Second Edition

CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

R. Ian Freshney

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Alan R. Liss, Inc., New York

Cover Illustrations. *From the top: Vero cells growing on microcarriers; suspension culture vessels; primary explant from human mammary carcinoma; human glioma cells.*

Address all Inquiries to the Publisher
Alan R. Liss, Inc., 41 East 11th Street, New York, NY 10003

Printed in the United States of America

First Edition published 1983
Second Printing, April 1984
Third Printing, November 1984

Second Edition published 1987

Library of Congress Cataloging-in-Publication Data

Freshney, R. Ian.
Culture of animal cells.

Bibliography: p.
Includes index.
1. Tissue culture. 2. Cell culture. I. Title.
[QH585.F74 1987] 591'.07'24 87-3313
ISBN 0-8451-4241-0

Preface to the Second Edition

In revising Culture of Animal Cells I have tried to keep the emphasis on the practical aspects of cell culture and have discussed the theoretical background only when it seemed necessary to the understanding of the technique or the status of the culture. For example, cell transformation and some of its implications are dealt with more fully to help the reader to appreciate the phenotypic properties that these cells might be expected to express and the roles that they might usefully play in experimental studies and commercial exploitation.

Major changes have been introduced in the presentation of serum free medium formulations as these have gained more general acceptance, and some have become commercially available, since publication of the first edition. In parallel with this, and in many cases as a direct consequence, the culture of specific cell types such as epidermal keratinocytes, melanocytes, and breast epithelium have become more feasible, so a number of protocols are included for such specialized cultures.

To enable these areas to be covered more effectively, I have enlisted the help of experts in each respective field to present protocols from their own experience where I feel my own expertise is insufficient. These specialist protocols cover areas of new technology, such as somatic hybridization and production of hybridomas as well as the culture of specific cell types, and have been presented in the same style as the previous protocols. I am very grateful to these new contributors and feel that they have extended the scope of the text more than I could have hoped to do alone. In some cases these protocols will be sufficient for readers to fulfill their needs without further recourse to the literature, but to satisfy those whose demands are greater, or where the technique is more complex, the appropriate references are provided.

A more extensive treatment has also been given to cytotoxicity assay and the culture of tumor cells, particularly from human tumors, in line with the emphasis that these techniques are currently being given in hospitals, basic research laboratories, and the biotechnology and drug industry.

In addition to the contributors of specialized protocols referred to above, I am again indebted to my colleagues in the Department of Medical Oncology including Jane Plumb, Stephen Merry, Carol McCormick, Alison Mackie, and Ian Cunningham, and a succession of graduate and undergraduate students including John McLean, Alison Murray, Jim Miller, Iain Singer, Barbara Christie, and Alan Beveridge who have provided data and ideas. While trying to answer their questions, I was stimulated into thinking more about the potential needs of the reader.

My thanks are also due to Mrs. Rae Fergusson for typing new material faster than I could generate it and handling my poor handwriting and illegible corrections with unbelievable accuracy.

Most of all I would like to thank my wife and family for their continuing help and encouragement. They provided much practical help, advice, and moral support. In particular my wife's many hours collating, referencing, and proof reading, have spared me many hours of often tedious work.

Preface to the First Edition

Tissue culture is not a new technique. It has been in existence since the beginning of this century and has passed through its simple exploratory phase, a later expansive phase in the 1950's, and is now in a phase of specialization concerned with control mechanisms and differentiated function. Matching the current trends, recent additions to the range of available tissue culture books have been concerned with specialized techniques and the result of this is that the basic procedures have become a little neglected.

It has been my objective in preparing this book to provide the novice to tissue culture with sufficient information to perform the basic techniques. It is anticipated that the reader will have a fundamental grasp of elementary anatomy, histology, cell physiology, and the basic principles of biochemistry, but will have had little or no experience in tissue culture. This book should prove useful at the advanced undergraduate level for technicians in training, for graduate studies, and at the post-doctoral level. It is intended as an introduction to the theory of the technique, and biology of cultured cells as well as a practical, step-by-step guide to procedures, and should be of value to anyone without any, or with little, prior experience in tissue culture. Of necessity, some of the more exciting developments in recent years, e.g., production of monoclonal antibodies by hybridoma cultures, can only be described briefly and references provided to further reading.

A list of reagents and commercial suppliers is located at the end of the book. Occasionally, a supplier's name is incorporated in the text but in most cases reference should be made to the trade index. Other reference materials included at the rear of the book are a glossary, a list of cell banks, a subject index, and the literature references cited in the text.

It is inevitable when preparing a text such as this that, in addition to my own experience, I have called upon the help and advice of many others both during the preparation of the book and in the twenty years or so since I was first introduced to the field. As with many other similar techniques, there is much of tissue culture that is never documented, but passed on by word of mouth at meetings, or, more often, in moments of conviviality after meetings. Hence there may be occasions when I have reproduced advice or information as if it were my own, without due acknowledgment to published work, because I have been unable to trace a reference, or none exists. In all such cases I would like to thank those who have contributed consciously or unconsciously to my own accumulated experience in the field.

While it would be impossible to recall all of those with whom contact over the past two decades has influenced my current understanding of the field, there are those of whom I must make special mention. First among these is Dr. John Paul, who introduced me to the field and whose sound common sense and practicality were a good introduction to what can, in the correct hands, be a very precise discipline. I owe him my sincere gratitude, as his one-time student and now associate and friend.

In my years with the Beatson Institute I have had the privilege to work with many people, both resident and visitors, and share in their experience in the development of techniques to which I would otherwise not have been exposed. In some cases they are acknowledged in the text or figure legends, but I hope any who are not mentioned by name will still recognize my gratitude.

Among others who should be named are those who have worked most closely with me in recent years, helped in my own research activities, and generated some of the data that appear on these pages. They include Ms. Diana Morgan, Mrs. Elaine Hart, Mrs. Margaret Frame, Mr. Alistair McNab, Mrs. Irene Osprey, and Miss Sheila Brown. Although my wife and I do not work together usually, I have had the benefit of her skilled assistance at times, and, in addition, her experience in the field has added greatly to my own. Others who have worked with me for shorter periods, elements of whose work may be reported here in part, are Mohammad Hassanzadah, Peter Crilly, Fadik Akturk, Metyn Guner, Fahri Celik, Aileen Sherry, Bob Shaw and Carolyn MacDonald.

I have also been indebted to many people in Glasgow and elsewhere for helpful advice and collaboration. Among many others, these include David G.T. Thomas, David I. Graham, Michael Stack-Dunne, Peter Vaughan, Brian McNamee, David Doyle, Rona MacKie, Kenneth

C. Calman, and the late John Maxwell Anderson, with whom I had my first introduction to clinical collaboration.

I must also record my good fortune to have been able to spend time in other laboratories and learn from the approaches of others such as Robert Auerbach, Richard Ham, and Wally McKeehan.

I am also grateful to Flow Laboratories for their help and collaboration in running basic tissue culture courses and the resultant opportunity to broaden my knowledge of the field.

I would like to express my gratitude to Paul Chapple who first persuaded me that I should write a basic techniques book on tissue culture, and to numerous others, including Don Dougall, Wally and Kerstin McKeehan, Peter del Vecchio, John Ryan, Jim Smith, Rob Hay, Charity Waymouth, Sergey Federoff, Mike Gabridge, and Dan Lundin for help and advice during the preparation of the manuscript.

I would also like to thank Miss Donna Madore for converting my often illegible manuscript into typescript, Mrs. Marina LaDuke for expert photography, Miss Diane Leifheit for further help with the illustrations, and Ms. Jane Gillies for preparing the line drawings. These four ladies spent many hours on my behalf and their patience and skill is greatly appreciated. My thanks are also due to Mrs. Norma Wallace for completing the final retype quickly, efficiently, and at very short notice.

It would not be fitting for me to conclude this preface without further major acknowledgment to my wife, Mary, my daughter, Gillian, and son, Norman. Not only did I enjoy their sympathy and understanding at home, when I am sure, at times, I did not deserve it, but I also benefitted from the fruits of their labors during the day: drawing graphs, collecting references, researching and tabulating methods and information. My wife's experience in the field, plus countless hours of reading, revising, and collecting information, made her share in this work indispensible.

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Chapter 1 Introduction

BACKGROUND

Tissue culture was first devised at the beginning of this century [Harrison 1907, Carrel, 1912] as a method for studying the behavior of animal cells free of systemic variations that might arise in the animal both during normal homeostasis and under the stress of an experiment. As the name implies, the technique was elaborated first with undisaggregated fragments of tissue, and growth was restricted to the migration of cells from the tissue fragment, with occasional mitoses in the outgrowth. Since culture of cells from such primary explants of tissue dominated the field for more than 50 years, it is not surprising that the name "tissue culture" has stuck in spite of the fact that most of the explosive expansion in this area since the 1950s has utilized dispersed cell cultures.

Throughout this book the term "tissue culture" will be used as the generic term to include organ culture and cell culture. The term "organ culture" will always imply a three-dimensional culture of undisaggregated tissue retaining some or all of the histological features of the tissue in vivo. "Cell culture" will refer to cultures derived from dispersed cells taken from the original tissue, from a primary culture, or from a cell line or cell strain, by enzymatic, mechanical, or chemical disaggregation. The term "histotypic culture" will imply that cells have been reassociated in some way to recreate a three-dimensional tissue-like structure, e.g., by perfusion and overgrowth of a monolayer, reaggregation in suspension, or infiltration of a three-dimensional matrix such as collagen gel.

Harrison chose the frog as his source of tissue presumably because it was a cold-blooded animal, and consequently incubation was not required. Furthermore, since tissue regeneration is more common in lower vertebrates, he perhaps felt that growth was more likely to occur than with mammalian tissue. Although his technique may have sparked off a new wave of interest in cultivation of tissue *in vitro*, few later workers were to follow his example in the selection of species. The stimulus from medical science carried future interest into warm-blooded animals where normal and pathological development are closer to human. The accessibility of different tissues, many of which grew well in culture, made the embryonated hen's egg a favorite choice; but the development of experimental animal husbandry, particularly with genetically pure strains of rodents, brought mammals to the forefront as favorite material. While chick embryo tissue could provide a diversity of cell types in primary culture, rodent tissue had the advantage of producing continuous cell lines [Earle et al., 1943].

The demonstration that human tumors could also give rise to continuous cell lines [e.g., HeLa: Gey et al., 1952], encouraged interest in human tissue, helped later by Hayflick and Moorhead's classical studies with normal cells of a finite life-span [1961].

For many years the lower vertebrates and the invertebrates have been largely ignored though unique aspects of their development (tissue regeneration in amphibia, metamorphosis in insects) make them attractive systems for the study of the molecular basis of development. More recently the needs of agriculture and pest control have encouraged toxicity and virological studies in insects, and the rapidly developing area of fish farming has required more detailed knowledge of normal development and pathogenesis in fish.

In spite of this resurgence of interest, tissue culture of lower vertebrates and the invertebrates remains a very specialized area, and the bulk of interest remains in avian and mammalian tissue. This has naturally influenced the development of the art and science of tissue culture, and much of what will be described in the ensuing chapters of this book reflect this, as well as my own personal experience. Hence advice on incubation and the physical and biochemical properties of media refers to homiotherms and guidance on the appropriate modification for poikilothermic animals will require recourse to the literature. This will be discussed in a little more detail in a later chapter.

Many of the basic techniques of asepsis, preparation and sterilization, primary culture, selection and cell separation, quantitation, and so on, apply equally to poikilotherms and will require only minor modification; on the whole the principles remain the same.

The types of investigation that lend themselves particularly to tissue culture are summarized in Figure 1.1.: (1) Intracellular activity, e.g., the replication and transcription of deoxyribonucleic acid (DNA), protein synthesis, energy metabolism; (2) intracellular flux, e.g., movement of ribonucleic acid (RNA) from the nucleus to the cytoplasm, translocation of hormone receptor complexes, fluctuations in metabolite pools; (3) "ecology," eg., nutrition, infection, virally or chemically induced transformation, drug action, response to external stimuli, secretion of specialized products; and (4) cell-cell interaction, e.g., embryonic induction, cell population kinetics, cell-cell adhesion.

The development of tissue culture as a modern sophisticated technique owes much to the needs of two major branches of medical research: the production of antiviral vaccines and the understanding of neoplasia. The standardization of conditions and cell lines for the production and assay of viruses undoubtedly provided much impetus to the development of modern tissue culture technology, particularly the production of large numbers of cells suitable for biochemical analysis. This and other technical improvements made possible by the commercial supply of reliable media and sera, and by the greater control of contamination with antibiotics and clean air equipment, has made tissue culture accessible to a wide range of interests.

In addition to cancer research and virology, other areas of research have come to depend heavily on tissue culture techniques. The introduction of cell fusion techniques [Barski, et al., 1960; Soreuil and Ephrussi, 1961; Littlefield, 1964; Harris and Watkins, 1965] and genetic manipulation established somatic cell genetics as a major component in the genetic analysis of higher animals including man, and contributed greatly, via the monoclonal antibody technique, to the study of immunology, already dependent on cell culture for assay techniques and production of hemopoietic cell lines.

The insight into the mechanism of action of antibodies, and the reciprocal information that this provided about the structure of the epitope, derived from monoclonal antibody techniques [Kohler and Milstein, 1975] was, like the technique of cell fusion itself, a prologue to a whole new field of studies in genetic manipulation. This has supplied much basic information on the control of gene transcription and a vast new technology has grown from the ability to insert exploitable genes into prokaryotic cells. Cell products such as human

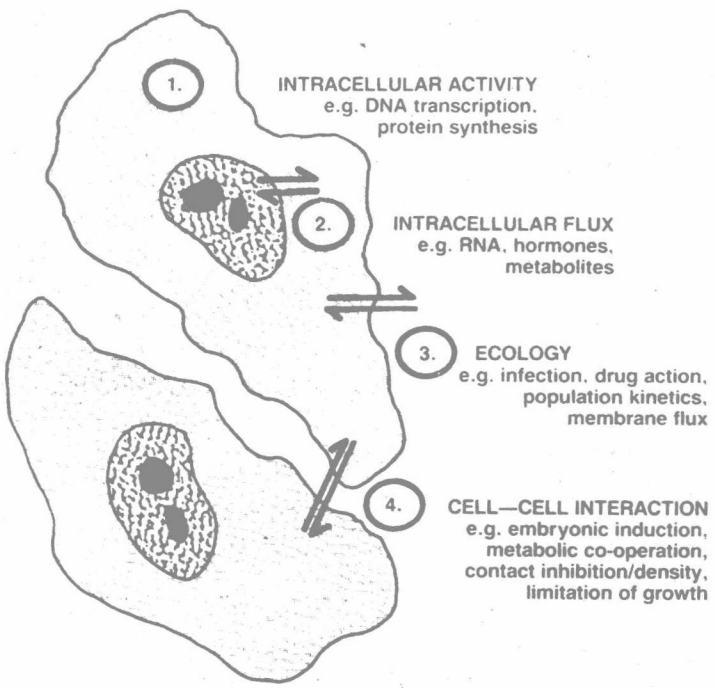

Fig. 1.1. *Areas of interest in tissue culture.*

growth hormone, insulin, and interferon have been genetically engineered, but the absence of post-transcriptional modifications, such as glycosylation, in bacteria suggest that mammalian cells may provide more suitable vehicles. The insertion of the appropriate genes into normal human cells (1) to make them continuous cell lines (see Chapter 2) and (2) to make them produce pharmaceutically viable drugs will have profound effects on the drug industry, which can only be overshadowed by radical innovations in organic chemical synthesis that are, as yet, not apparent. Other areas of major interest include the study of cell interactions and intracellular control mechanisms in cell differentiation and development [Auerbach and Grobstein, 1958; Cox, 1974; Finbow and Pitts, 1981] and attempts to analyze nervous function [Bornstein and Murray, 1958; Minna et al., 1972; Kingsbury et al., 1985]. Progress in neurological research has, however, not had the benefit of working with propagated cell lines as propagation of neurons has not so far been possible *in vitro* without resorting to the use of transformed cells (see Chapter 15).

Tissue culture technology has also been adopted into many routine applications in medicine and industry. Chromosomal analysis of cells derived from the womb by amniocentesis (see Chapter 23) can reveal genetic disorders in the unborn child, viral infections may be assayed qualitatively and quantitatively on monolayers of appropriate host cells, (see Chapter 23), and the toxic effects of pharmaceutical compounds and potential environmental pollutants can be measured in colony-forming and other *in vitro* assays (see Chapter 19).

Further developments in the application of tissue culture to medical problems may follow from the demonstration that cultures of epidermal cells form functionally differentiated sheets in culture [Green et al., 1979], and endothelial cells may form capillaries [Folkman and Haudenschild, 1980], suggesting possibilities in homografting and reconstructive surgery using an individual's own cells [Pittelow and Scott, 1986].

It is clear that the study of cellular activity in tissue culture may have many advantages; but in summarizing these, below, considerable emphasis must also be placed on its limitations, in order to maintain some sense of perspective.

ADVANTAGES OF TISSUE CULTURE

Control of the Environment

The two major advantages, as implied above, are the control of the physiochemical environment (pH, temperature, osmotic pressure, O_2, CO_2 tension), which may be controlled very precisely, and the physiological conditions, which may be kept relatively constant but cannot always be defined. Most cell lines still require supplementation of the medium with serum or other poorly-defined constituents. These supplements are prone to batch variation [Olmsted, 1967; Honn et al., 1975] and contain undefined elements such as hormones and other regulatory substances. Gradually the essential components of serum are being identified, making replacement with defined constituents more practicable [Birch and Pirt, 1971; Ham and McKeehan, 1978; Barnes and Sato, 1980; Barnes et al., 1984; Maurer, 1986] (see also Chapter 7).

Characterization and Homogeneity of Sample

Tissue samples are invariably heterogeneous. Replicates even from one tissue vary in their constituent cell types. After one or two passages, cultured cell lines assume a homogeneous, or at least uniform, constitution as the cells are randomly mixed at each transfer and the selective pressure of the culture conditions tends to produce a homogeneous culture of the most vigorous cell type. Hence, at each subculture each replicate sample will be identical, and the characteristics of the line may be perpetuated over several generations, or indefinitely if the cell line is stored in liquid N_2. Since experimental replicates are virtually identical, the need for statistical analysis of variance is reduced.

Economy

Cultures may be exposed directly to a reagent at a lower and defined concentration, and with direct access to the cell. Consequently, less is required than for injection *in vivo* where >90% is lost by excretion and distribution to tissues other than those under study.

Screening tests with many variables and replicates are cheaper, and the legal, moral, and ethical questions of animal experimentation are avoided.

DISADVANTAGES

Expertise

Culture techniques must be carried out under strict aseptic conditions, because animal cells grow much less rapidly than many of the common contaminants such as bacteria, molds, and yeasts. Furthermore, unlike microorganisms, cells from multicellular animals do not exist in isolation, and consequently, are not able to sustain independent existence without the provision of a complex environment, simulating blood plasma