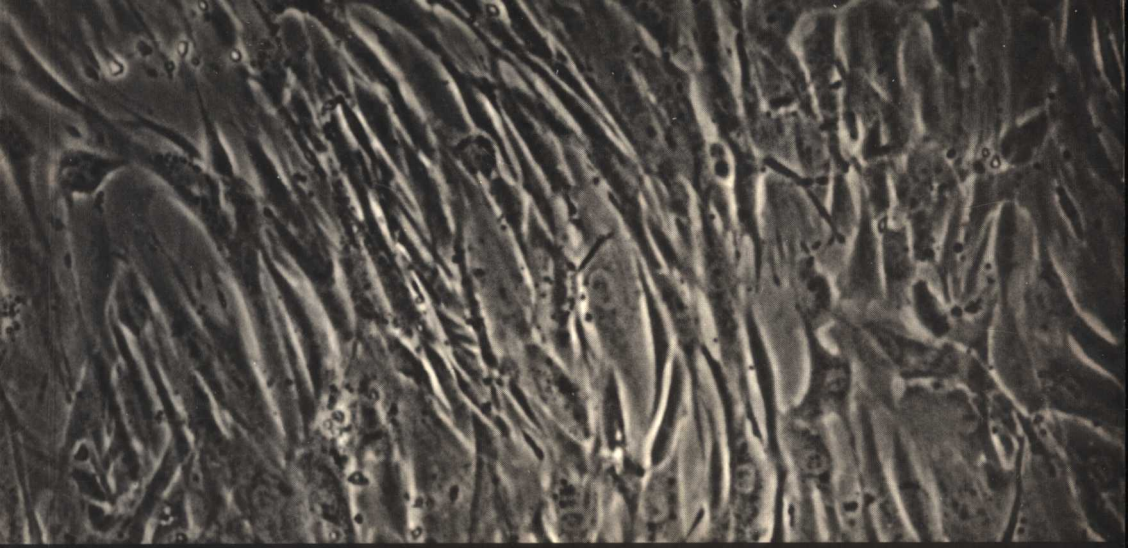
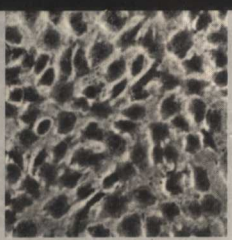
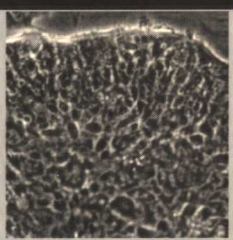


HANDBOOKS IN PRACTICAL ANIMAL CELL BIOLOGY


General techniques of cell culture

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General techniques of cell culture

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Preface

Handbooks in Practical Animal Cell Biology is a series aimed to provide practical workbooks on specific primary cell lineages. Contributing authors to each volume have assumed that their readers will have a basic level of skill and knowledge of general cell culture techniques. The role of this volume on general cell culture techniques is two fold: First, to provide a grounding in the basics of cell culture techniques and second, to give practical details of setting up a cell culture facility from scratch or improving an existing one. The authors run a large and efficient cell culture facility at the Imperial Cancer Research Fund, London; however, the procedures and equipment that they describe are equally applicable to the laboratory with one laminar flow hood and one cell culture incubator. Cell culture novices would be well advised to gain competence in the culture of a few robust long-term cell lines prior to attempting the culture of one of the more demanding primary cell types described in the individual cell lineage volumes. Those with ample experience of cell culture techniques will find this volume most valuable for troubleshooting and for ideas on how to improve their cell culture facility.

Ann Harris
Oxford October 1996

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Contents

1

Introduction

The aim of this volume is to provide a guide to the basic essentials of tissue culture, progressing from the equipment needed, through useful media and sera to the handling of different types of cells, their growth and storage, how to recognize and deal with contamination, and to provide some pointers towards good quality control and safe handling procedures. Detailed protocols on specialized applications are outside the scope of this book, although a chapter is included which covers the basics of some of the more widely used special techniques.

Individual volumes in this series deal with the specific cultures of primary cell types, but before commencing on a project necessitating more specialized skills it may be useful to experience the pleasures and pitfalls of basic tissue culture methods, which will be applicable to primary systems, through handling cell lines. Cell banks and smaller cell production facilities exist largely for the purpose of issuing cell lines and during the culturing of a few selected varieties, anyone of average dexterity and with a good grasp of sterile technique will soon develop a basic expertise.

Tissue culture developed from some of the embryology techniques used in the last century, which involved maintaining the medullary plate of a chick embryo in warm saline. Attempts were also made to maintain pieces of human skin *in vitro*. This was followed by attempts to maintain leukocytes from the salamander in hanging droplets (Jolly, 1903). From this early work, the traditional tissue culture techniques were rapidly devised. The term 'tissue culture' includes both cell and organ culture, although within the confines of this book we will be discussing only the former.

Harrison, in 1907, worked with frog tissues, since they require no incubation and since tissue regeneration is more common in lower vertebrates, there was a possibility that growth was more likely to occur than with mammalian tissue. Burrows's and Carrel's work (Burrows, 1910; Carrrel and

Burrows, 1912) showed that animal cells could be grown *in vitro*. A history of somatic cell genetics will provide a more detailed account of the origins of the subject (Harris, 1995).

The rudiments of tissue culture as we know it today came predominantly from Dr Earle's group at the National Cancer Institute among others. This group grew cells directly on glass, propagated cultures from single cells, and were successful in growing suspension cultures. Numerous workers followed this with investigations into the factors in culture medium that are necessary for growth. Some of these researchers have given their name to media in common use today, e.g. Fischer, Waymouth, Eagle. The development of tissue culture as a useful tool in the modern laboratory arose largely because of the needs of (a) the production of antiviral vaccines and (b) cancer research. Now that there are standard procedures for the production of large numbers of cells, and sera and media may be obtained commercially, tissue culture lends itself to a whole range of investigative fields including all aspects of cell biology, physiology and biochemistry.

The advantages of tissue culture include the ability to control the environment of the cell (pH, temperature, osmotic pressure, O_2 and CO_2). Additionally, after one or two passages cultured cell lines assume a uniform constitution, and as the cells are randomly mixed at each passage, there is a tendency for the culture conditions to produce a similar type of cell in as much that the most vigorously growing cells will predominate. The other noteworthy advantage is that, in numerous instances, tissue culture may be used to replace experimentation on live animals, which aside from ethical preferences is cheaper and quicker.

Cell markers

Characterization of cell lines is important. It is naturally a necessity to be able to correlate a culture with its original tissue from the point of view of its descent and its stage of advancement during the course of the development of the mature cell type. During the maintenance of cell lines there may be instability in the phenotype and genotype caused by variations in culture conditions, selective overgrowth or genetic changes. It is therefore important that conditions are standardized and a seed stock is preserved to be able to return to at intervals to maintain consistency. It is essential that cells are checked regularly for cross contamination (see Chapter 8). Stable markers are needed for characterizations, and culture conditions may need to be modified so that these markers are expressed.

For details of markers of other cell lines the reader should refer to other books in the Handbooks in Practical Animal Cell Biology series.

In general, the most obvious technique used to identify cells is morphology. However, it should be remembered that cellular morphology can appear different according to the culture conditions, e.g. Mouse 3T3 cells have a fibroblastic appearance at low cell density but when confluent become epithelial-like. Over the years the terms 'fibroblastic' and 'epithelial' have come to apply to the appearance of the cells rather than their origin. If this criterion is used, a cell which has length more than twice its width could be called fibroblastic, whilst a monolayer cell with more regular dimensions could be said to be epithelial.

Growth cycles, which give an idea of the population doubling time and the time it takes for a monolayer to become confluent or a suspension culture to reach saturation density should remain constant so that a change in the expected times, assuming that culture conditions have remained stable, could indicate cross contamination, or senescence (see Chapter 5). However, such changes may also indicate transformation (possibly due to viral infection) or contamination.

Types of cells

Cells may be loosely divided into two main types, those that grow as a suspension and those which, as the name suggests, grow as an adherent cell culture. Primary cultures and the evolution of cell lines will be discussed in Chapters 5 and 6.

Propagation of cells becomes possible due to cell proliferation. When cells are selected from a culture, the subculture is termed a 'cell strain' and usually strains will be selected for a particularly detailed characterization.

Suspension cultures are derived from cells which can divide and survive without being attached to a substrate, e.g. cells of haemopoietic lineage, whereas adherent cultures or monolayers must adhere to a substrate to survive.

It is possible to culture many different cell types of various lineages, e.g. epithelial cells including keratinocytes, mammary gland duct cells, cervical cells, cells from the gastrointestinal tract, liver, pancreas, kidney, bronchial and tracheal cells; mesenchymal cells which include connective, adipose and muscle tissue, cartilage and bone. Neuroectodermal cells include glia and endocrine cells, and the haemopoietic system includes macrophages and lymphoid cells. Specialized techniques for the culture of these cell types will be found in other books in this series.

Sources of cells

Aside from preparing cultures from the original tissues (see Chapter 6), the most usual sources of cells are cell banks or cell stocks from other workers in the field.

A number of reliable organizations produce good-quality controlled cell cultures, which have been subjected to tests for viability, the absence of contamination including mycoplasma, karyotypic and isoenzyme analysis, DNA fingerprinting, and in some cases tests for viral susceptibility, tumourgenicity, biochemical traits and drug susceptibility. Cell banks will also accept deposits of useful cell lines. It may be useful, at this point, to briefly describe the scheme for banking cells, as a similar procedure scaled down may be adopted for banking cells in a laboratory/department.

When a new cell line is brought into the laboratory, there should be defined procedures on how to handle it. Cultures should be handled in Class II safety cabinets in a quarantine laboratory away from the main tissue culture area. A small amount should be frozen as soon as possible and quality control tests carried out. When the quarantine conditions (see Chapter 8) have been satisfied, the new cultures may be transferred to the main laboratory for production of master and working banks.

An outline is given of the general plan for running a cell bank in Figure 1.1.

If an organization is attempting to run a cell bank on a smaller scale, i.e. producing cultures for that particular organization only, it will usually not be possible to fulfil the criteria as laid out in the plan above and amendments must be made to take into account such things as space available for the storage of frozen cells, the practicalities of returning samples to the originator for verification and the pressures of having to produce cultures in the shortest possible time. However, it would be foolhardy in the extreme to cut out any contamination checks (particularly for mycoplasma) or species verification.

The cell banks listed at the end of this chapter produce catalogues of material held, and access to up-to-date information is available using on-line data systems. (The American Type Culture Collection (ATCC) database is available on-line via the Cambridge, UK based MSDN computer network using electronic mail systems.) Both the ATCC and ECACC catalogues are accessible on the world-wide web:

ATCC http://www.Atcc.org/

ECACC http://www.biotech.ist.unige.it/cldb/descat5.html

Both these cell banks have begun a project to generate a European directory of cell line resources available in European laboratories.

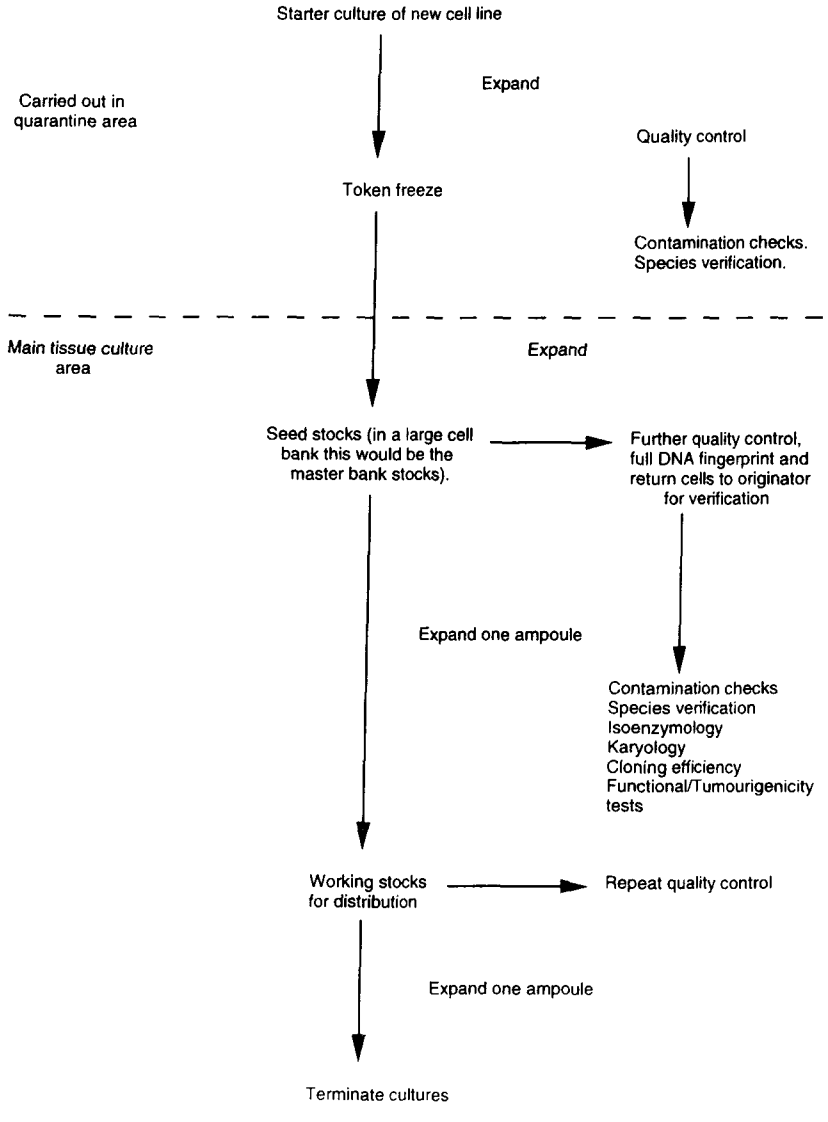

Fig. 1.1. Procedures for handling a new cell line.

Addresses of useful cell banks:

American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, USA Tel: (301) 991 2600. Telex: 908768 ATCC ROVE. Fax: (301) 231 5826

The European Collection of Animal Cell Cultures, PHLS Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire SP4 OJG, UK Tel: 01 980 610391. Telex: 47683 PHCAMR G. Fax: 01 980 611315 E-mail: Telecom Gold, 75:DB1 0222

NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, 401 Haddon Avenue, Camden, NJ 08103, USA Tel: (609) 757 4848. Fax: (609) 964 0254

References

Burrows, M. T. (1910). The cultivation of tissues of the chick-embryo outside the body. *J. Am. Med. Assoc.*, **55**, 2057–8.

Carrel, A. & Burrows, M. T. (1912). Cultivation of adult tissue and organs outside the body. *J. Am. Med. Assoc.*, **55**, 1379–81.

Harris, H. (1995). *The Cells of the Body: A History of Somatic Cell Genetics.* Cold Spring Harbor Press, USA.

Harrison, R. G. (1907). Observations on the living developing nerve fibre. *Proc. Soc. Exp. Biol. (NY)*, **4**, 140.

Jolly, J. (1903). Sur la durée de la vie et de la multiplication des cellules animals en dehors de l'organisme. *CR Soc. Biol. (Paris)*, **55**, 1266.

2

Equipment

In its broadest definition, equipment includes the laboratory in which cell culture work is undertaken. Some are fortunate enough to occupy purpose-built cell culture facilities but many use existing laboratories which require varying degrees of adaption to house a successful cell culture area. When planning a laboratory for cell culture, six main functions have to be accommodated. These can be neatly divided into two main groups: sterile handling and support services. Sterile handling includes a cell culture and manipulation area which should be adjacent to an incubation and a storage area. Support services include washing-up, preparation (repackaging) and sterilization. These three functions should also be adjacent to each other and provision made to extract the large amounts of heat and steam associated with this type of operation. It is not essential for the services to be adjacent to the sterile handling area but they should be within the same building. By far the most important consideration is to minimize the chances of microbiological contamination of cell cultures. One of the main causes of contamination can be sudden draughts of room air crossing the work surface from opening doors, the passage of staff behind the operator, open windows or wall-mounted air-conditioning units. Where a laboratory has opening windows, it is vital they are kept closed whenever cell culture work is in operation. Wall-mounted air-conditioning units have no place in a cell culture laboratory because the damp internal conditions harbour and support a source of microbiological contamination readily circulated by the forced movement of air from the unit. It is very important therefore, that the area designated for handling and manipulating cells should be as far away as practicable from the laboratory entrance and from the main passage of staff within the laboratory. Much of the other equipment required for cell culture work will be common to most laboratories, e.g. various types of glassware (pipettes, beakers, flasks, bottles, measuring cylinders, etc.) all of which need

to be sterile; water baths, centrifuges, balances, etc. The number of items of specialized equipment required are comparatively few.

Large equipment

Laminar flow cabinet

Although it is possible to culture cells on an open bench employing careful aseptic technique with or without the aid of a gas burner, the use of a laminar flow cabinet significantly reduces the chance of contamination and eliminates the need for a gas burner. If using a laminar flow cabinet, the minimum requirement is for a horizontal laminar flow cabinet primarily designed to protect the work from the operator but not the operator from the work. With ever-increasing improvements in health and safety regulations in some countries this type of cabinet is now considered to be insufficiently protective of the operator and therefore cannot be recommended. Much better protection for both the operator and the work is provided by a Class II vertical laminar flow (Fig. 2.1) cabinet and this type of cabinet is used widely in cell culture laboratories. Two main variants are available, both employing high efficiency particulate air (HEPA) filters with an efficiency of 99.999%: (a) Class II microbiological safety cabinet (MSC) designed to BS5726 (1992) (UK), NSF 49 (USA), DIN 12950(Germany), NFX 44-201 (France) or AS2252 (Australia) suitable for working with Category 2 pathogens. These are part-open fronted cabinets with double HEPA filters that give sufficient protection to permit them to be used without external ducting. Air is drawn in through a grill at the front lower edge of the cabinet and circulates through a HEPA filter before passing vertically down across the internal front face of the cabinet as a curtain of air at 0.4m/s thereby protecting both the operator and the work. In this type of cabinet, 70% of the air is recirculated through one HEPA filter whilst the remaining 30% is discharged through a second HEPA filter to the external atmosphere. (b) Tissue culture cabinet specifically designed for cell culture and other low risk operations but not suitable for work with pathogens. These are less expensive than the Class II MSC but still meet the safety regulation requirements.

All cabinets should be performance tested at least once a year by a competent person who will advise on filter changing. Some laboratories consider it preferable to leave cabinets running all the time, but, where they are not, the cabinet should be isolated by closure of the operator access, i.e. by replacing the removable front panel.

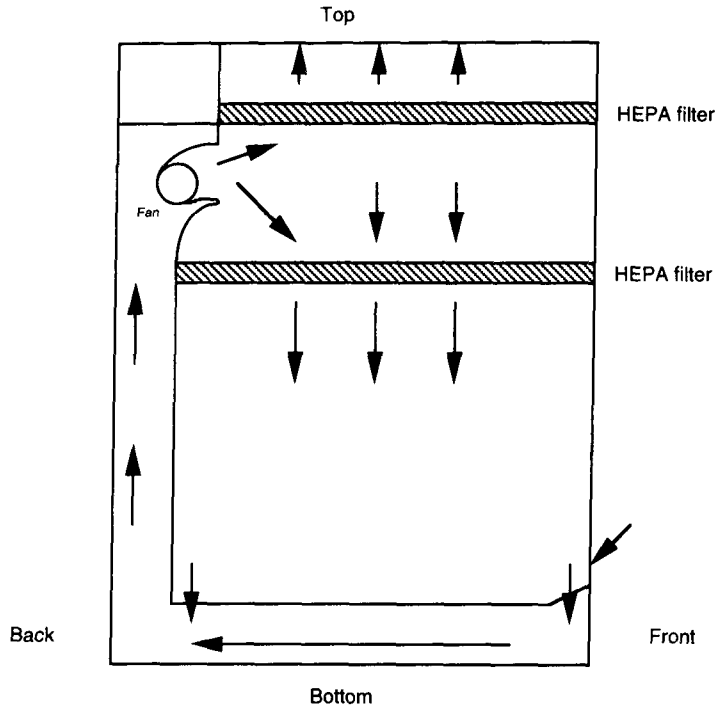

Fig. 2.1. Side view of a typical laminar flow cabinet design in which 70% of the air is recirculated. Room air is drawn in at the bottom front edge of the cabinet, mixed with air being drawn from the working area and circulated round the rear ducting to the upper chamber. Here, 30% of the air is returned to the room through one set of HEPA filters and the remaining 70% recirculated into the working chamber through a second set of HEPA filters,

Manufacturers of laminar flow cabinets

Astec Environmental Systems, 30–31 Lynx Crescent, Weston-Super-Mare, Avon BS24 9BP, UK Tel: 01934 418685 Fax: 01934 419033

The Baker Co. Inc., PO Drawer E, Sanford, Maine 04073, USA. Tel: (207)-324-8773 Fax: (207)-324-3869

Bassaire Ltd, Duncan Road, Swanwick, Southampton, Hampshire SO3 7ZS, UK Tel: 01489 885111 Fax: 01489 885211

Bigneat Ltd, 5 Pipers Wood, Brambles Farm Industrial Estate, Waterlooville, Hampshire PO7 7XU, UK Tel: 01705 266400 Fax: 01705 263373

Coy Laboratory Products Inc., 14500 Coy Drive, Grass Lake, Michigan 49240, USA Tel: (313)-475-2200 Fax: (313)-475-1846

Envair Ltd, York Avenue, Haslingden, Rossendale, Lancashire BB4 4HX, UK Tel: 01706 228416 Fax: 01706 831957

Gelaire – supplied by ICN Ltd.

Heraeus Instruments GmbH, Heraeusstrasse 12–14, PO Box 15 53, D-6450 Hanau 1, Germany Tel: (06181)35-465 Fax: (06181)35-749

Heto-Holten Laminair A/S, Gydevang 17–19, DK-3450 Allerod, Denmark. Tel: (+45) 48 14 27 77 Fax: (+45).42.27.46.55

ICN Biomedicals Ltd, Unit 18, Thame Business Centre, Wenman Road, Thame, Oxfordshire OX9 3XA, UK Tel: 01844 213366 Fax: 01844 213399

ICN Pharmaceuticals Inc., 3300 Hyland Avenue, Costa Mesa, California 92626, USA Tel: (714)-545-0113 Fax: (800)-334-6999

Jouan Inc., 110B Industrial Drive, Winchester, Virginia 22602, USA Tel: (540)-869-8623 Fax: (540)-869-8626

Jouan Ltd, Merlin Way, Quarry Hill Road, Ilkeston, Derbyshire DE7 4RA, UK Tel: 0115 944 7989 Fax: 0115 944 7080 E-mail: jouan.co.uk

Jouan SA, CP.3203 – 44805 Saint-Herblain Cedex, France Tel: 40.16.80.00 Fax: 40.94.70.16

Labcaire Systems Ltd, 15 Hither Green, Clevedon, Avon BS21 6XU, UK Tel: 01275 340033 Fax: 01275 341313

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Microflow Dent and Hellyer (MDH), Walworth Road, Andover, Hampshire SP10 5AA, U.K Tel: 01264 36211 Fax: 01264 356452

Nu-Aire Inc., 2100 Fernbrook Lane, Plymouth, Minnesota 55447, USA Tel: (612)-553-1270 Fax: (612)-553-0459

Incubators

Culturing cells from any organism requires the provision of environmental conditions that mimic as closely as possible those experienced by cells *in vivo*. For mammalian cells, this includes incubation at a temperature as close as possible to the normal temperature of the animal species from which the cells were taken. Generally, this ranges from 33 °C to 43 °C depending on the ambient temperature. In practice, most incubators used for cell culture are set to 35 °C to 37 °C which, for most types of mammalian cell, is satisfactory. Incubators range from what is essentially a simple insulated metal box with a door and basic temperature controls to water-jacketed CO_2 incubators with sophisticated electronic controls. The CO_2 incubator, which is designed specifically for cell culture in open vessels, e.g. Petri dishes, microtitre plates, etc. is provided with a supply of CO_2 either from a piped system or from gas cylinders. The electronic controls on the incubator ensure the atmosphere within the incubator chamber is maintained