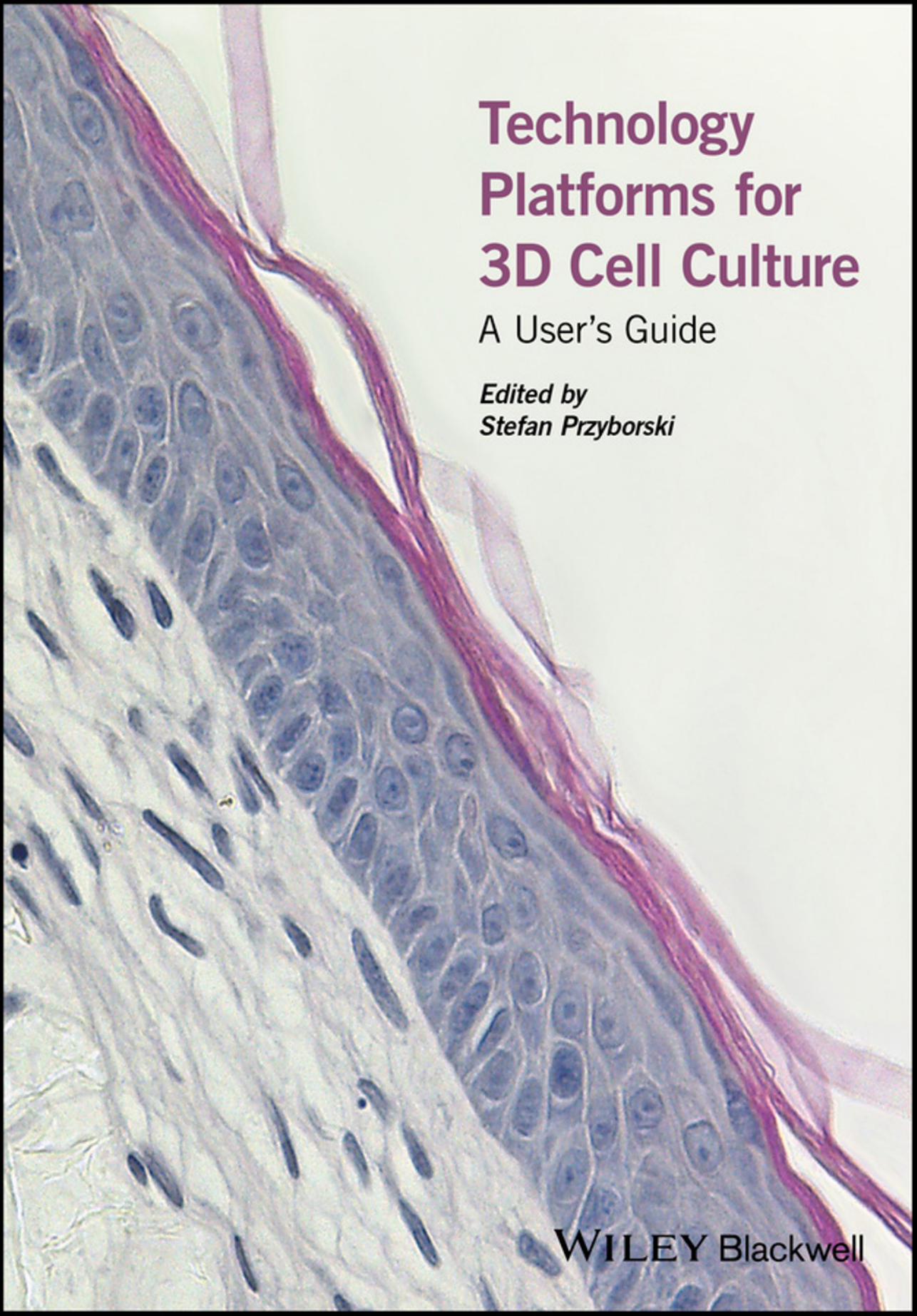

Technology Platforms for 3D Cell Culture

A User's Guide

Edited by
Stefan Przyborski

WILEY Blackwell

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Preface

Understanding basic cellular biology relies on research involving cell-based *in vitro* assays that are also used to model disease, test and screen compounds and assess the safety of chemicals. Most often, the investigation of such biological processes is based on studying homogeneous populations of mammalian cells cultured as monolayers on flat, two-dimensional (2D) polystyrene substrates. However, cells naturally exist within a complex three-dimensional (3D) tissue microenvironment composed of mixed cell populations, extracellular proteins and both physical and chemical signals from multiple sources.

It should be recognised that many important discoveries have been made from conventional 2D culture approaches. This reductionist view to understanding basic biological processes has value but is limited since cells growing on 2D substrates do not always reflect the true physiological behaviour of their native counterparts in real tissues. When forced to grow on a flat 2D substrate, cells adapt and radically change their shape, proliferate in an aberrant fashion and often lose their differentiated phenotype, resulting in abnormal cellular behaviour. It is widely appreciated that such structural modifications to the physical environment can result in changes to gene transcription and protein translation, remodelling of the cytoskeleton and irregular cell signaling.

Accordingly, the anatomy of a cell, i.e. its structure and form, are inextricably linked to its physiological function. Technologies are now becoming available that enable researchers to culture cells in 3D that in turn enhance the value of such cell-based assays and the generation of more accurate and physiologically relevant results. The growth of tissue-like structures in 3D in combination with media perfusion/circulation creates a dynamic system which advances cell-based models still further towards the recreation of more '*in vivo*-like' conditions.

Maintaining the natural 3D architecture of a cell is therefore considered one of the fundamental steps toward enhancing the value of cell-based assays. There are now several technologies available that promote solutions for 3D cell culture. In general, these fall into one of three categories: hydrogels (e.g. Matrigel™, collagen gels); cell aggregates (e.g. hanging drop methods, low adherence plates); and scaffolds (e.g. porous physical supports). There is no panacea and no one solution is suitable for all 3D culture needs. The availability of these technologies from commercial sources now allows the investigator to select the most appropriate method suitable for their experimental requirements. Moreover, 3D cell culture technology is often combined with new developments in dynamic media

perfusion, to further enhance cell growth and physiological relevance of the model. There are numerous advanced technologies that enable media perfusion encompassing cleverly designed devices and mini-benchtop bioreactors.

The aim of this text is provide the reader with a review of the types of technology available and examples of where such methods may be applied. This has been divided into descriptions of 3D cell culture technologies by certain commercial developers representative of these key areas and the users of such methods. The reader will learn about the options available to perform 3D culture, explore the options for media perfusion, from where such 3D technologies can be acquired, how they work and how they can be used. Any cell biologist considering 3D cell culture and aiming to enhance the physiological relevance of their cell-based assay should consult this text as a guide to getting started with such methods.

Key features

- A review of the current state-of-the-art technologies available for 3D cell culture and media perfusion models.
- Contributions from leading developers and researchers active in 3D cell technology and advanced cell-based assays.
- Instruction and guidance on performing specific 3D culture methods and media circulation systems.
- Examples of where such technologies have been successfully applied.
- Guidance on resources and technical support to help get started using 3D culture methods with options of dynamic media circulation.
- Relevance to multiple fields including stem cells, tissue engineering, cell-based screening assays, etc.
- Examples of advanced physiologically relevant *in vitro* models, including use of 3D culture and perfusion technology, organotypic models, co-cultures, etc.

Primary readership

Interest in 3D cell culture and the ability to create more tissue-like constructs are developing rapidly in the scientific community. Researchers recognise its value and are keen to apply such technology to their experimental systems. This book will be especially topical given the drive to improve the value of *in vitro* cell-based assays and generate more physiologically relevant data. A quick scan of the scientific literature will indicate that interest in this sector is developing rapidly. While many different approaches that enable 3D culture have been developed, most are based on research in academic labs and are published in scientific journals. While of value, they are not always technologies readily available to the majority of investigators interested in 3D cell culture. Several approaches to culture cells in 3D have now been commercialised. Numerous

small and large companies have undertaken the process of translating research into the creation of marketable products. These organisations are the pioneers of a new era in cell culture methods and have made such technology available to the greater scientific community through the development of bespoke products and applications. This was not possible until now and the time is right for this book to bring together the different approaches that are readily available and to support this rapidly growing sector of 3D cell culture.

Any cell biologist practising conventional 2D cell culture will be interested in this book as an opportunity to enhance the value of their cell-based assays and perform 3D culture methods. Specific sectors of interest include cancer cell biology, stem cells, tissue engineering, *in vitro* alternatives to animal use, liver toxicology, neuroscience and those requiring specialised cell culture models (e.g. co-culture, organotypic models). Cell culture as a technique is very general and is performed in academic, industrial and government laboratories worldwide. It is anticipated that many will benefit from this text as a comprehensive guide to technologies that are readily available, to act as a reference and assist in the selection of technology and guidance for use. Therefore the primary readership will be the practising bench scientists (postgraduate and postdoctoral students, research fellows, research scientists and the like). The secondary readership may be managers of cell culture facilities/departments, R&D heads of section, technical supervisors, advisors, etc. who would recommend methods to perform. Fringe interests include parties interested in cell biology in general and methods associated with the field.

The pharmaceutical industry is particularly interested in developing new approaches to enhance its ability to discover new compounds and has identified 3D culture as a priority area. Similarly, contract research organisations are now using 3D culture methods to provide additional information to their clients. Collectively, these are substantial opportunities. Societies interested in alternatives to animal research, cell biology, tissue engineering, cancer biology, etc. should all find this text of value.

Stefan Przyborski
Professor of Cell Technology
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January 2017

List of abbreviations

2D	Two-dimensional
3D	Three-dimensional
5-FU	5-Fluorouracil
A1AT	α1 Antitrypsin
ADME Tox	Absorption, distribution, metabolism, excretion and toxicity
ALI	Air-liquid interface
ASC	Adipose-derived stem cells
ATP	Adenosine-5′-triphosphate
ATRA	All trans-retinoic acid
BBB	Blood–brain barrier
bFGF	Basic fibroblast growth factor
BME	Basement membrane extracts
BNDF	Brain-derived neurotrophic factor
CAF	Cancer-associated fibroblast
CD	Cytochalasin D; cluster of differentiation
CDI	1,1′-Carbonyldiimidazole
CDM-HD	Chemically defined medium for high-density cell culture
C/EBPa	CCAAT-enhancer-binding proteins
CHO	Chinese hamster ovary
CLL	Chronic lymphocytic leukaemia
CNS	Central nervous system
CP	Cortical plate
CTG	CellTracker green; CellTiter-Glo
CV	Coefficient of variation
CYP1A1	Cytochrome P450, family 1, subfamily A, polypeptide 1
CYP2E1	Cytochrome P450 2E1
CYP3A4	Cytochrome P450 3A4
d4T	2′,3′-Didehydro-3′deoxythymidine
DNA	Deoxyribonucleic acid
DCIS	Ductal carcinoma *in situ*
DLS	Dynamic light scattering
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethyl sulfoxide
DNP	Dinitrophenol
DOX	Doxorubicin
DPMK	Drug metabolism and pharmacokinetics
DRG	Dorsal root ganglia
DTT	Dithiothreitol
EthD	Ethidium homodimer-1

EB	Embryoid body
ECM	Extracellular matrix
ECS	Extracapillary space
EDTA	Ethylenediamine tetra-acetic acid
EEF1	Elongation factor 1 complex
EFP	Evoked field potential
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
EHS	Engelbreth-Holm-Swarm
EHT	Engineered heart tissue
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-mesenchymal transition
ESC	Embryonic stem cell
ET	Engineered tissue
EVS	Extravascular space
FA	Focal adhesion
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FDA	Food and Drug Administration; fluorescein diacetate
GABA	γ-Aminobutyric acid
GAG	Glycosaminoglycan
GFAP	Glial fibrillary acid protein
GFP	Green fluorescent protein
GMP	Good Manufacturing Practice
GSH	Glutathione synthase
GSSG	Oxidised glutathione
HA	Hyaluronic acid
HARV	High aspect ratio vessel
HCA	High-content analysis
HCV	Hepatitis C virus
HCVpp	Hepatitis C virus pseudoparticles
HD	Hanging drop
HDMEC	Human dermal microvascular endothelial cell
HDP	Hanging drop plate
H&E	Haematoxylin and eosin
HF	Hollow fibre
HFBR	Hollow fibre bioreactor
HFIM	Hollow fibre infection model
HIPE	High internal phase emulsion
HiPSC	Human induced pluripotent stem cell
HIV	Human immunodeficiency virus
HMEC	Human microvascular endothelial cell
hMSC	Human mesenchymal stem cell
HNF4α	Hepatocyte nuclear factor 4 α
HPC	Hydroxypropylcellulose
HSC	Haematopoietic stem cell
HS	Heparan sulfate

HTS	High-throughput screening
HUVEC	Human umbilical vein endothelial cell
ICM	Inner cell mass
ID	Internal diameter
IDC	Invasive ductal carcinoma
IgG	Immunoglobulin G
iPSC	Induced pluripotent stem cell
LC-MS	Liquid chromatography-mass spectrometry
LCST	Liquid crystal solution temperature
LDH	Lactate dehydrogenase
NF	Neurofilament
M-dPEG-NHS	Maleimide-dPEG8-N-hydroxysuccinimide ester
Mal-PVA	Maleimide-functionalised polyvinyl alcohol
MAP	Microtubule-associated protein
MCR	Multicellular resistance
MCTS	Multicellular tumour spheroid
MDCK	Madin–Darby canine kidney
MEA	Microelectrode array
MeHg	Methyl-mercury
mESC	Murine embryonic stem cell
MRP2	Multidrug-resistant associated protein 2
MPLSM	Multiphoton laser scanning microscopy
MSC	Mesenchymal stem cell
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MWCO	Molecular weight cut-off
NAPQI	N-acetyl-p-benzoquinone imine
NASA	National Aeronautics and Space Administration
NCP	NanoCulture Plate
NE	Neuroepithelium
NGF	Nerve growth factor
NIH	National Institutes of Health
NMR	Nuclear magnetic resonance
NPC	Neural precursor cells
PA	Polyacrylamide
PBS	Phosphate buffered saline
PCL	Poly-ε-caprolactone
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDMS	Poly-dimethyl-siloxane
PEG	Polyethylene glycol
PEGDA	Polyethylene glycol diacrylate
PEGSSDA	Polyethylene glycol diacrylate with internal disulfide bonds
PEGnor/I2959	Polyethylene glycol norbornene/Irgacure 2959
PET	Polyethylene terephthalate
PFA	Paraformaldehyde
PGA	Polyglycolic acid

PGLA	Poly-D, L-lactide-co-glycolide
Pgp	P-glycoprotein
PHA	Polyhydroxyl alkanoate
PI	Propidium iodide
PK/PD	Pharmacokinetic/pharmacodynamic
PLA	Polylactic acid
PLLA	Poly-L-lactide
PolyHEMA	Polyhydroxyethylmethacrylate
PPAR	Peroxisome proliferator-activated receptor
PPI	Paired pulse inhibition
PrC	Prostate cancer
PS	Polysulfone
PSC	Pluripotent stem cell
PTFE	Polytetrafluoroethylene
PVA	Polyvinyl alcohol
PVDF	Polyvinylidene fluoride
QV	Quasi Vivo
RCCS	Rotary Cell Culture System
RFP	Red fluorescent protein
RGD	Arginine-glycine-aspartate
RGDS	Arginine-glycine-aspartate-serine
RIG-I	Retinoic acid-inducible gene 1
RLU	Relative luminescence unit
ROCK	Rho-associated coiled-coil protein kinase
RPM	Revolutions per minute
RT	Room temperature
RTK	Receptor tyrosine kinase
RT-PCR	Real-time polymerase chain reaction
RWV	Rotating wall vessel
SCARB1	Scavenger receptor class B, member 1
SEM	Scanning electron microscopy
shRNA	Short hairpin RNA
STA	Staurosporine
STLV	Slow turning lateral vessel
SU	Single use
TAX	Taxol
TCEP	Tris (2-carboxyethyl) phosphine
TCP	Tissue culture polystyrene
TEER	Transepithelial electrical resistance
TGF-β2	Transforming growth factor-β2
TMRE	Tetramethylrhodamine ethyl ester
TMTC	Trimethyltin chloride
TPZ	Tirapazamine
ULA	Ultra-low attachment
UV	Ultraviolet
XPS	X-ray photoelectron microscopy

CHAPTER 1

An introduction to the third dimension for routine cell culture

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Introduction

In recent years, the advent of three-dimensional (3D) cell culture technologies has led to a paradigm shift in our understanding of eukaryotic cell culture. The challenge of reproducing the complexity of whole tissues *in vitro* is being addressed through various approaches incorporating biological parameters known to influence cellular behaviour. As such, the increasing number of publications utilising these technology platforms is evidence of the transition into 3D cell culture. This book juxtaposes these efforts and successes with the shortcomings of culturing mammalian cells with conventional methods. However, full adoption of these techniques for routine mammalian cell biology research will require their validation. This book therefore serves as a guiding tool for researchers who seek to shift towards more advanced cellular assays that recreate *in vivo*-like conditions, compiling readily available techniques for 3D cell culture.

Two-dimensional (2D) *in vitro* models have been vital to understand biological processes and mechanisms in cellular biology. For decades, cellular monolayers have been used to model disease, screen and assess the efficacy and toxicity of chemical compounds and develop anticancer treatments. Although valuable, it should be recognised that these conventional cell culture approaches are a simplistic method, overlooking important biological parameters that influence cellular behaviour. 2D cell culture does not provide an *in vivo*-like environment where physical cues, cell-cell and cell-matrix communication and the interplay of different cell types can be reproduced. This results in a poor reflection of physiological cellular behaviour, as well as limited potential to form more complex tissue-like structures. These disadvantages become more significant in the context of drug testing, where monolayers of cultured cells fall short in reflecting how drugs interact with target molecules *in vivo*. The lack of inclusion

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of the signalling context as part of the cell culture system hinders the predictive value of traditional cell-based drug screening methods (Bhadriraju & Chen, 2002; Sun *et al.*, 2006).

Cells naturally exist within a complex 3D tissue environment composed of heterogeneous cell populations, extracellular proteins, forming an intricate system of physical and chemical cues that impact the natural response of cells. Replicating the native environment is a fundamental step towards making these models more physiologically accurate and enhancing the value of the results drawn from these culturing systems. Here, we examine specific areas where 2D cell culture fails and anticipate the areas of improvement that 3D cell culture seeks to tackle.

Structure and cell adhesion

Culturing cells in 2D imposes physical constraints that impede cells from organising naturally and spreading vertically (Figure 1.1). For cells to adopt their native morphology, they need to form integrin-mediated adhesions with the extracellular matrix (ECM). Flat polystyrene or glass substrates cannot faithfully capture the topographically complex extracellular environments, and therefore they tend to force an apical-basal cell polarity on all cells. This characteristic polarity, seen in monolayer-cultured cells, may be relevant to epithelial cells but it impedes mesenchymal cells in acquiring their characteristic stellate morphology. In turn, cell shape and tissue architecture will probably affect the growth of 2D-cultured mesenchymal cells and thus their differentiation. Similarly, apical-basal polarity and the formation of 3D structures are means by which tumour cells develop resistance to apoptosis (Weaver *et al.*, 2002). In this way, the inability of cells to establish integrin-induced cell polarity in monolayers prevents the

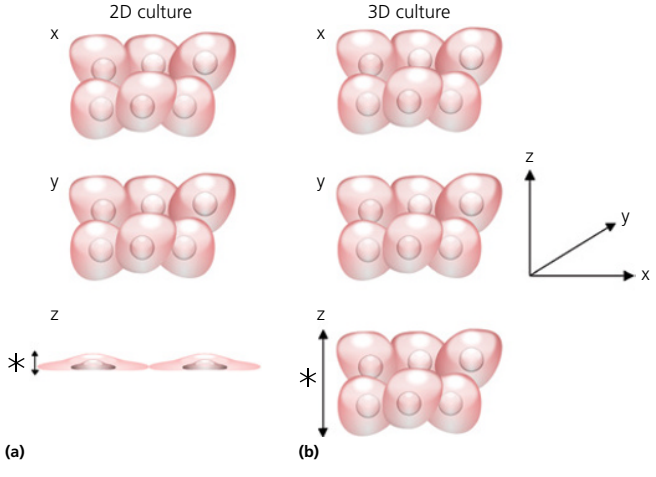

Figure 1.1 Cell flattening. This schematic shows how cells remodel in a flat, 2D environment (a). 3D cell culture (b) ensures cell integrity is preserved maintaining a more physiological shape and form.

study of the mechanisms by which tumour cells can escape extrinsic control. 3D models that support growth in the vertical dimension will be able to recapitulate such mechanisms and study tumourigenesis accordingly. Amongst other features, such models must incorporate the tumour microenvironment, as it has been identified as a key component driving tumour progression (Castelló-Cros & Cukierman, 2009).

Finally, when only 5% of anticancer candidate compounds in preclinical development are licensed after undergoing successful phase III testing (Hutchinson & Kirk, 2011), it is evident that there is an urgent need for more robust and higher quality models to assess these agents. Even if these 3D models delay the time it will take for drugs to reach phase III trials, ultimately it will be a more cost-effective approach to deliver more predictable results.

Mechanotransduction

The patterning of cell-adhesive ligands on more complex substrates and the development of 3D platforms, ranging from solid scaffolds to the manipulation of fluids at a microscale with microfluidics, have become popular avenues of advanced cell culture. These examples corroborate that cell adhesion and structure are two salient features of 3D cell culture (Baker & Chen, 2012). Three-dimensionality, however, has become a generalised statement for all discrepancies between traditional cell culturing systems and newer technology platforms for 3D cell culture. As such, there are other important features of advanced culturing systems, which reside in mechanotransduction and the impact of cells adapting to their surroundings through mechanosensing.

Cells are naturally exposed to mechanical stresses that can influence biological processes such as mitosis, cell migration, stem cell differentiation and self-renewal (Eyckmans *et al.*, 2011). This occurs via adhesion-mediated signalling, which is the mechanism whereby the cells' contractile ability and response to these pressures are transduced into biochemical signals, modifying their behaviour. The machinery behind mechanotransduction involves several cytoskeletal proteins, spanning long distances enabling mechanical continuity and acting as mediators of force transmission (Wang *et al.*, 1993). Whilst intermediate filaments, made of vimentin, keratin and laminin monomers, establish the intracellular structure, actin and myosin form contractile filaments that bind to a cluster of proteins connecting the cytoskeleton to the ECM through transmembrane integrin receptors (Eyckmans *et al.*, 2011). Focal adhesions (FAs) are found amongst this group of proteins and are a key and well-documented unit in cell-ECM adhesion (Kuo, 2014). When force is applied to this unit and cells undergo mechanical deformation, the intracellular structure and organelle positioning in the cell will be disrupted because of the interconnectedness of the cytoskeleton with the cell membrane. Force transmission is also reciprocal; in normal circumstances, cells can also exert forces towards the extracellular space. The continuous polymerisation and depolymerisation of microtubules coupled with

the engagement of myosin II pulling actin filaments during contraction creates mechanical forces that are transmitted to focal adhesions (Eyckmans *et al.*, 2011). In turn, this force can remodel the ECM, depending on intracellular activity.

Knowing that these forces are constantly reshaping cells and their exterior, the question then becomes: how do these forces transduce into biochemical signals? One mechanism is through restructuring of the ECM resulting in the exposure of new sites for signalling molecules to engage with or release of growth factors bound to the matrix. Mechanical forces are known to release and activate transforming growth factor (TGF)-β1, which in turn can induce the transdifferentiation of fibroblasts into myofibroblasts and affect developmental processes, wound healing and tumourigenesis (Wipff *et al.*, 2007).

In this way, flat polystyrene or glass substrates for cell culture will inherently lead to the remodelling of cellular architecture (Vergani *et al.*, 2004), providing an inexact representation of native tissue. Along with the flattening of the cell, force transmission through focal adhesions will alter the shape of the cell nucleus, modifying gene expression and therefore protein synthesis (Thomas *et al.*, 2002). Moreover, the rigidity of the substrate where cells reside can enhance cell proliferation but inhibit cell differentiation due to limited cell-cell and cell-matrix interactions (Cukierman *et al.* 2002). To successfully model physiological responses, *in vitro* experiments have to embrace these variables to choose a suitable platform that acknowledges cell integrity, tissue organisation and the impact of mechanotransduction on cell behaviour.

Crosstalk and effector transport

3D cell culture is an enabling technology, bringing the possibility of studying the intricate developmental processes occurring in early embryogenesis as well as the instances when these go awry (Yamada & Cukierman, 2007). The study of branching morphogenesis entails being able to reproduce tubular structures (Fata *et al.*, 2004). Along with structural support, it is necessary to create an information-rich environment with the necessary signalling molecules to facilitate development and differentiation into more elaborate structures. By having a more natural spread of receptors and adhesion molecules distributed across the cell surface, cells not only can achieve this but also engage in a dialogue with neighbouring cells and the supporting stroma (Cukierman *et al.*, 2002). The increase in these interactions enhances intercellular signalling and preserves the transmission of instructive signals for tissue homeostasis.

The ECM is also responsible for laying out the compartments for dispersal of nutrients. It establishes the tissue architecture where gradients of nutrients, oxygen, pH and waste products can manifest. Biological gradients are essential in exerting pressures that can stimulate or inhibit cellular activities, and thus regulate processes such as cell migration and homing (Baker & Chen, 2012). Accurate modelling of the events occurring *in vivo* will have to consider the ECM as a spatial organiser. For example, recapitulating the full picture of cancer

means that we need to abandon the reductionist approach of monolayers. To understand how multicellular drug resistance arises, the topography of the tissue or organ needs to be acknowledged. Often, therapeutic agents fail to target all cancerous cells because they lie in inaccessible or deeper areas of the tissue. Similarly, drug resistance can also be attributed to hypoxia, which is why it must be considered as an important factor of the microenvironment when emulating *in vivo* conditions (Asthana & Kisaalita, 2012). Deficient early cell-based models cannot reproduce different oxygen concentrations, and therefore it is no surprise that therapeutic agents slip through the screening, ultimately failing at later stages of the drug development pipeline.

For these reasons and the shortcomings of 2D cell culture, 3D cell technologies seek to fix the discrepancy between the events occurring *in vivo* and the conventional methods used in tissue culture.

Technology platforms for 3D cell culture are predominantly categorised into scaffold-based and scaffold-free systems. Scaffold-based technologies provide physical support in the form of matrices made from natural or synthetic materials to create a suitable microenvironment for optimal cell growth, differentiation and function. Hydrogels are a popular 3D culture method that falls into this category; they work on the same principle of preserving native cellular shape and tissue architecture, enabling a more physiologically relevant function through multiple applications. Conversely, scaffold-free culture systems do not rely on an exogenous input acting as a cellular framework. These technologies encourage the formation of multicellular masses, often referred to as aggregates or spheroids. In this way, spheroids can form their own ECM and then assemble into 3D microtissues.

Finally, these different technologies can be combined in a cleverly designed manner to create another set of platforms for 3D cell culture. Seemingly complex and robust, mini-bioreactors with perfusion flow are mainly concerned with maintaining a constant or controlled supply of biochemical and mechanical cues to improve the quality of engineered tissues.

Aggregate-based technologies

Aggregate-based technologies consist in coaxing cells into forming 3D tissue-like masses or spheroids, by exploiting the biophysical variables acting on the media in which they are grown. Aggregates have the advantage of secreting their own ECM and self-organising into microtissues with multiple cell-cell interactions. Along with self-assembly, other virtues of these technologies include maintaining a consistent spheroid size, not requiring additional materials for culturing, and being compatible with high-throughput screening (HTS). Overall, spheroids offer a simple transition into 3D cell culture and are rapidly becoming an attractive tool in tissue engineering for developing banks of mini-organoids to be

used in personalised medicine, drug screening and regenerative therapy (Barker, 2014; van de Wetering *et al.*, 2015; Yui *et al.*, 2012).

Different methods have been developed to generate this type of cell culture for routine use. Cells can be initially cultured in a drop of medium, which is then suspended on the lid of a cell culture dish (Figure 1.2). The lack of surface to attach to encourages cells to aggregate at the apex of these hanging droplets to then form spheroids. Hanging drop plates have multiple concave wells where the cell suspension is distributed, reproducing this phenomenon and maximising the production of tissue-like masses. Moreover, hanging drop plates are covered with a lid that prevents evaporation, maintaining a humid and sterile environment. These suspension cultures are adequate for cells that can proliferate in a non-adhesive environment where aggregation is favoured (Jo & Park, 2000).

An alternative to the hanging drop technique involves using attachment-resistant cell surfaces, which are coated with hydrophilic polymers that inhibit cell adherence (Jo & Park, 2000). This mechanism forces cells to float in the medium, stimulating them to coalesce into spheroids. The surface of the bottom of the plates can also be modified to control spheroid shape. Whilst flat bottoms result in irregular morphology and size, U- or V-shaped surfaces have been optimised to promote formation of single clusters of cells for use in high-throughput studies. Still, there are other constraints that can limit the use of low-adherence substrates. For example, coating the substrate is a time-consuming procedure that can delay the cell seeding procedure. Similarly, the production of 3D spheroids is cell type dependent, which can limit the applications of all scaffold-free

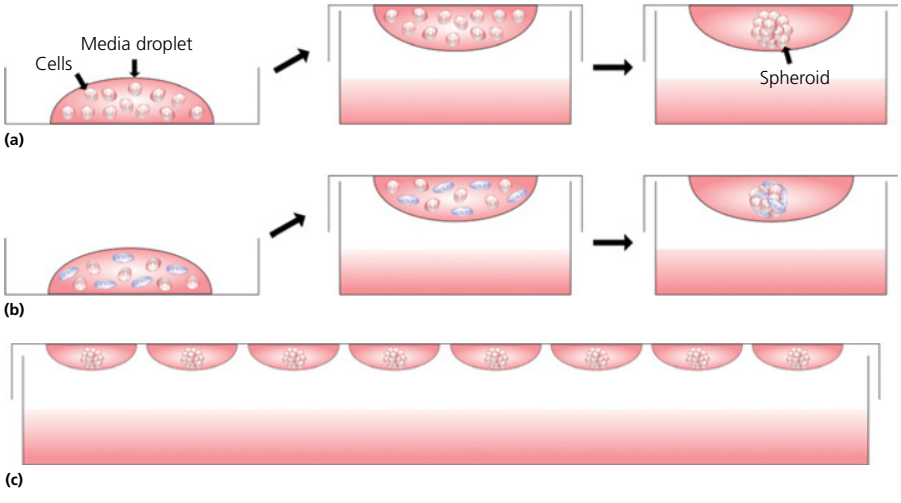

Figure 1.2 3D cell aggregates. (a) Formation of 3D microtissues using the hanging drop technique. Droplets of cell suspension are placed on the lid of a Petri dish, which is gently inverted and placed on top of the dish containing medium to maintain a humid atmosphere. Suspended cells come together in the apex of the droplet, forming a compact 3D aggregate. (b) Co-culture alternative cell types within each technology. (c) Multiple 3D cell aggregates can be produced in a single dish.

3D cell culture technologies. Furthermore, a third technique to encourage spheroid formation by reproducing the native microenvironment of cells focuses on micropatterned surfaces. Microcontact printing methods can engineer surfaces with defined simple tessellations such as square or honeycomb patterns that can also generate spheroids (Yoshii *et al.*, 2011). Although obtaining uniform size is difficult, this method is another interesting prospect in this area of 3D cell culture technologies.

Regardless of how spheroid formation is achieved, these methods make it possible to scale down experiments and work with smaller volumes. This can reduce the cost of exogenous molecules used when studying the influence of growth factors on cellular function, for example. Similarly, suspension cultures are also advantageous since they keep a high local concentration of endogenous factors improving tissue function (Szczepny *et al.*, 2009). In this way, aggregate-based cultures are highly suited for building more realistic models that permit co-culturing methods, where different cell types are grown in the same droplet. Co-culture with other cell types can establish a signal-rich environment which can be used to study the effect of paracrine signalling in real tissue, as well as cellular interactions. Varying configurations of co-cultures allow for adjusting the ratio of cell types to accurately model their native context or merging two spheroids to form a 'Janus spheroid' (Hsiao *et al.*, 2012; Torisawa *et al.*, 2009).

By working in conjunction with stem cell biology, the *in vitro* differentiation of pluripotent stem cells (PSCs) using this type of culture results in cell aggregates referred to as embryoid bodies (EBs). Regarding morphology, these 3D masses can resemble morula-like structures or they may develop into cystic EBs akin to embryos in the blastula stage (Abe *et al.*, 1996). These spheroids have the potential to form tissues from different germ layers within one single EB (Ader & Tanaka, 2014). The lack of available human tissue of this kind and the need for a 3D model to study early developmental processes have favoured EBs as a platform to study organogenesis and test inductive factors and lineage decisions. The size of these 3D masses is known to affect their potential for differentiation (Bratt-Leal *et al.*, 2009) and often the steps in the generation of these aggregates can cause cell loss and size variation. These difficulties have prompted the use of microcarriers to propagate human pluripotent stem cells (hPSCs), avoiding the manual cutting of the monolayer to induce spheroid formation and scaling up the production of evenly sized EBs (Lam *et al.*, 2015).

Spheroids are particularly useful to simulate low nutrient conditions such as hypoxia, but this has also obstructed their use in tissue engineering. This is because, as the size of the spheroid increases, oxygenation becomes problematic due to poor vasculature and thus the centres of these 3D tissue-like masses develop necrosis. Oxygen is known to diffuse across 100–200 µm of tissue thickness (Griffith & Swartz, 2006), which is why if an organoid exceeds these measurements, it can be rendered unviable for implantation. Regenerative medicine has striven to bypass this barrier and engineer larger tissues by maintaining

an optimal size. Conversely, there are instances where the risk of hypoxia is in fact welcomed. Low oxygen concentrations are physiologically relevant when modelling embryogenesis and tumour progression. Hypoxia is known to induce angiogenesis and instruct the release of growth factors from the tumour stroma and infiltrating immune cells, which ultimately play a role in tumour development (Cukierman & Yamada, 2007). In this way, the interdependence between microenvironment factors, such as size and oxygen levels, should be included in the experimental design and in the selection of a platform to more closely represent the *in vivo* environment of cells (Ashtana & Kisaalita, 2012).

Spheroid culture has served as an instrument to grow rudimentary structures that imitate the anatomy and physiology of real organs. In recent years, these organoids have garnered significant attention as they offer a wide spectrum of opportunities, from disease models and drug screening tools to grafts with therapeutic potential. Part of their success stems from exploiting cells' biological ability to self-organise into structures of higher complexity. These, however, are not perfect; organoids suffer from batch-to-batch variation, may lack certain cell types or may not fully mimic all stages of organ development. Inclusion of native signalling cues in the culture system has improved the outcomes of these organotypic cultures. For example, culturing intestinal stem cell Lgr5+ cells to induce greater levels of Wnt signalling, Noggin and epidermal growth factor (EGF) signalling has resulted in enhanced intestinal crypt physiology (Sato *et al*. 2009, 2011).

Building on this optimised method to culture intestinal organoids, a biobank was developed from colorectal carcinoma patients as a strategy to delve further into the genetic alterations found in this epithelial cancer (van de Wetering *et al*., 2015). Aggregate-based technologies offer a uniform and reproducible tool to analyse the genotype-phenotype correlations in intestinal carcinoma. Likewise, these 'miniguts' can be tested against the available anticancer drugs and push forward the case for personalised medicine and cancer genetics. These possibilities exhibit the versatility and vast potential of this platform for 3D cell culture. As with the rest of these innovative systems, their strengths lie in their specific approach to solving the lack of three-dimensionality and restricted portrayal of living tissue through traditional culturing techniques.

Scaffold-based technologies

Ranging from hydrogels and microcarriers to microfluidic surfaces and solid scaffolds, this category encompasses the broadest spectrum of platforms for 3D cell culture. The unifying characteristic is that the platform serves as an artificial matrix that allows cell growth in a new dimension in order to escape the geometrical limitations of monolayer cultures. Based on this principle, different technologies have been developed to satisfy various niches in biological research.

Hydrogels

Hydrogels are moderately different from solid scaffolds. A first evident distinction is in the strength of the physical support they give. Hydrogels are loose scaffolds consisting of crosslinked natural or synthetic materials for cell encapsulation (Figure 1.3). In this way, these superabsorbent matrices are better suited to modelling soft tissue because of their tissue-like flexibility and viscoelasticity (Tibbitt & Anseth, 2009). Recreating the stem cell niche with only hyaluronic acid as a matrix supporting the growth of hESCs does not reflect the natural complexity of the ECM (Gerecht *et al.*, 2007). The addition of specific proteins, however, can significantly influence the differentiation of hESCs in 3D models. Coating hydrogels with ECM molecules enables the cultured cells to engage in the cross-talk of *in vivo*-like cues. Success with these platforms hinges on a combination of signalling via chemical and molecular pathways and biomechanical properties.

In a similar fashion to scaffolds, these gels can have a porous architecture facilitating the mass transfer of drugs, nutrients and oxygen to reach all areas. The idea of the choice of material determining the application and benefits of the technology is also very present in these platforms. Hydrogels can be derived from a wide variety of sources that in turn affect their compatibility and properties. For example, animal-derived hydrogels mainly use collagen, which is the most abundant protein in the ECM, making it a natural biological ligand for integrin attachment. Matrigel® is a popular commercially available hydrogel composed of tumour extract derived from Engelbreth-Holm-Swarm (EHS)

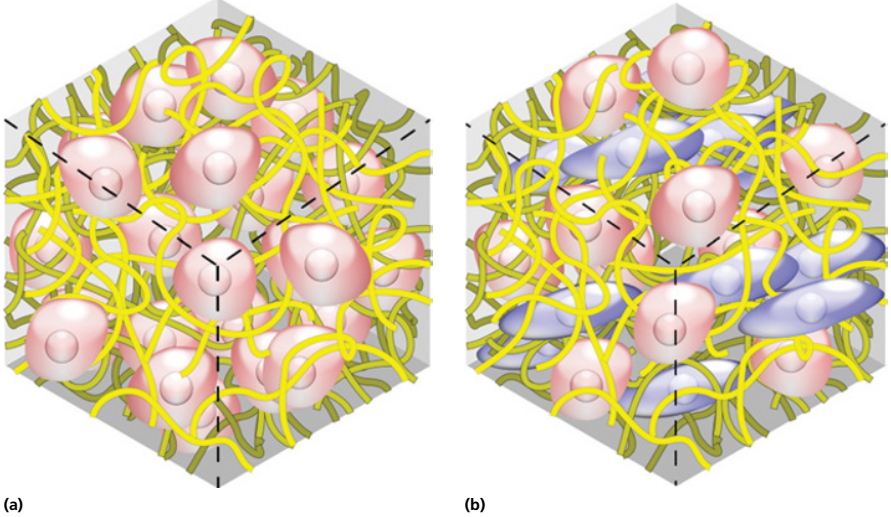

Figure 1.3 Hydrogels. 3D culture using hydrogel technology. (a) The cartoon shows cells within a matrix of protein molecules that create a nano-scale microenvironment mimicking the structure of the extracellular matrix. Cells are embedded within the proteinaceous 3D framework within an aqueous-based gel. (b) Co-culture of alternative cell types using hydrogel technology.

mouse sarcoma. It is known to contain various growth factors, a rich protein mix including collagen IV, laminin and enactin and other undefined constituents (Vukicevic *et al.*, 1992). Though these may result in batch-to-batch variation, hindering reproducibility, Matrigel can promote cellular functions that would otherwise remain unseen by providing the necessary endogenous factors (Benton *et al.*, 2014). Moreover, plant-derived hydrogels have been developed by crosslinking alginate monomers (Zimmermann *et al.*, 2007). Despite having no adulteration with animal proteins, they cannot escape from biological variation, rendering them unviable for HTS. Synthetic hydrogels solve these issues by using inert materials, whilst still being able to be supplemented with bioactive molecules to enhance their use.

Exploiting synthetic hydrogels with careful manipulation of their properties has resulted in the creation of injectable hydrogels. By controlling the gelation time and degradation of these materials, synthetic hydrogels can be utilised as a delivery mechanism of cultured cells to sites that would otherwise require an invasive procedure (Temenoff & Mikos, 2000). Other practical uses have seen these matrices employed to investigate developmental processes such as branching and vascular morphogenesis (Lo *et al.*, 2012).

Microcarriers are another system that can incorporate hydrogels that use these matrices as the basis to build microscopic spheres (90–500 µm in diameter) for culturing entrapped cells in 3D. With a high surface area to volume ratio, this technology also allows the culturing of anchorage-dependent cells (van Wezel, 1967). The main application of this system has been as a high-yield culture for the production of biologics in industry (Wu *et al.*, 2004). These spheres usually have a magnetic core, allowing control during media changes (Justice *et al.*, 2009).

Overall, synthetic hydrogels have vast potential as a culturing technique that can have research, therapy and industrial applications. Notwithstanding, the general obstacles faced by hydrogels include short culture periods due to diffusion of nutrients across the hydrogel. Also, using ultraviolet (UV) light to cure the gel is believed to be damaging to cells (Nicodemus & Bryant, 2008).

Solid scaffolds

Solid scaffolds were originally devised for transplantation applications in wound healing. Seeding cells in biodegradable scaffolds enabled creation of 3D cellular structures, which could be incorporated into living tissue where the exogenous framework would eventually degrade and be replaced by healthy natural tissue. In recent years, however, there has been a growing interest in introducing *in vitro* scaffolds for routine use in cell culture.

The materials used in the fabrication process are very important in shaping the purpose of the scaffold-based technology. Components of the native ECM including collagen, fibrin and hyaluronic acid (HA) (Gerecht *et al.*, 2007; Matsiko *et al.*, 2012) have been used effectively to create 3D matrices to support cell growth. These constituents have the benefit of being biocompatible and possessing readily available adhesion sites that can increase the complexity of the tissue.

Decellularised scaffolds are an example of a natural matrix where the native composition and architecture of tissue are fully preserved. Organs and tissue sections can undergo physical, chemical and enzymatic treatment to remove all cellular antigens whilst still preserving the ECM (Song & Ott, 2011). The preparation of such scaffolds can involve ionic detergents, which circumvents the problems of enzymatic treatment and collagen degradation (Gilbert *et al.*, 2006). Ensuring collagen remains intact also conserves its bioactive sites, facilitating the culture of cells in this decellularised matrix. Depending on the purposes of this type of scaffold, the recovery and processing techniques will vary to achieve optimum use.

Similarly, scaffolds can be produced from naturally derived materials such as alginate, silk and gelatin (Zimmermann *et al.*, 2007). Both decellularised matrices and these types of scaffold share the advantage of being biodegradable, which makes them suitable for growing grafts or to lay the foundations for new functioning cells to repopulate damaged tissue.

Despite being beneficial in the context of tissue engineering, working with biological materials in the laboratory affects consistency. A partial solution has been to use biodegradable polymers such as polyglycolic acid, polylactic acid and their co-polymer polylactic-*co*-glycolic acid (Mikos *et al.*, 1993). This is not ideal because their degradation results in the release of unwanted by-products that can alter cell behaviour. The build-up of lactic acid, for example, is known to cause suboptimal culturing conditions for embryonic stem cells (ESCs), decreasing pluripotency markers and inducing spontaneous differentiation (Ouyang *et al.*, 2006). The added variability coupled with short shelf-life and problematic storage make biodegradable materials unsuitable for standard use in 3D cell culture. In light of these shortcomings, synthetic scaffolds with defined composition have risen as a more consistent alternative. Inert and non-degradable materials such as polymers, titanium and ceramic-based platforms can be carefully tweaked to capture the cellular niche, creating scaffolds suitable for cell culture (Boccaccini & Blaker, 2005; van den Dolder *et al.* 2003).

The methodology behind the making of these matrices separates them into two categories: fibrous and porous scaffolds. One example of how fibrous scaffolds are manufactured is through electrospinning, a technique by which polymer jets are passed through an electric field (Reneker & Chun, 1996). The electrospun fibres that accumulate in the collector plate are then used to form interlaced structures or aligned patterns in which cell positioning can be regulated. This technique is highly flexible, allowing a variety of substances, from biologically active to synthetic polymers, to be used as jetting materials. In fact, it is possible to use two or more materials to produce heterogeneous scaffolds (Yang *et al.*, 2005).

Porous scaffolds, on the other hand, make available a controllable 3D space where cells can enter and grow, forming contacts and interactions with adjacent cells (Figure 1.4). The dimensions of the voids are known to affect cell seeding as well as how cells behave and grow in the scaffold (Knight & Przyborski, 2014).

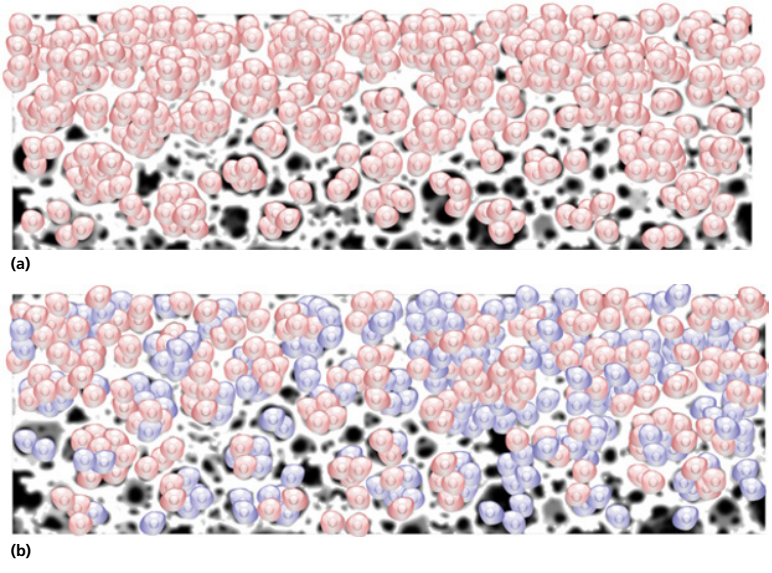

Figure 1.4 Solid scaffolds. (a) Porous solid scaffold supporting 3D cultured cells. Cells enter the porous framework of the solid scaffold where they do not flatten, they maintain their 3D structure and they bind to one another forming 3D tissue-like masses. (b) Co-culture of alternative cell types using scaffold technology.

Voids are interconnected by small pores, which prevent cells from being isolated within the 3D microenvironment and also contribute to greater cell infiltration. Achieving pore formation is an elaborate process that can be carried out through different techniques. Particulate leaching is a physical process in which a polymer is cast around soluble beads known as porogens (Reignier & Huneault, 2006). Popular porogens include sugar, salt and paraffin wax. Although this method has the advantage of tight control over pore size, it has limited connectivity amongst these spaces, which may result in heterogeneous cultures. An alternative procedure ensuring greater interconnectivity through multiple pores is emulsion templating. This method for fabricating solid scaffolds incorporates polymerisation by high internal phase emulsion (HIPE). This biphasic emulsion consists of an aqueous and a non-aqueous monomer/surfactant phase, which results in a highly porous scaffold linked by interconnecting pores (Barbetta *et al.*, 2000). A third method is gas foaming technology, which can generate large internal volume and 3D spaces by agitating polymers to create foam. Phase separation ensues from these conditions, causing the dissolved gas to split from the polymer. The free gas molecules then join to reduce free energy forming clusters and in turn leave porous structures, suitable for cell growth (Harris, 1998). Regulating the agitation and the use of high-pressure gases facilitates control of the porosity of the scaffold, although the low pore interconnectivity still remains a problem (Salerno *et al.*, 2009).

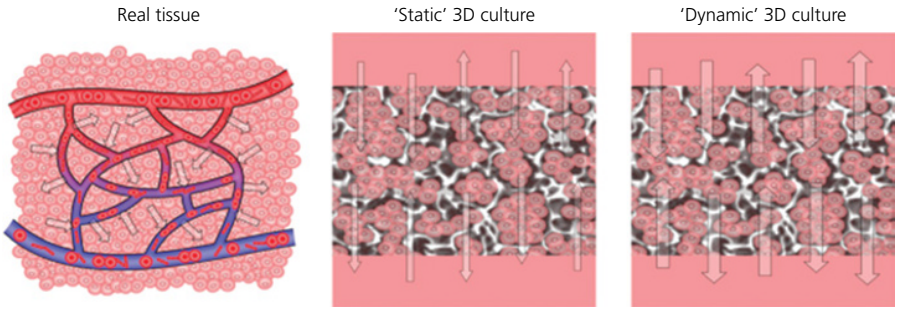

Figure 1.5 Perfusion model. Unlike real tissue, 3D cell culture models lack a vascular and capillary bed. Exchange of gases, nutrients and waste products occurs by diffusion, most often in a static culture where unstirred layers can build up in stagnant media. Dynamic 3D culture involves perfusion and movement of the media to reduce unstirred layers and increase exchange..

The lack of biological activity and natural cell adhesion sites can be overcome by coating these substrates with ECM proteins such as laminin and fibronectin (Knight & Przyborski, 2014). Despite providing physical support in the form of 3D spaces where cells can proliferate, these voids have poor mass transfer since these cultures are static systems. For these reasons, scaffolds are usually engineered as thin membranes (200 µm) that permit sufficient exchange of nutrients and waste products. This in turn enriches the physiological accuracy of these models, allowing researchers to study *in vivo* phenomena in a controlled *in vitro* setting.

3D bioreactors

Perfusion flow culturing systems can be identified as another division in 3D cell culture. These systems focus on replicating continuous circulation of nutrients and waste in cells and tissues (Figure 1.5). In addition, microfluidic culture systems and 3D bioreactors serve to model dynamic biological processes and the consequences of *in vivo* forces such as shear stress and fluid turbulence. Pulsating blood flow, for example, causes a mechanical stretch on endothelial and smooth muscle cells, which in turn can trigger cell signalling pathways, altering their behaviour (Tzima *et al.*, 2005). Similarly, flow rates are known to favour certain developmental decisions such as an arterial versus a venous phenotype in vasculogenesis (Le Noble *et al.*, 2004).

Regarding the culture type, microfluidics are often recognised as a scaffold-based platform in the literature whereas 3D bioreactors would largely fall under scaffold-free technologies since they generally produce suspension cultures aided by a constant agitation maintaining cells in suspension. This classification overlooks the fact that the concept behind these platforms can be implemented on both scaffold-based and scaffold-free technologies. Therefore, it is also possible to consider them as a separate category of 3D culture systems, borrowing aspects from both, and thus exemplifying how this field of science is in fact multidisciplinary.

Directional flow type technologies that primarily involve pumping of media over cultured cells can be performed on a variety of scales, including large bioreactors composed of complex tubing arrangements, smaller scale bench-top versions and micro-scale fluid control devices. Microfluidics consist of the engineered manipulation of fluids at a micro-scale (Sackmann *et al.*, 2014). These lab-on-a-chip microtechnologies exploit fluid behaviour at the submillimetre scale because the rules controlling forces such as laminar versus turbulent flow, surface tension and capillary forces are vastly different compared to the macro scale (Sackmann *et al.*, 2014). The fabrication of these intricate systems uses processes such as microcontact printing, photolithography and replica moulding (Ito *et al.*, 1997; Park & Shuler, 2003; Sun *et al.*, 2012). The mechanism behind this culturing system involves an array of pillars that support the growth of cells. These micropillars also immobilise cells, preventing fluids from displacing them and ensuring a controlled transient or continuous flow of media circulating through the culture system. Passing a collagen matrix creates a thin layer surrounding the cell, which establishes cell-matrix interactions, introducing more complexity to the model. These upgrades contribute to an inexpensive and efficient model for drug screening, compatible with automation where single cell manipulation is feasible. The potential to quantitatively and qualitatively examine the impact of fluid forces acting on cells, while minimising reagent volume, makes this platform very attractive in medical and biological research.

Also referred to as agitation-based approaches, these culturing systems' principal purpose is to recreate biophysical cues experienced by cells in live tissue. By ensuring constant movement in the culture system, cells are prevented from adhering to the walls of the container and instead they are encouraged to form cell-cell interactions (Breslin & O'Driscoll, 2013). These systems, such as rotational and spinner flask bioreactors, form spheroids by continuous rotation or stirring, respectively. Constant motion and perfusion flow allow for transfer of nutrients and waste to and from the suspension culture. These bioreactors are well equipped for large production and long-term maintenance of cell aggregates, aided by easy media changes to suit these purposes (Rodday *et al.*, 2011). Disadvantages of bioreactors include larger media requirements since the culture system operates with greater volumes. Whilst the shear force can affect cell physiology (Lin & Chang, 2008), it can also exert pressures that are constantly occurring in an *in vivo* context. Moreover, size variation and poor uniformity in morphology are problems that can be addressed by combining this approach with other aggregate-based technologies more suitable for culturing multiple spheroids with consistent dimensions. In this way, suspension cultures can be initially generated through this technique but later transferred to rotational culture systems or spinner flask bioreactors. The enhanced environment of bioreactors will complement the model and facilitate long-term culturing. However, using these culture systems for drug screening would require replating spheroids into suitable plates that can ensure one spheroid per well and maintain a uniform size for HTS (Breslin & O'Driscoll, 2013).

Barriers to adoption and future directions

Amongst the obstacles discussed in each section, this rapidly growing multidisciplinary field faces the difficulty of trying to incorporate the various biological parameters into one single platform. The ideal 3D cell culture platform is imagined as a system comprising multiple cell types in a chamber that recreates the *in vivo* forces acting on cells. These would include structure and surface modifications, cellular interactions between adjacent cells and the ECM, mechanical and fluid flow forces. The problem, however, is that tissues are widely diverse and have a variable set of needs, which obstructs the efforts of designing an all-encompassing technology that meets every biological requirement. Experimental models need to show the different facets of the same tissue in a reproducible, measurable and reliable manner and in certain cases this is not easy, straightforward or possible. For these reasons, scientists have moved away from this approach. Rather than a panacea for culturing cells *in vitro*, this is a matter of utilising various aspects of 3D cell technology, depending on the biological question to be explored.

An anatomical or histological view of disease would argue that pathological conditions normally reflect an alteration of the tissue organisation or an insult to the cellular structure. In order to fully grasp the progression from one state into another, it is necessary to replicate such modifications. At times, mimicking the *in vivo* forces influencing cell behaviour can be conflicting. For example, static cultures may be adequate to establish gradients that in turn can allow for a close study of avascular tissues, such as tumours. On the other hand, lack of perfusion flow and circulation of nutrients makes it difficult to build 3D systems with vascularised tissue. This example illustrates the issues researchers encounter and the importance of weighing these factors when planning an experiment to address their proposed biological inquiries. Commercialised 3D cell culture technologies provide an array of accessible solutions that are flexible and easily adaptable to different experimental set-ups. These platforms can enhance cell-based models by bridging the gap between traditional monolayer cultures and animal models, ultimately expanding our understanding of cellular biology. By working in concert with other modern resources in cell biology, such as human stem cells, these technologies can create robust tissue mimetics by introducing a 3D component that enables cell differentiation into more complex structures. Creating human tissue *in vitro* offers exciting possibilities for advancing drug discovery with an enhanced predictive accuracy of drug candidate compounds, as well as furthering regenerative medicine. The success of platforms for 3D cell culture technology explored hereafter will depend on overcoming the barriers to adoption and validating their potential for routine use.

3D culture systems face various challenges before full adoption becomes a reality. Even when their worth is undeniable, conclusions from one system may not be true for another. Whilst using synthetic materials and platforms compatible with HTS can mitigate the problem of variable results, these methods may fail to mimic some characteristic of an *in vivo* setting or be unsuited to carrying

out downstream analyses. These drawbacks hint at a wider issue in 3D cell culture. There are multiple answers to the question of reproducing a more *in vivo*-like setting. These technologies will invariably impact cell culture in different ways, making it difficult to see the path towards advanced cell culture as a single step to improve the biological relevance of cell-based assays. For these reasons, culturing systems are shifting their focus towards investigating the interdependence of factors known to enrich the representation of physiological phenomena. This, however, is often prohibited by the nature of the 3D culturing system that cannot be subjected to other conditions, such as oxygen concentrations and mechanical forces. Even though these platforms may lack flexibility, 3D cell culture will innovate and seek to examine the synergy of microenvironment factors, offering a higher degree of complexity and moving a step closer to reflecting true physiological behaviour.

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PART I

Aggregate-based technologies

CHAPTER 2

Gravity-enforced microtissue engineering

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Introduction

The hanging drop technology

The hanging drop (HD) technology is used to culture cells and tissues originated from embryology as a means to investigate basic developmental processes that would be otherwise restricted to growing embryonic stem cells on flat culture surfaces (Keller, 1995). The classic hanging drop culture comprises a small droplet of cell culture medium placed on a sterile surface such as the lid of a bacterial dish which is then inverted to generate the hanging droplet. The hanging drop is stabilised by its surface tension, preventing dispersion across the surface (Kelm *et al.*, 2003). This allows contact-free tissue culture as well as the reassembly of microtissues or embryoid bodies without the use of supporting materials.

The inventor of the HD method was Ross Granville Harrison who adapted HD technology to grow frog neuronal tissue. Utilising this technology, he was the first to observe the development of growth cones in developing neurons and provided the basis for the discovery of nerve growth factor (NGF) by Rita Levi-Montalcini, for which she received the 1986 Nobel Prize for Physiology or Medicine (Levi-Montalcini, 1964). Currently, HD technology is not only extensively used in stem cell biology and growing whole embryos, but it has become one of the major technologies used to reassemble spherical tissue structures (Kelm *et al.*, 2005). The ability to resolve three-dimensional (3D) structures, endogenous extracellular matrix and cell-cell contacts is an important advance that made the hanging drop a widely used tissue culture method. However, besides improving the cell's biology, a decisive step towards industrial application is the development of automation-compatible technologies enabling assay automation with a high throughput. Moreover, the lack of any supporting materials allows compatibility with a vast variety of currently established endpoints.

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