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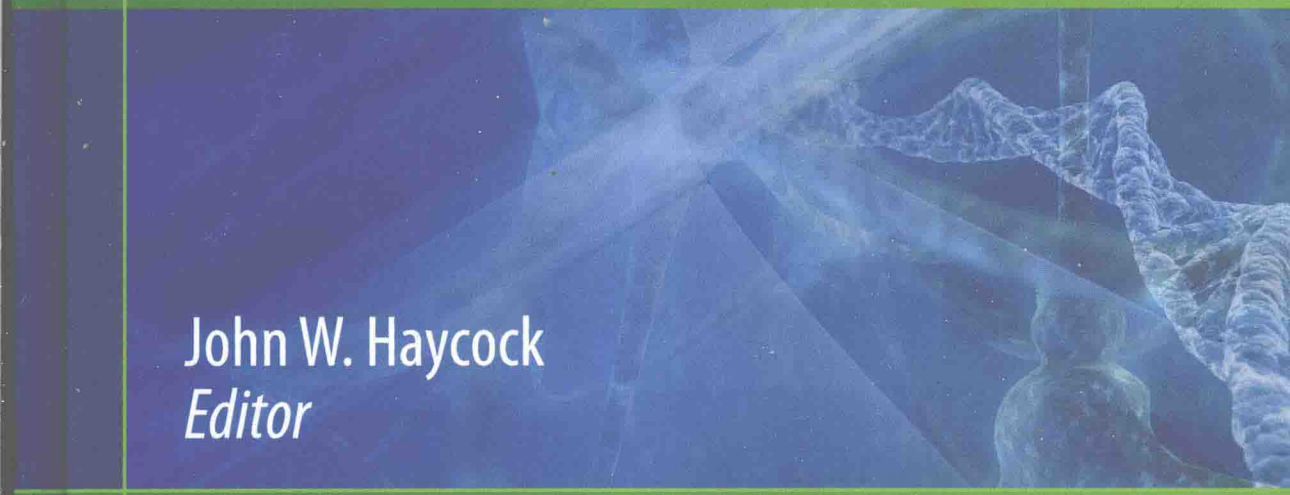

John W. Haycock
Editor

3D Cell Culture

Methods and Protocols

Humana Press

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Edited by

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Preface

Since the advent of routine eukaryotic cell culture more than 40 years ago, the most common substrates for supporting cell growth have been made from polystyrene or glass and have taken the form of a flat two-dimensional (2D) surface. A tremendous number of studies have been performed using cells in 2D culture, and a frequently raised point is exactly how relevant are such studies for interpreting the information gained when compared to the complexities of real tissue physiology? In answer to this, a number of three-dimensional (3D) models have now been developed for a range of tissues where the culture environment takes into account the spatial organization of the cells therein. A common goal of many of these studies is to bridge the gap between in vivo studies at one extreme with that of simple cell monolayers at the other.

For this, it is therefore necessary to create a growth environment that mimics the native tissue as closely as possible. To achieve this, 3D culture models rely inherently on a number of key areas, in particular materials science, cell biology, and bioreactor design. The integration of these approaches is more important than ever, given the practical and applied directions of such work; we frequently hear of tissue engineering and regenerative medicine in the news, with the promise to treat conditions associated with an aging population. For this to become a reality, accurate and relevant 3D culture models are essential and underpin the development of such technologies, be it growing nerves for treating neuronal injuries or skin for burns patients.

While the ultimate goal might be to create an identical tissue ex vivo, many strategies have made tremendous gains by focussing on a single aspect such as biomaterial design, an appropriate cell source, or the bioreactor environment. For tissue engineering, a more common approach has not always been to make an exact copy of living tissue but quite often to generate a "nucleating environment" in which nascent 3D structures have sufficient information for permitting cellular adhesion, proliferation, and differentiation into a functional tissue construct.

3D culture models can be grouped into the study of whole animals and organotypic explant cultures (including embryos), cell spheroids, microcarrier cultures, and tissue-engineered models. While not all 3D culture models require a scaffold, the use of scaffolds for 3D models has increased considerably in the past 10 years, especially due to advances in biomaterial science and processing technologies. Methods for accurately shaping and forming polymers such as microstereolithography hold considerable promise for creating scaffolds quickly with micrometer resolution. Of particular interest is the "re-birth" of traditional processing techniques such as electrospinning and wet spinning, which are seeing a rapid increase in their use for generating 3D culture scaffolds.

The promise of stem cell technology is also highly relevant when practicing 3D culture, not only for understanding the basic processes of differentiation but also for therapeutic purposes. The importance of being able to isolate cells with the capacity to renew, mitotically divide, and differentiate into a diverse range of cell types with control is self-evident. However, many stem cell studies are conducted using 2D environments, and so

the relevance of a 3D environment is imperative to understand in detail how one differentiates a stem cell toward a desired lineage, especially if intended for therapeutic purposes.

3D Cell Culture: Methods and Protocols contains a number of basic and applied methodologies taken from a breadth of scientific and engineering disciplines. Many of the topics deal with direct applications of 3D culture models, most notably in the formation of tissues for clinical purpose. I hope that this book will serve as a basic manual for laboratory-based scientists who not only need to have a comprehensive range of techniques contained within a single text but also require techniques described using a standard format. The chapters have all been written by practicing scientists and engineers who provide careful detail for the reproduction of a variety of 3D cell culture models.

The book starts with two review chapters which give an overview of the biological and materials scaffold requirements for successfully creating 3D models. Thereafter, there are 18 chapters which cover key areas for the construction of 3D culture models. These include general scaffold design and fabrication techniques, models for bone, skin, cartilage, nerve, bladder, and hair follicles, and chapters on bioreactor design, imaging, and stem cells. Topics include tissue engineering, where reconstruction in 3D is primarily for clinical purposes, the use of 3D cultures for in vitro models, where work is intended to have an impact in areas such as drug screening, and on tools and technologies, for underpinning the successful development of 3D models. There is also a focus on the design and use of bioreactors.

I hope that readers of *3D Cell Culture: Methods and Protocols* will find this book a valuable reference manual for their day-to-day use in the laboratory. I am personally indebted to all of the international experts who have very kindly contributed chapters and taken enormous trouble to carefully prepare their contributions for this volume of *Methods in Molecular Biology*.

Sheffield, UK *John W. Haycock*

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Contents

Chapter 1

3D Cell Culture: A Review of Current Approaches and Techniques

John W. Haycock

Abstract

Cell culture in two dimensions has been routinely and diligently undertaken in thousands of laboratories worldwide for the past four decades. However, the culture of cells in two dimensions is arguably primitive and does not reproduce the anatomy or physiology of a tissue for informative or useful study. Creating a third dimension for cell culture is clearly more relevant, but requires a multidisciplinary approach and multidisciplinary expertise. When entering the third dimension, investigators need to consider the design of scaffolds for supporting the organisation of cells or the use of bioreactors for controlling nutrient and waste product exchange. As 3D culture systems become more mature and relevant to human and animal physiology, the ability to design and develop co-cultures becomes possible as does the ability to integrate stem cells. The primary objectives for developing 3D cell culture systems vary widely – and range from engineering tissues for clinical delivery through to the development of models for drug screening. The intention of this review is to provide a general overview of the common approaches and techniques for designing 3D culture models.

Key words: Cell culture, Bioreactor, Biomaterials, Tissue engineering, Imaging, Scaffold, Stem cells

1. Introduction

Since the advent of routine eukaryotic cell culture more than 40 years ago, the most common substrates for supporting cell growth have been made from polystyrene or glass and have taken the form of a flat two-dimensional surface (1). Thousands of published studies ranging from cancer drug screening through to developmental biology have relied on this format for the growth of adherent cells. A major criticism of these studies, however, is an assumption that animal physiology can be accurately reproduced using a cellular monolayer. Clearly, the presentation of a eukaryotic cell to a two-dimensional glass or polystyrene

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substrate is not an accurate representation of the extracellular matrix found in native tissue. As a result, many complex biological responses arising such as receptor expression, transcriptional expression, cellular migration, and apoptosis are known to differ quite significantly from that of the original organ or tissue in which they arise.

The role of a normal cell from division, through proliferation to migration and apoptosis, is an accurately controlled series of events that inherently relies on the principles of spatial and temporal organisation. The culture of cells in two dimensions is arguably far too simple and overlooks many parameters known to be important for accurately reproducing cell and tissue physiology. These include mechanical cues, communication between the cell and its matrix, and communication between adjacent cells. On the point of intercellular communication, many two-dimensional culture experiments fail to consider the interplay between different cell types, with the vast majority of cultures being of a single cell type. 2D co-cultures overcome some of these shortfalls, but are some way off in accurately reproducing cellular function observed within a tissue.

2. Three-Dimensional Cell Culture

In answer to these problems, a number of three-dimensional methods have been developed for a range of tissues where the culture environment takes into account the spatial organisation of the cell (2–5). A common goal for many of these studies is to bridge the gap between the use of whole animals at one end of the spectrum, with cellular monolayers at the other. It is therefore necessary to create a growth environment that mimics the native tissue as closely as possible, and a simple starting point is the introduction of cells into a porous biocompatible scaffold. However, the complexity of 3D systems then becomes apparent with a number of parameters to consider. Important criteria include the choice of material for the scaffold, the source of cells, and the actual methods of culture, which in practice varies considerably according to the tissue of study. A number of common approaches exist, but so too does the opinion of investigators – from the precise design of scaffolds through to the sourcing of a particular cell type. For example, does one use naturally derived or synthetic materials for a scaffold? Does one use autologous or adult-derived stem cells? Does one invest time and money fabricating an accurate nanostructured scaffold, or produce a microstructured scaffold with an approximate geometry for maintaining cell growth?

3. Three-Dimensional Culture Models

Three-dimensional culture models can be grouped into the study of whole animals and organotypic explant cultures (including embryos), cell spheroids, microcarrier cultures, and tissue-engineered models (6). Not all three-dimensional culture models require a scaffold; however, the use of scaffolds for 3D models has certainly increased considerably in the past ten years. Whole animal and organotypic explants are principally used in studies where an absolute requirement for tissue-specific information is needed (7). These models enable data where the cell is physically located within its native environment. Examples include *drosophila melanogaster* (fruitfly) and the use of zebrafish and mouse embryos. Experimental versatility in terms of environmental conditions is permissible for non-mammalian models such as the fruitfly and zebrafish, but maintaining cellular viability for mouse embryos is an absolute necessity, and so culture conditions such as pH, temperature, and O_2 levels must be very carefully controlled for these models (6, 8). Organ explantation for culture has largely been pioneered in the areas of brain and neural physiology. Here, explants can be maintained in vitro in gels or on semi-permeable membranes in the presence of an isotonic or nutrient medium. Advantages include the maintenance of tissue architecture and importantly the presence of differentiated cells within the tissue (6). Technical demands for these models include the time available for maintaining specimen integrity and the need to image deeply into samples.

Cellular spheroids are simple three-dimensional models that can be generated from a wide range of cell types and form due to the tendency of adherent cells to aggregate. They are typically created from single culture or co-culture techniques such as hanging drop, rotating culture, or concave plate methods (6, 9, 10). Spheroids do not require scaffolds and can readily be imaged by light, fluorescence, and confocal microscopy. Consequently, spheroids have seen a use in modelling solid tumour growth and metastasis studies and are also used in a multitude of therapeutic studies, e.g. for high throughput screening (11). An analogous approach is in the development of epithelial tissues to form polarised sheets, such as the epidermis of skin (12). Normal human keratinocytes can be isolated from skin and cultured on supports such as collagen gels, synthetic polymer membranes, microfibre meshes, or de-epidermalised human dermis (DED) (12). The use of DED involves removing the dermis of its original cellular components, but, importantly for 3D cell culture, it maintains many of the native basement membrane proteins (e.g. collagen type IV). The presence of these proteins in the matrix is an absolute necessity for the reconstructive adhesion and growth of keratinocytes thereafter (13).

4. Biomaterial Scaffolds for Fabricating Structure and Shape

As one increases the size and complexity of a three-dimensional model, the need for a scaffold becomes apparent. Cellular aggregates require the careful exchange of nutrients and gases in addition to spatial control, and problems with cell death arise when aggregate thicknesses of 1–2 mm arise through a lack of mass transfer, principally through a limited exchange of nutrients and waste metabolites (6, 14). This has been addressed by the use of highly porous scaffolds where basic designs consider shape, cell adhesion sites and the flow of gases, nutrients, and metabolites (4). Different cell types are embedded within matrices possessing distinctly different properties and shapes. For example, if engineering peripheral nerve, one must consider the native structure where axons are surrounded by a soft uniaxially aligned lipoprotein myelin sheath. In contrast, osteoblasts adhere to a hard surface of bone within cuboidal sheets. Consequently, the design of scaffolds must reflect the tissue of interest and a tremendous diversity exists in the design of scaffolds for the engineering of tissues (4). An important consideration is the intended application and use. Clinical work that requires a functioning implant may require just a temporary biodegradable scaffold, which after implantation is remodelled by the body and replaced by native tissue to restore original function. In this instance, the scaffold must support cell growth and differentiation, and a physical match must exist between the size of the scaffold and that of the defect. Furthermore, the scaffold should break down into metabolites without a toxic or immunogenic response. Alternatively, scaffolds may be intended as a 3D in vitro model, e.g. to further our understanding in a fundamental aspect of tissue biology or to generate systems for drug and cosmetics screening (15). Here, there is a need to accurately reproduce the native tissue structure containing cells at a given stage of differentiation, and arguably there is a greater need to image these models for cell function and response. The absolute size of the scaffold for these models and the need for hydrolysis or degradation may not be quite so important.

The choice of bulk materials to be used for scaffold fabrication includes metals, glasses, polymers, and ceramics (4). Polymers are commonly used due to an ability to control their chemical and structural properties, in combination with methods for fabrication. They are typically grouped into synthetic and natural derivatives (4). Synthetic polymers include materials such as poly glycolic acid (PGA) and poly lactic acid (PLA), whereas natural polymers include materials such as chitosan and collagen. A general requirement for all biomaterial scaffolds is to reproduce an extracellular matrix environment for supporting cell growth outside of the body.

The bulk chemical composition of a biomaterial must therefore be the first consideration when designing a scaffold, with biocompatibility being a priority for implantation (3). In particular, a material must be selected that avoids triggering an immune response or the development of a fibrous capsule. A degradable scaffold should ideally be used for clinical purposes and most degradable synthetic scaffolds such as PGA undergo hydrolysis in situ. Consequently, the body must be able to metabolise the monomeric products released during breakdown without a toxic or inflammatory response. For systems such as PGA/PLA, degradation rates can be readily tuned by the composition of PGA versus PLA, where a higher PGA content degrades faster. Natural scaffolds such as collagen are degraded by enzymolysis and consequently less control is possible on tailoring the breakdown rate. However, natural scaffolds tend to exhibit better biocompatible properties over synthetic materials – but their clinical use is concerned with potential disease transmission (4, 12), a situation avoided by the use of synthetic scaffolds.

The surface chemical properties of a biomaterial are fundamental for dictating the adhesion and spreading of living cells (16). Such properties are not necessarily governed by the bulk chemistry, in particular due to surface modification with soluble proteins derived either from the growth medium or from the cells themselves (17). Surface chemistry is predominantly controlled by charge and polarity, which in general terms control the attractiveness of proteins in solution to diffuse and adsorb at the surface. The rate at which this happens is determined by the Vroman effect, whereby highly mobile proteins in a heterogeneous mixture will reach a surface quickly, but in time may be replaced by more slowly moving proteins with a higher affinity (18, 19). This arises in particular for serum proteins, where fibrin will adsorb to a polymer surface rapidly leading to fibronectin depletion in vivo. A relationship exists between the extent of charge at a surface and the proportion of proteins that are adsorbed. This is known to correlate with the tendency of cells to adhere to a biomaterial, where the cell interacts via an adsorbed protein layer, rather than directly to the biomaterial surface (16).

Optimising the surface chemistry of biomaterials can therefore be controlled either to increase or decrease protein adsorption and in turn cellular attachment. A good example of an approach for increasing cellular attachment is given in chapter 10, where Schwann cell adherence to aligned PLA microfibres is improved by the deposition of a plasma acrylic acid layer (20). Here an increase in the negative surface charge of acid groups is associated with an increase in cell attachment and proliferation. Conversely, the deposition of allyl amine serves to prevent Schwann cell attachment. Although both layers contain surface

charges, the surface chemical groups must also dictate not just the extent of protein adsorption, but the folding conformation of the protein. At a molecular level, the interaction between acid or amine groups in the plasma polymer with amino acids containing polar, non-polar, and charged groups will dictate how a protein interacts and folds at the surface. This determines whether the adsorbed protein presents adhesive ligands permissive for binding to receptors such as integrins. A number of similar studies using plasma deposition are reported in the literature where the aim was to optimise cell adhesion and growth, e.g. the adhesion of human keratinocytes to polymer sheets for clinical delivery (21). Conversely, non-fouling surfaces such as polyethylene glycol serve to minimise protein adsorption (or fouling) and in turn cellular adhesion. The theory as to why PEG surfaces are non-fouling is highly complex – indeed the mechanisms are still being investigated. Predominant reasons suggest that chain mobility and a steric stabilising force are important, with protein-resistant properties arising through both a mixing interaction and excluded volume component (22). Thus, when a protein approaches a PEG layer, the available volume per glycol unit is decreased resulting in a repulsive force, due to a decrease in conformational entropy. In addition, the compressive force of a protein into a PEG layer reduces the total number of confirmations originally available to the chain, which creates an osmotically repulsive force, effectively pushing the protein away from the PEG layer (22).

Cell adhesion can also be controlled by integrating precise structural motifs into a biomaterial. Original work from Massia and Hubbell in 1991 reported that the alpha-V-beta-3 integrin adhesion ligand RGD, when covalently attached to a surface with a critical spacing of 440 nm, was permissive for the attachment of fibroblast cells in vitro (colloquially known as the "Hubbell limit") (23). If the separation distance between ligands was decreased to 140 nm, then fibroblast stress fibre and focal contact formation was observed. This has led many investigators to conjugate RGD-like ligands for attachment into and onto biomaterial surfaces for controlling cellular adhesion (4). However, many direct conjugation methods, while elegant, are confined predominantly to cell culture in 2D. In contrast, surface modification techniques such as plasma vapour phase deposition have proved to be effective for influencing cell adhesion in 3D scaffolds (20, 21). For example, Barry et al. report on the use of an allyl amine plasma polymer specifically for encouraging fibroblast cell attachment, morphology, and metabolic activity into 3D P(DL)LA porous scaffolds without changing the bulk characteristics of the scaffold (24). A major advantage of this approach is in the rapidity, reproducibility, and chemical control possible for modifying 3D scaffolds.