving applied field of biotechnology. An introduction to the basic procedures is available in many laboratories and frequently features as an integral part of undergraduate study in the biological sciences. Several text books are already available (1 – 3) to assist the complete novice during his or her introduction to the basic principles of preparation, sterilisation, and cell propagation, so this book will concentrate on certain specialised aspects, many of which are essential for complete understanding and correct utilisation of the technique.

This chapter will review some of the general aspects of cultured cells, their biology, derivation and characterisation, and set out some of the basic assumptions and definitions.

2. BIOLOGY OF CELLS IN CULTURE

2.1 Origin and Characterisation

The list of different cell types which can now be grown in culture is quite extensive, and includes connective tissue elements such as fibroblasts, skeletal tissue (bone and cartilage), skeletal, cardiac and smooth muscle, epithelial tissues (e.g., liver, lung, breast, skin, bladder and kidney), neural cells (glia and neurones, though neurones do not proliferate), endocrine cells (adrenal, pituitary, pancreatic islet cells), melanocytes and many different types of tumour [for further information see (4)].

The use of cell type specific markers (see Chapter 4) has made it possible to determine the lineage from which many of these cultures were derived, but what is not entirely clear, in many cases, is the position of the cells within the lineage. For the cells to proliferate, it is likely that they represent a precursor cell type rather than a fully differentiated cell which would not normally proliferate. However, the population need not be uniform or of a fixed phenotype. Some cultures, e.g., epidermal keratinocytes, contain stem cells, precursor cells and keratinised squames. There is constant renewal from the stem cells, proliferation and maturation in the precursor compartment, and terminal and irreversible differentiation releasing squames into the culture medium. Other cultures, such as fibroblasts, contain a fairly uniform population of proliferating cells at low cell densities ($\sim 10^4$ 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 trypsinised or scraped to reduce the cell density or create a free edge.

Nutritional factors like serum or Ca^{2+} ions (5), hormones, cell (6) and matrix interactions (7), in addition to the density of the culture (8), can all affect differentiation and cell proliferation, often inversely. Hence, it is not only essential to define the lineage of cells being used, but also characterise and stabilise the stage of differentiation, by controlling cell density and the nutritional and hormonal environment, to obtain a uniform population of cells.

Because the dynamic properties of cell culture are sometimes difficult to control, and the appropriate cell interactions found *in vivo* difficult to recreate *in vitro*, many people have forsaken the idea of serial propagation in favour of retaining the structural integrity of the original tissue. Such a system is called histotypic or organ culture and is dealt with in Chapter 7. Attempts have also been made to recreate tissue like structures *in vitro* by reaggregating different cell types and culturing at high density as multicellular spheroids (9), perfused multilayers on glass or plastic substrates (10) or floating cultures on collagen (11) or synthetic microporous filters (12).

2.2 Differentiation

As propagation of cell lines requires that the cell number increases continually, culture conditions which have evolved over the years have been selected to favour maximal cell proliferation. It is not surprising that these conditions are not often conducive to cell differentiation where cell growth is severely limited or completely abolished. Those conditions which favour cell proliferation are low cell density, low Ca^{2+} concentration (13) ($100-600\ \mu M$) and the presence of growth factor such as epidermal growth factor (EGF), fibroblast growth factor (FGF), and platelet derived growth factor (PDGF). High cell density ($>10^5$ cells/cm^2), high Ca^{2+} concentration ($300-1500\ \mu M$) and the presence of differentiation inducers [hormones such as hydrocortisone (14), glia maturation factor (15), nerve growth factor (16), retinoids (17) and polar solvents, such as dimethyl sulphoxide (18)], will favour cytostasis and differentiation.

The role of serum in differentiation is not entirely clear and depends on the cell type and medium used. While a low serum concentration promotes differentiation in oligodendrocytes (19), a high serum concentration causes squamous differentiation in bronchial epithelium (20). In the latter case, the active principle is a molecule closely resembling or identical to tumour derived growth factor β isolated from platelets. The use of defined media will hopefully help to resolve this question.

The establishment of the correct polarity and cell shape may also be important, particularly in epithelium. Many workers have shown that growing cells to high density on a floating collagen gel allows matrix interaction, access to medium on both sides, the possibility of establishing correct polarity with respect to the basement membrane and the adoption of the correct cell shape due to the plasticity of the substrate (21).

Different conditions are required, therefore, for propagation and differentiation and hence an experimental protocol may require a growth phase to increase cell number and allow for replicate samples, followed by a non-growth maturation phase to allow for increased expression of differentiated functions.

Table 1. Respective Advantages of Cell and Organ Culture.

Organ culture	*Cell culture*
Histology	Propagation and expansion
Differentiation	Cloning, selection and purification
Cell interaction, homotypic and heterotypic	Characterisation and preservation
Matrix interaction	Replicate sampling and quantitation

3. CHOICE OF MATERIALS

The primary determinant in selecting a tissue or cell line for further study is the nature of the observations that have to be carried out. General cellular processes such as DNA synthesis, membrane permeability, or determination of cytotoxicity may be feasible with any cell type, while the study of specialised properties such as myotube fusion, antibody production or regulation of urea cycle enzymes will require cell types expressing these specific functions.

3.1 Organ Culture or Cell Culture?

Originally tissue culture was regarded as the culture of whole fragments of explanted tissue with the assumption that histological integrity was maintained, at least in part. Now 'tissue culture' has become a generic term and encompasses 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 by spontaneous migration from an explant and may be propagated as a cell suspension or attached monolayer.

In adopting a particular type of culture the following points should be taken into account. Organ culture (see Chapter 7) will preserve cell interaction, retain histological and biochemical differentiation for longer, and, after the initial trauma of explantation and some central necrosis will generally remain in a non-growing steady state for a period of several days and even weeks. They cannot be propagated, generally incur greater experimental variation between replicates, and tend to be more difficult to use for quantitative determinations due to minor variations in geometry and constitution.

Cell cultures on the other hand, are usually devoid of structural organisation, 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. They can, however, be propagated and hence expanded and divided into identical replicates, they can be characterised and a defined cell population preserved by freezing, and they can be purified phenotypically by growth in selective media (Chapter 2), physical cell separation (Chapters 5,6) or cloning (Chapters 4,8) and genotypically to give a characterised cell strain with considerable uniformity.

These properties are summarised in *Table 1*

3.2 Source of Tissue

3.2.1 *Embryo or Adult?*

In general, cultures derived from embryonic tissues will survive and grow better than

those from the adult. This presumably reflects the lower level of specialisation and presence of replicating precursor or stem cells in the embryo. Adult tissues will usually have a lower growth fraction and a high proportion of non-replicating specialised 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.

Embryonic or foetal tissue has many practical advantages, but it must always 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 characterisation.

Examples of widely used embryonic cell lines are the various 3T3 lines (mouse embryo fibroblasts) and MRC-5 and other human foetal lung fibroblasts. Mesodermally derived cells (fibroblasts, endothelium, myoblasts) are also easier to culture than epithelium, neurones or endocrine tissue but this may reflect the extensive use of fibroblast cultures during the early years of the development of culture media together with the response of mesodermally-derived cells to mitogenic factors present in serum. A number of new selective media have now been designed for epithelial and other cell types (see Chapter 2) and with some of these it has been shown that serum is inhibitory to growth and may promote differentiation (20).

3.2.2 *Normal or Neoplastic?*

Normal tissue usually gives rise to cultures with a finite lifespan while cultures from tumours can give continuous cell lines (see below), although there are several examples of continuous cell lines (MDCK dog kidney, 3T3 fibroblasts) which are non-tumorigenic.

Normal cells will generally grow as an undifferentiated stem cell or precursor cell and the onset of differentiation is accompanied by a cessation in cell proliferation which may be permanent. Some normal cells, e.g., fibrocytes or endothelium, are able to differentiate and still dedifferentiate and resume proliferation and in turn redifferentiate, while others, e.g., squamous epithelium and many haemopoietic cells, once initiated into differentiation are incapable of resuming proliferation.

Cells cultured from neoplasms, however, can express at least partial differentiation, e.g., B16 mouse melanoma, while retaining the capacity to divide. Many studies of differentiation have taken advantage of this fact and used differentiated tumours such as the minimal deviation hepatomas of the rat (22) and human and rodent neuroblastomas (23), although whether this can be taken as normal differentiation is always in doubt.

Tumour tissue can often be passaged in the syngeneic host, providing a cheap and simple method of producing large numbers of cells, albeit with lower purity. Where the natural host is not available, tumours can also be propagated in athymic mice with greater difficulty but similar advantages.

Many other differences between normal and neoplastic cells are similar to those between finite and continuous cell lines (see below) and indeed the importance of immortalisation in neoplastic transformation has been recognised (24).

3.3 Subculture

Freshly isolated cultures are known as *primary cultures* until they are passaged or

subcultured. They are usually heterogeneous, and have a low growth fraction, but are more representative of the cell types in the tissue from which they were derived and in the expression of tissue specific properties. Subculture allows the expansion of the culture (it is now known as a *cell line*), the possibility of cloning (see Chapters 4 and 8), characterisation and preservation (Chapter 4) and greater uniformity but may cause a loss of specialised cells and differentiated properties unless care is taken to select out the correct lineage and preserve or reinduce differentiated properties (see below).

The greatest advantage of subculturing a primary culture into a cell line is the provision of large amounts of consistent material suitable for prolonged use.

3.3.1 *Finite or Continuous Cell Lines?*

After several subcultures a cell line will either die out (*finite cell line*) or 'transform' to become a *continuous cell line*. It is not clear in all cases whether the stem line of a continuous culture pre-exists masked by the finite population or arises during serial propagation. Because of the time taken for such cell lines to appear (often several months) and the differences in their properties it has been assumed that a mutational event (chromosomal rearrangement, translocation, partial or total non-disjunction, or point mutation) occurs, but the pre-existence of immortalised cells, particularly in cultures from neoplasms, cannot be excluded.

The appearance of a continuous cell line is usually marked by an alteration in cytomorphology (smaller cell size, less adherent, more rounded, higher nucleo:cytoplasmic ratio), an increase in growth rate (population doubling time decreases from 36–48 h to 12–36 h), a reduction in serum dependence, an increase in cloning efficiency, a reduction in anchorage dependence (i.e., an increased ability to proliferate in suspension as a liquid culture or cloned in agar), an increase in heteroploidy (chromosomal variation between cells) and aneuploidy (divergence from the donor, euploid, karyotype) and an increase in tumorigenicity. The resemblance between spontaneous *in vitro* transformation and malignant transformation is obvious but nevertheless the two are not necessarily identical although they have much in common. Normal cells can 'transform' to become continuous cell lines without becoming malignant and malignant tumours can give rise to cultures which 'transform' and become more (or even less) tumorigenic but acquire the other properties listed above.

The advantages of continuous cell lines are their greater growth rates to higher cell densities and resultant higher yield, their lower serum requirement and general ease of maintenance in simple media, and their ability to grow in suspension. Their disadvantages include greater chromosomal instability, divergence from the donor phenotype, and loss of tissue specific markers.

3.3.2 *Propagation in Suspension*

Most cultures, including primaries, are propagated as a monolayer, anchored to a glass or plastic substrate. Some cultures, principally transformed cells, haemopoietic cells and ascites tumours can be propagated in suspension. This has the advantage of simpler propagation (subculture only requires dilution, no trypsinisation), no requirement for increasing surface area with increasing bulk, ease of harvesting and the possibility of achieving a 'steady-state' culture if required (see Chapter 3).