Three-dimensional cell culture: a rapidly emerging technique for drug discovery

The past decades have witnessed significant efforts toward the development of three-dimensional (3D) cell cultures. Today, 3D cell cultures are emerging not only as a new tool in early drug discovery, but also as potential therapeutics to treat disease.

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Cell culture is a fundamental technique in both medical research and drug discovery, and for decades, two-dimensional (2D) culture has been the preferred method, due to the ease with which cell monolayers can be induced to proliferate on planar surfaces.

However, early pioneering work in cell culture utilised tissue explants grown in three-dimensions (3D). It was evident that such 3D cultures maintained many attributes that resembled cell growth and differentiation *in vivo*[1]. Today, the limitations of 2D culture, the development of advanced laboratory products and sophisticated detection instrumentation and analytic software, as well as the emergence of stems cells as powerful research tools, has led to the growing adoption of 3D culture models in many phases of drug discovery, including target validation, lead identification and preclinical optimisation.

In the past, 3D cell culture models were mostly developed for oncology research, given that tumours exist as 3D entities *in vivo* and, therefore, should be better approximations of the tumour microenvironment[2]. Consequently, several 3D cell tumour models now exist, from multicellular layers on porous membranes coated with collagen, to matrix-embedded cultures, hollow fibre bioreactors and multicellular spheroids. A more recent approach, yet to have a major impact on drug discovery, has been the growing use of dish-based organogenesis, using technologies from stem cell research and mixed cell culture techniques[3]. This approach has a high degree of physiological relevance and results in the development of organoids with realistic micro-anatomy.

Taken together, 3D cell culture techniques are no longer confined to the research space, but are emerging as a powerful new tool in preclinical drug discovery. Recent advances in cell biology, microfabrication techniques and tissue engineering have enabled the development of a wide range of 3D cell culture technologies. These include multicellular spheroids, organoids, scaffolds, hydrogels, organs-on-chips, and 3D bioprinting, each with its own advantages and disadvantages (see **Table 1** for a summary). Furthermore, since patient-specific cells can also be grown in 3D, the exciting possibility exists for drug discovery to be undertaken on cells

Table 1: Advantages and disadvantages of different 3D cell culture techniques

TECHNIQUE	ADVANTAGES	DISADVANTAGES
Spheroids*	Easy-to-use protocol Scalable to different plate formats Compliant with HTS/HCS[a] Co-culture ability High reproducibility	Simplified architecture
Organoids	Patient specific In vivo-like complexity In vivo-like architecture	Can be variable Less amenable to HTS/HCS Hard to reach in vivo maturity Complication in assay Lack vasculature May lack key cell types
Scaffolds/hydrogels	Applicable to microplates Amenable to HTS/HCS High reproducibility Co-culture ability	Simplified architecture Can be variable across lots
Organs-on-chips	In vivo-like architecture In vivo-like microenvironment Chemical, physical gradients	Lack vasculature Difficult to be adapted to HTS
3D bioprinting	Custom-made architecture Chemical, physical gradients High throughput production Co-culture ability	Lack vasculature Challenges with cells/materials Difficult to be adapted to HTS Issues with tissue maturation

* *Discussion is limited to low adhesion plates*

[a] *HTS: High Throughput Screening; HCS: High Content Screening*

with very precise pathophysiologies. Finally, 3D cell culture is growing in the area of bioproduction, notably as a means to scale-up abundant and reproducible numbers of cells as potential therapeutics.

3D cell culture in drug discovery

Cellular assays are routinely used in compound screening and optimisation, with growing evidence showing that compound library hits and optimised leads translate into better candidates for clinical evaluation[4,5]. The majority of cell-based screening is performed using 2D culture technologies, due in part to the demands of the automation and detection instrumentation in use. However, cells grown in 3D better reflect drug-target interactions *in vivo*[6,7]. Additionally, drug sensitivity in 3D culture models differs markedly from that obtained using 2D culture (**Figure 1**). In particular, cell-based screening technology has been pivotal in the area of imaging technologies as used in high content screening (HCS) assays. 3D culture technologies, therefore, coupled with confocal optical imaging, allow for drug screening in protocols that are unfeasible with 2D cultures. Further, many of the current technologies that enable 3D cell culture also support co-culture conditions, allowing for multiple cell types to be integrated into a 3D model that more closely mimics the *in vivo* microenvironment. These co-culture models are important for drug discovery, as the presence of certain cell types within tumour spheroids can greatly shift drug responsiveness (**Figure 2**).

Advances in hydrogels and scaffolds for creation of spheroids and organoids

3D culture often involves embedding cells in either extracellular matrix (ECM) gels or solid scaffolds. To date, more than 100 types of matrices and scaffolds have been developed, most of which are optimised to the growth of the specific cells under investigation.

Naturally-derived ECMs are widely used in 3D cell culture. These basement membrane hydrogels can provide the appropriate microenvironment needed for morphogenesis and organogenesis of cells possessing intrinsic developmental programmes. Immortalised cell lines and tissue fragments form structures that recapitulate key tissue features when

embedded in ECM gels and exposed to appropriate growth factors. Stem cell-derived organoids have been developed from embryonic stem cells (ESCs), induced pluripotent stem cells (IPSCs) or from primary stem cells purified from organs[3].

The possibility now exists for patient-derived organoids that potentially enable personalised approaches to identify the mechanisms underlying human diseases[8-10], and to evaluate the efficacy and predict toxic potential of drugs prior to administration. This transformative approach may help identify the best therapies for individual cancer patients over the course of their disease.

Cellular spheroids embedded in ECMs present several features, including a defined geometry, optimal physiological cell-cell and cell-ECM interactions, and better gradients of nutrients, growth factors and oxygen, upon which transport occurs for several hours or even days[7]. These attributes facilitate screening assays for compounds to modulate tumour growth, invasion and angiogenesis. Cellular spheroids can be generated from many types of cells; those formed include embryoid bodies, mammospheres, tumour spheroids, hepatospheres and neurospheres.

Naturally-derived hydrogels for 3D culture comprise proteins and other ECM components, including collagen, laminin and fibrin. A major advantage of 3D culture using a naturally-derived hydrogel is that the protocols are robust and simple. However, they have certain disadvantages due to their origin as undefined, complex material of variable compositions. Furthermore, naturally-derived hydrogels may lack the mechanical properties provided by endogenous ECMs. Finally, their non-human origin can preclude their use in human regenerative or transplantation therapies.

The microenvironment is clearly critical to complete organ development; but this feature has, nonetheless, been difficult to reconstitute completely in many 3D cultures. The development of synthetic structures or scaffolds using naturally-derived ECM, synthetic hydrogels or other biocompatible materials may address this issue, as they are designed to either replace or complement naturally-derived ECMs with clinical-grade materials. Tissue organoids in 3D ECMs have been developed for mammary, stomach, intestinal, liver, brain, salivary, kidney, lung and pancreatic ductal epithelium[3,15]. However, the thick ECM gel can limit optical imaging and cell recovery is more complex.

To overcome some of these limitations, several lab consumables and methods have been developed to take advantage of spontaneous cell-cell interactions, which occur when cells are in an environment that promotes greater attraction towards

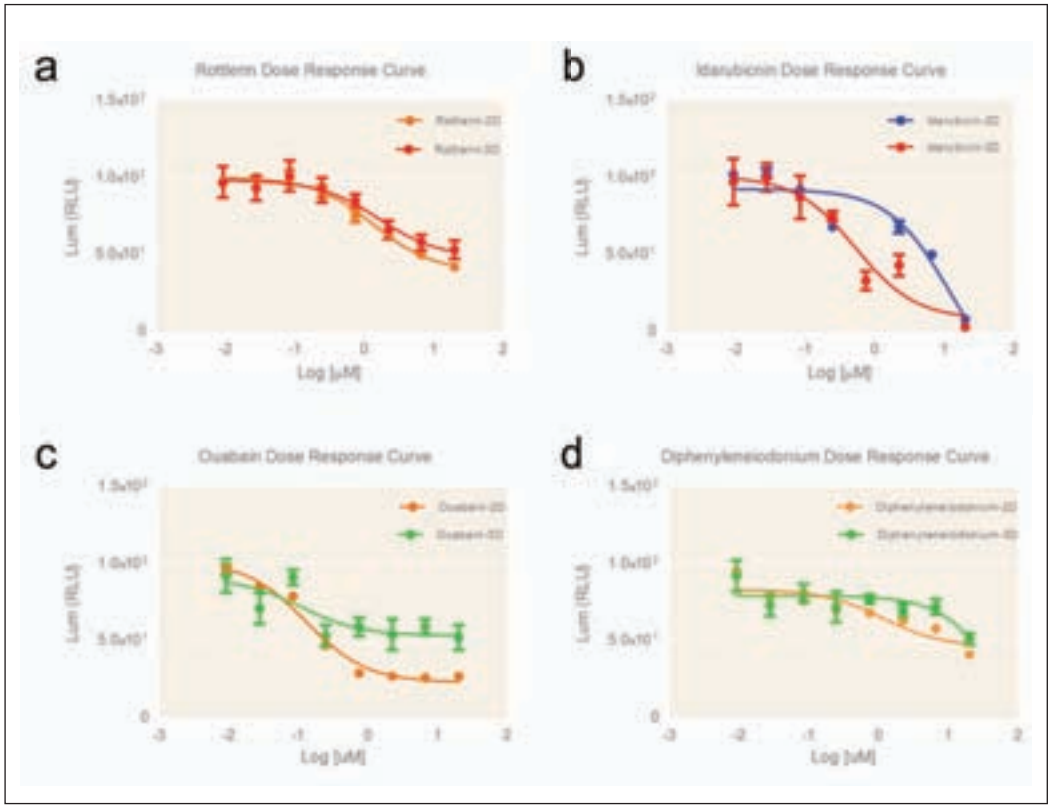

Figure 1
Different drug sensitivity of BT-474 cells in 2D monolayer versus 3D spheroidal culture. 2D culture was achieved by seeding 5,000 cells per well in a Corning 384-well flat bottom tissue-culture treated microplate and 3D spheroidal culture was achieved by seeding 1,000 cells per well in a Corning 384-well spheroid microplate. Cells were cultured for 48 hours, followed by 48 hours of drug treatment with either (a) rottlerin; (b) idarubicin; (c) ouabain; or (d) diphenyleneiodonium, prior to viability assessment using Promega CellTiter-Glo® 3D Cell Viability Assay. Data represents mean ± SD (n=7). Results show that while rottlerin displayed comparable sensitivity in 2D and 3D formats, idarubicin displayed a ~20-fold left-shift in potency in 3D compared to 2D, whereas ouabain and diphenyleneiodonium displayed larger or more potent cytotoxic effects in 2D compared to 3D

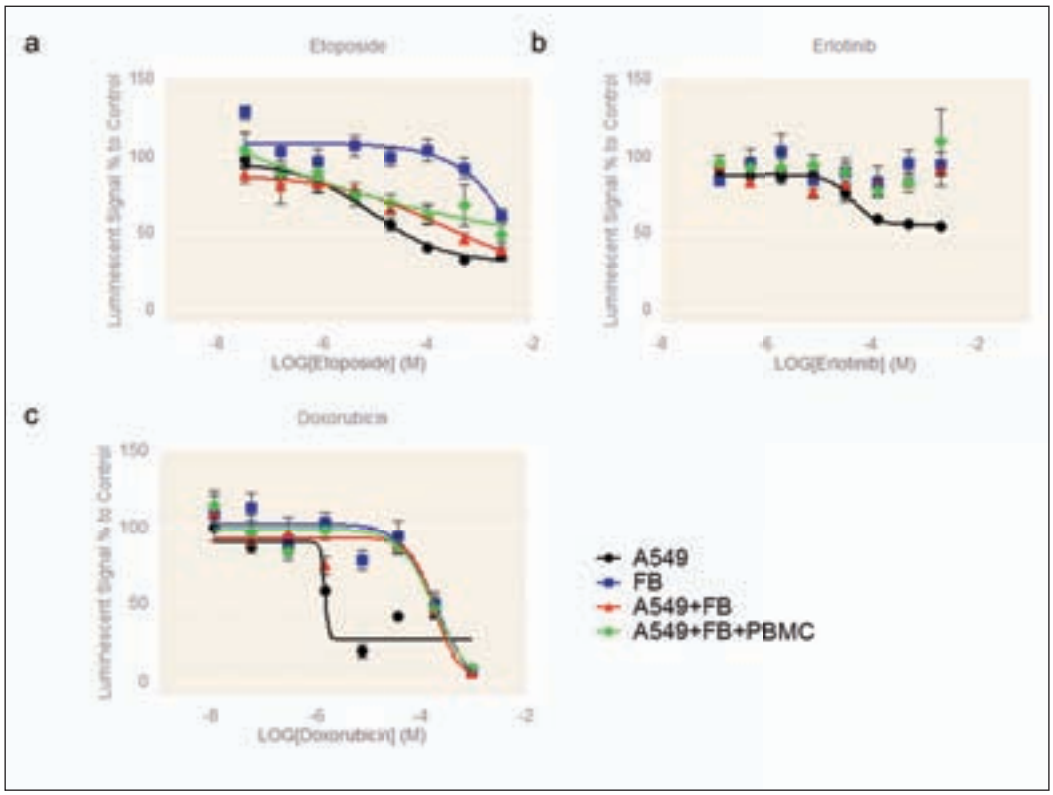

Figure 2
Shifts in chemotherapeutic compound sensitivity of mono-, co- and tri-culture 3D spheroids. Chemotherapeutic compounds were applied for 48 hours to 3D spheroids, previously formed for 48 hours using monoculture A549 cells (black), monoculture primary human lung fibroblasts (blue), co-culture A549 cells with fibroblasts (red) and tri-culture A549 cells with fibroblasts and peripheral blood mononuclear cells (green). Viability was assessed using Promega CellTiter-Glo® 3D Cell Viability Assay. The presence of fibroblast and immune cells in the spheroids affected the potency of several compounds, resulting in right-shifts in potency of etoposide (a); erolotinib (b); and doxorubicin (c). Data represents mean ± SEM (n=6)

each other than to any available surface. Of the techniques developed to take advantage of the phenomenon of cell aggregation, the hanging drop[13] and low-attachment methods[14] (**Figure 3**) are widely used due to their compatibility with automated screening instrumentation and detection systems. Additionally, technologies to circumvent the issues of vascularisation have been reported with microfluidic systems and/or organ-on-chip platforms (see below).

A novel approach to cell therapy

Cell therapy and tissue engineering not only offer new hope for patients with injuries, end-stage organ failure, or other clinical issues, but will eventually transform our lives. However, it is becoming clear that realising the full potential of cell therapy and tissue engineering requires advances in cell culture technologies to meet the demand in quantity, quality and process robustness for commercialisation and clinical trials. 3D cell cultures offer not only a solution for cell scale-up production, but also a new form of therapeutics for treating many different diseases.

Stem cells are widely used as a cell source for regenerative medicine and cell therapy applications. However, conventional 2D culture techniques, in combination with the current best practice, may be ineffective in the expansion of stem cells for clinical applications. This is reflected by the fact that 2D cultures are inadequate to reproduce the *in vivo* microenvironment of stem cells[16]. In addition, clinical observations show that the beneficial effects of stem cell-based therapeutics seen in initial small-scale clinical studies are often not validated by large, randomised clinical trials[17,18]. In fact, mesenchymal stem cells (MSCs) often decrease their replicative ability, colony forming efficiency and differentiation capabilities over time when culturing and passaging in 2D adherent monolayer[19,20].

In contrast, MSCs cultured in spheroids display a morphology that is significantly different from 2D culture[21]. Furthermore, compared with 2D culture, MSCs cultured in spheroids have different gene expression patterns, with up-regulation of many genes that are associated with hypoxia, angiogenesis, inflammation, stress response and redox signalling[22].

Spheroid cultures have been reported to improve the efficacy of MSC-based therapeutics. Compared with 2D cultures, MSC spheroid cultures were also found to have additional benefits, such as enhanced anti-inflammatory and tissue regenerative and reparative effects, as well as better post-transplant survival of MSCs[22]. Furthermore, compared with

2D cultured cells, spheroids of human adipose-derived MSCs produced higher levels of ECM proteins, exhibited stronger antiapoptotic and antioxidative capacities and increased the paracrine secretion of cytokines[23].

When considering transplantation, organoids could provide a source of autologous tissue, as organoid research advances rapidly. For instance, renal organoids derived from pluripotent stem cells were successfully transplanted under the renal capsules of adult mice[24]. Here, the organoid reconstituted the 3D structures of the kidney *in vivo*, including glomeruli with podocytes and renal tubules with proximal and distal regions and clear lumina. Furthermore, the glomeruli were efficiently vascularised upon transplantation, which is a promising step toward kidney replacement strategy.

Although early in development, organoid-based replacement may find applications in other diseases, such as retinal organoids obtained from human ESCs for treating certain types of retinal degeneration and blindness[25], intestinal organoids for replacement of damaged colon after injury or following removal of diseased tissue[26] and gene-corrected organoids for replacement of damaged organs with gene defect(s)[25].

In addition to the advantages of 3D culture techniques for expanding stem cells for therapeutic use and transplantation, there is also promise for stronger *in vitro* screening models applicable to immune cell therapy. While the use of immune cells in cancer treatment has shown great success, especially for B cell malignancies, with best case clinical outcome of complete remission, targeting solid tumours has added complications. Evaluating immune cell homing, efficacy and selectivity against tumour spheroids may be advantageous in helping to overcome these challenges, as the invasion and cytotoxic activity of immune cells against tumour cells cultured in 3D is quite different than that of a 2D adherent monoculture (**Figure 4**).

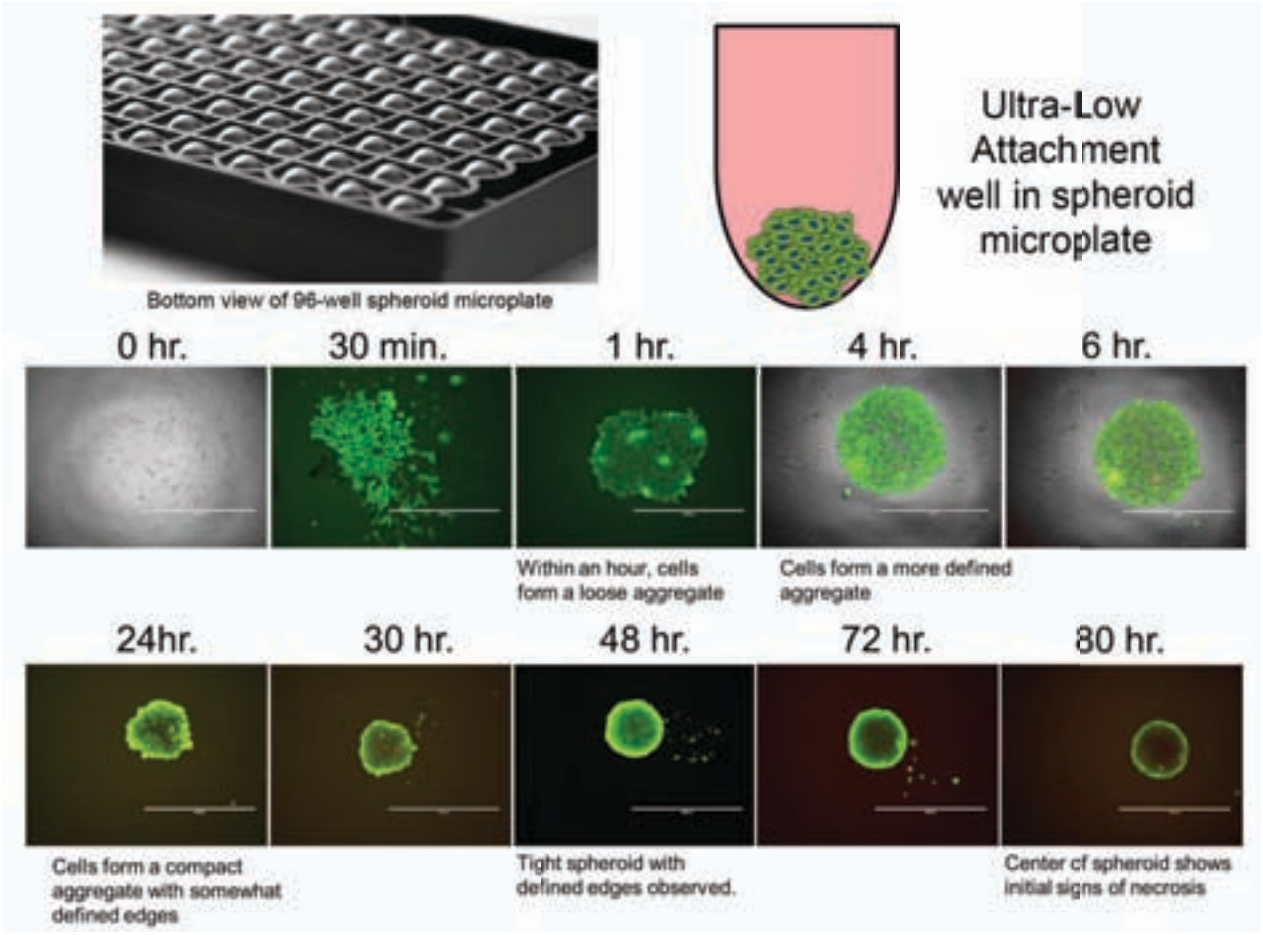

Figure 3
The formation and growth pattern of 3D spheroids of HT-29 cells in Ultra-Low Attachment, round-bottomed microplate. 5,000 cells were seeded in each well of a Corning 96-well spheroid microplate (that is, Ultra-Low Attachment, round-bottomed microplate), and cells were stained using Live/Dead viability/cytotoxicity kit (Life Technologies, Cat # L3224) and then imaged using an EVOS fluorescence microscopy at specific times. Scale bar: 1,000µm

What the future may hold: bioprinting and organ-on-chip

As we move into the future of cell culture, 3D bioprinting is an emerging technology currently utilised to create complex 3D tissue models for biomedical research[27]. Bioprinting technologies may enable biofabrication of organ structures accompanied by defined positioning of a range of cell types and with optimal ECM components. This may provide a more optimal cell/organoid architecture, topology and functionality that are highly representative of the *in vivo* organ. Furthermore, automated systems may provide layer-by-layer deposition of biological materials that can ensure good reproducibility of the bioprinted structures.

Recent examples of bioprinted tissues include muscle fibres, printed with human myoblasts and tenocytes that exhibit contractile movement in multiwell devices containing physical posts for attachment[28]. These devices offer the possibility of identifying compounds that modulate fibre contractility. In a different model, human lung endothelial and epithelial cells, bioprinted in a 3D air-blood barrier on multiwell permeable support system[29], offer the potential to screen for aerosolised toxic compounds.

Organs-on-chips represent a family of miniature models of human organs on plastic chips. These micro-engineered models leverage novel technologies including microfabrication, microfluidics, tissue architecture engineering and sensors.

Microfabrication techniques (such as soft lithography, photolithography and contact printing) enable the creation of well-defined structures, patterns and scaffolds to control the position, shape, function and physical microenvironment of the cells in culture[30]. Microfluidics permits the precise control of spatiotemporal gradients of chemical and physical cues to the cells[31]. Tissue architecture engineering allows for designing practical and simple tissue models to recapitulate the tissue-tissue

interfaces, as well as the chemical and physical microenvironments found *in vivo* for drug testing[32]. Furthermore, sensors can also be incorporated and provide in-process controls for pH, O_2, temperature, flow velocity, metabolic status and organ viability and functionality[33].

Currently, organs-on-chips are designed to reconstitute the structural and functional complexity of human organs and clinically-relevant disease phenotypes and pharmacological responses. The past decade has witnessed dramatic expansion of different types of organs-on-chips, including lung, heart, brain, liver, kidney, intestine, fat, muscle and bone marrow (**Table 2**).

Human organs-on-chips hold promise in replacing animal models for assessing drug safety, efficacy and pharmacokinetics in human, as results from animal models often fail to predict human responses[34-38].

Limitations and commercial implications

There are, of course, some limitations to using 3D cell culture in drug screening. One of these lies in the technical aspects that relate to assay protocols. These include the need to optimise and standardise procedures for cell harvesting, cell lysis, production scale up, as well as control of pH and temperature to reduce well-to-well and lot-to-lot variations.

Since 3D culture can be more heterogeneous than 2D culture, interpretation of data is more challenging. In addition, the potential for compound non-specificity may be increased due to the more complex culture conditions used, as well as physicochemical issues, including compound access to the cells within the 3D complex.

Nonetheless, as occurred in the adoption of 2D cell culture procedures in HTS/HCS, defined protocols – and novel instrumentation – are now being developed for 3D culture that could circumvent these limitations.

3D cell cultures have a clear role in drug discovery and development. There is enormous potential to model development and disease, as advanced cell models under development may fully capture the *in vivo* functions of organs and tissues. Furthermore, the development of screening-compatible 3D cell cultures would transform the drug discovery process, as it becomes possible to obtain early the physiologically-relevant efficacy and toxicity data. In addition, the optimisation of 3D cell cultures for scaling-up cell production would improve quality, quantity and efficacy, thus making cells as therapeutics a reality.

Today, the market is rapidly adopting 3D cell culture technologies, and the commercial implications for the industry are starting to become clear. According to a recent HTStec global benchmarking survey, 70% of respondents are currently performing 3D cell cultures, and the median annual budget allocated to spheroid culture consumables is growing – from US$1,000-$2,500 per laboratory in 2015 to an estimated US$5,000-$10,000 per laboratory in 2018. In addition, 3D spheroids are expected to be the customary 3D platform for drug research within the next three to five years. HTStec has assessed the 2016 market for 3D spheroid culture/generation consumables at approximately US$33 million[39]. If the predicted growth numbers are accurate, advances in 3D cell culture technology will have a significant impact on laboratory spend in the next few years.

The growing interest in using cells in an environment that authentically replicates their function *in*

Figure 4
The invasion of CAR-T cells into 3D tumour spheroids. HCC827 3D tumour spheroids cultured for 48 hours were exposed to ProMab Biotechnologies CAR-T cells affinity tuned to target EGFR expressing cells. HCC827 tumour cells were stained for cytokeratin-7 (green) and EGFR CAR-T cells for CD3e (red), using Hoescht nuclei counterstain (blue). As the effector to target ratio (E/T) is increased from 10:1 to 40:1, invasion of the CAR-T cells into the HCC827 tumour spheroid and subsequent tumour cell lysis are visible. Images obtained using a Thermo Fisher CellInsight CX7 in confocal mode using 10X objective

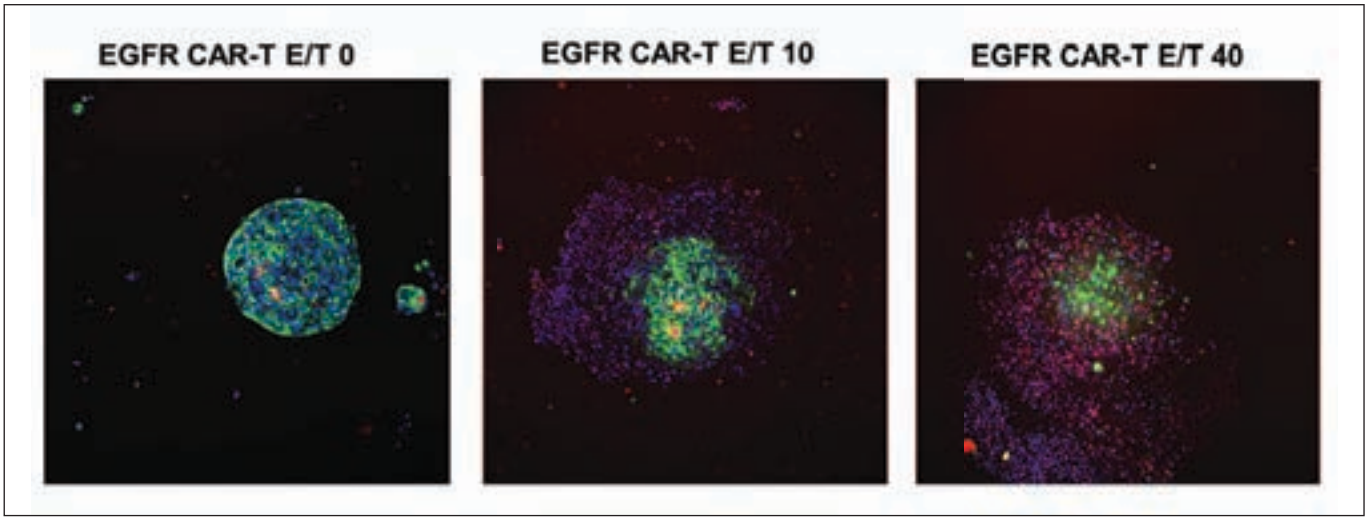

Table 2: Representative organ-on-a-chip models

ORGANS	CELL TYPES	FUNCTIONS	REFERENCE
Lung	Alveolar epithelial cells Vascular endothelial cells	Alveolar-capillary barriers Surfactant production Lung inflammation Extrapulmonary absorption	34
Liver	Hepatocytes Vascular endothelia cells Fibroblasts	Serum protein synthesis	35
Kidney	Rat inner medullary collecting duct cells	Molecular transport	36
Gut	Intestinal epithelial cells	Intestinal absorption Villus differentiation Drug metabolism	37
Heart	Cardiomyocytes	Cardiotoxicity	38
Breast	Mammary epithelial cells Mammary fibroblasts Vascular endothelial cells	Cancer research	39
Brain	Neural progenitor cell	Alzheimer's disease model	40
Bone marrow	Hematopoietic stem and progenitor cells	Toxicity	41

vivo, yet is amenable to manipulation and experimentation, has driven the adoption of 3D culture in both medical research and drug discovery. Technologies are rapidly advancing in the area, with many protocols being reduced to routine practice, as well as for use in the automation systems employed in compound screening and drug evaluation. The use of cultured primary human cells can help doctors choose the most appropriate medicine for specific patients, as well as provide compound screening systems to develop novel therapeutics.

Indeed, as the industry continues to adopt new 3D cell culture technologies, the future of discovery looks bright.

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