Published in final edited form as:
Bioanalysis. 2012 June ; 4(12): 1509–1525. doi:10.4155/bio.12.133.

Microfluidic 3D cell culture: potential application for tissue-based bioassays

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Abstract

Current fundamental investigations of human biology and the development of therapeutic drugs, commonly rely on two-dimensional (2D) monolayer cell culture systems. However, 2D cell culture systems do not accurately recapitulate the structure, function, physiology of living tissues, as well as highly complex and dynamic three-dimensional (3D) environments *in vivo*. The microfluidic technology can provide micro-scale complex structures and well-controlled parameters to mimic the *in vivo* environment of cells. The combination of microfluidic technology with 3D cell culture offers great potential for *in vivo*-like tissue-based applications, such as the emerging organ-on-a-chip system. This article will review recent advances in microfluidic technology for 3D cell culture and their biological applications.

Introduction

Cell cultures are integral to cell biology, biochemistry, drug discovery and development, pharmacokinetic studies, and tissue engineering [1]. The most common cell culture platform is two-dimensional (2D) monolayer cell culture in petri dishes or flasks. Although such 2D *in vitro* models are less expensive than animal models and are conducive to systematic, and reproducible quantitative studies of cell physiology (e.g., in drug discovery and development), the physiological relevance of the information retrieved from *in vitro* studies to *in vivo* system is often questionable [2]. It has now been widely accepted that three-dimensional (3D) cell culture matrix promotes many biological relevant functions not observed in 2D monolayer cell culture [3-6]. These discrepancies are primarily attributed to two reasons: (1) Cell growth *in vivo* is significantly affected by the diffusion-limited distribution of oxygen, nutrients and other molecules [7]. Oxygen, nutrients and other molecules are continuously consumed and produced by cells. Such dynamic distributions are not mimicked in conventional 2D cell culture [8,9]. (2) Cell growth and cell functions operate within a highly complex three-dimensional environment and under the influence of a myriad of regulatory interactions from other tissue cells (e.g., via signal transduction), the extracellular matrix (ECM), and other systemic factors, which are not recapitulated by the 2D cell culture. As such, the transition from 2D cell culture to 3D cell culture has gained momentum as an increasing number of reports have confirmed significant differences in the morphology, protein expression, differentiation, migration, functionality, and viability of cells between 3D and 2D cell cultures [10] [11] [12] [13] [14] [15] [16] [17] [18] [19] [3]

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[20] [21]. Therefore, countless efforts have been made to create 3D cell culture platforms to mimic the native *in vivo* biological systems.

Gel-based systems are widely used for conventional 3D cell culture, where cells are embedded in 3D matrix gels for cultivation. Conventional 3D cell culture is also performed on spheroid-based systems [10,22] and porous materials, such as poly (lactic-co-glycolic acid) PLGA and silk fibroin as scaffolds [23,24]. Spheroid-based systems take advantage of the natural tendency of many cell types to aggregate. Spheroids can be obtained from single culture or co-culture [10,22]. Nevertheless, conventional 3D cell cultures often face technical challenges in creating a highly complex, and well-controlled 3D dynamic environment as *in vivo* systems.

The microfluidic technology developed in the 1990s offers a unique opportunity for 3D cell culture and cell-based assays, creating a platform for engineering highly complex and dynamic microenvironments that are controllable, reproducible, and optimizable. The microfluidic technology, also called Lab-on-a-chip (LOC), or micro total analysis system (μTAS), has been widely used to study cell biology for biomedical applications [25,26] [27], including single-cell analysis [28-30], genetic assays [31,32], protein studies [33], intracellular signaling [28,34], multidrug resistance [35-37], drug toxicicity [38] [39], pathogen detection [33], cell culture [40,41], tissue engineering [42], and so on. The microfluidic technology has five significant features for 3D cell culture and cell-based assays: (1) Its micro-scale dimensions are compatible with those of many microstructures and environments native to *in vivo* systems. For example, the mean free path length between adjacent capillaries in many *in vivo* animal tissue models is in the micro-scale region (e.g. 24 μm for rat heart capillaries [43]). (2) Microfluidic devices can readily create complex dynamic micro-scale environments to mimic 3D *in vivo* environments, such as a complex chemical gradient. (3) It requires only a small amount of samples, and the reagent consumption is low, which significantly reduces costs in bioanalysis, drug discovery and development; (4) Some substrates like polydimethylsiloxane (PDMS) used in microfluidic devices are permeable to O_2, an important factor influencing cell proliferation; and (5) microfluidic technology can integrate multiple steps such as cell culture, cell sampling, fluid control, cell capture, cell lysis, mixing, and detection on a single device. Microfluidics provides a versatile platform for 3D cell culture and subsequent, more complicated cell-based assays. For example, the work of Jang *et al*, which combined microfluidics with 3D cell culture and subsequent bioassays, has shown that MC3T3-E1 cells cultured inside a 3D continuous-perfusion microfluidic channel allowed shear-stress induced osteoblast differentiation with dramatic increase (10 fold increase) in alklaline phosphatase (ALP) activity, an enzyme marker for osteoblasts, compared to 2D microarray cultured cells [13]. For an ideal 3D cell culture system, continuous nutrition and oxygen supply, and waste removal through the culture medium, must be ensured. The micro-environment provided by microfluidic systems should be able to mimic the one *in vivo*.

In this paper we review the advances of microfluidic technology to include various platforms and supporting matrices for 3D cell culture and its versatile bio-applications reported since 2006, and further highlight its potential applications in tissue-based bioassays.

Microfluidic platforms for 3D cell culture

Different microfluidic platforms have been used in 3D cell culture. We can categorize them as glass/silicon-based, polymer-based, and paper-based platforms, based on the substrates used for microdevice fabrication. Although the fabrication methods for these different platforms vary due to the difference in properties of the substrates, most fabrication processes are based on photolithography, initially developed in IC (i.e. integrated circuit)

microfabrication, which typically includes standard RCA cleaning, thin-film deposition, photolithography, wet HF etching, access hole forming, and chip bonding, as delineated in a book chapter in *Ewing's Analytical Instrumentation Handbook* and the book by Paul Li [44,45]. These two references also cover other microfluidic fundamentals, including flow control, concentration gradient generation and so on.

Glass/ Silicon-based platforms

Although silicon is rarely used as the substrate of microfluidic devices for 3D cell culture due to its complicated and costly fabrication process [46], the microfabrication process initially used in standard Si microfabrication was adapted in glass micromachining and mold mask fabrication for polymeric material-based microchips. For example, Ling *et al* microfabricated an SU-8 mold mask on a silicon wafer for casting an agarose-based microfluidic device, and demonstrated the importance of a perfused microchannel network for delivering nutrients and oxygen to maintain cell viability in large hydrogels [47].

Although PDMS devices are now the predominant form of 3D cell culture platforms, glass devices typically offer better optical properties useful in high-resolution fluorescence microscopy, since some polymeric materials have intrinsic fluorescence. In addition, glass-based devices have advantages for long-term monitoring and repeated experiments, because glass can provide a well-defined surface and a stable and reproducible electroosmotic flow. Jang *et al* fabricated a 3D continuous-perfusion microchip system with Tempax glass by photolithographic wet etching to culture osteoblasts, and this system was further applied for long-time drug screening (e.g., 10 days) using fluorescence microscope and thermal lens microscope (TLM) [13]. Ziolkowska *et al* presented a hybrid (PDMS/glass) microfluidic cell culture system (MCCS) integrated with a concentration gradient generator for cytotoxicity tests and cell's passaging [48]. This device is reusable, and can be used several times for cell culture and cytotoxic experiments. Recently, Lin *et al* reported an integrated microfluidic perfusion cell culture system which consisted of an indium tin oxide (ITO) glass-based microheater chip for micro-scale perfusion cell culture [49]. The use of transparent ITO glass enables not only heating on chip, but also real-time microscopic observation [49].

Since glass is impermeable to oxygen, this property has been used to create hypoxic environment for oxygen related cellular studies [50]. For instance, Webster *et al* reported a glass microfluidic device for tissue biopsy culture [50]. The response of normal colorectal tissue and neoplastic biopsies to hypoxia was assayed by measuring the release of vascular endothelial growth factor (VEGF) using this system.

Polymer-based platforms

The ease of soft lithography technique pioneered by George Whitesides *et al* [51], its permeability to oxygen, and low cost associated with polydimethylsiloxane (PDMS) and its fabrication, have made PDMS-based microfluidic devices the dominant microfluidic cell culture platform. Other polymeric materials, such as poly (methyl methacrylate) (PMMA), polycarbonate (PC) and polystyrene (PS) are also biocompatible materials for microdevice substrates [52,53]. Over the recent decades, numerous polymer-based devices have been designed and fabricated for microfluidic cell cultures, with different structural features, such as microchannels [5,13,19,47,54-58], micro-wells [3,11,59], micropillars [10,14,17,18,20,60-63] and cell retention chambers. Most of them were designed to optimize medium flow and oxygen perfusion throughout the cell culture and diminish the effect of medium-starved necrotic regions. The ease of design and fabrication of micro-scale features such as microfluidic channels and micropillars through the use of PDMS injection-molding

has allowed the harnessing of these features for dynamically-perfused 3D culture. In contrast to static 2D culture, microchannels allow the perfusion of cell culture medium throughout the cell culture during *in vitro* studies and as such offer a more *in vivo*-like physical environment. Generally, an inlet port allows injection of cell culture medium into a cell-laden microfluidic channel or chamber, thus delivering nutrients and oxygen to cells. An outlet port then permits the exit of remaining medium as well as harmful metabolic by-products. Figure 1 shows a PDMS-based microfluidic perfusion system for 3D cell culture [14]. Hydrodynamic parameters such as shear stress induced differentiation [13] and perfusion flow direction effect on capillary morphogenesis [18] in microfluidic channels have been documented.

Several devices have integrated medium-infused channels with micro-well features for cell culture [11] [3] [59]. In one work, a PDMS micro-bioreactor array (MBA) with twelve micro-wells was designed for culturing cells that were either attached to substrates (2D) or encapsulated in hydrogels (3D) [64]. The design of micro-well array has afforded the capability to spatially and temporally investigate many factors that regulate cell differentiation [64].

In addition to PDMS devices, natural polymers such as collagen, fibrin and agarose have been used to fabricate cell-laden microfluidic devices which provide an *in vivo*-like environment resembling living tissues. Ling *et al* have fabricated 1 cm thick cell-laden agarose replica molds by cooling a hot solution of agarose to 70 °C before mixing with a cell suspension and then pouring the agarose-cell mixture onto a silicon master for gellation [47]. Figure 2 shows the fabrication process of agrose-based microfluidic devices, based on soft lithography. This method allowed the perfusion of cell culture medium through the cell-laden agarose hydrogel device via a microchannel encased inside the hydrogel. Murine hepatocyte cell line AML12 was modeled in this construct, demonstrating the viability of natural extracellular matrix molecules such as agarose as a material for 3D cell culture devices.

Paper-based platforms

Because the microfabrication of Si/glass-based and polymer-based platforms often requires specialized engineering approaches and instrumentation not available in most biology laboratories, the Whitesides group initiated a simpler and inexpensive paper-based platform for 3D cell culture [40] [65].

Hydrophobic barriers were first patterned on individual layers of chromatographic paper by wax printing. Multiple layers of paper impregnated with suspensions of cells in extracellular matrix hydrogel (e.g., Matrigel) were then stacked to mimic the 3D oxygen and nutrient-gradient architecture *in vivo* (Figure 3) [40]. This stacked paper-based platform can be destacked, affording unique layer-by-layer molecular analysis after 3D cell culture. Culturing several carcinoma cell lines (e.g., MDA-MB-231), as well as endothelial cell lines was used to validate different cell proliferation profiles in oxygen and nutrient-gradient environment [40].

In more recent work from the same group, a 96 3D multi-layer zone array was fabricated by patterning 96 hydrophilic zones on layers of Whatman filter paper #114, spotting these zones with cell suspension and then stacking the layers to construct the paper-based device [65]. The characteristics of this stacked paper-based 3D culture were tested against various cell-based assays to analyze the 3D cell migration of MDA- MB-231 cells and provide a comparison of regulation of cell density, generation of complex gradients, and cell

proliferation in paper-supported multi-layered cultures versus the multicellular spheroid model.

Supporting matrices for 3D cell culture in microfluidic devices

Supporting matrix (also called scaffold) for 3D cell culture is also an important factor to be considered in 3D cell culture. Supporting matrices (e.g., collagen gel) used in traditional 3D cell cultures are adapted in many applications of microfluidic 3D cell culture. The microfluidic technology also enables gel-free 3D cell culture in microdevices. Hereafter we refer to "gel-supported" cultures as those where cells are embedded directly into hydrogel matrices, and "gel-free" as those cultures that do not require gels at all, or that use gels only as a coating material for initial cell adherence to various micro-structures.

Gel-supported 3D cell culture

Development of robust 3D tissue analogs *in vitro* is commonly limited by passive, diffusive mass transport. Perfused microfluidic-engineered tissue scaffolds hold the promise of eradicating mass transport limitations and promoting the development of complex and clinically relevant tissues. The use of hydrogels as scaffolding materials allows the encapsulation of cells into the hydrogel while also permitting diffusive permeability to oxygen and mass transport of nutrients to encapsulated cells. In an effort to recreate the *in vivo* microenvironment, a number of gel-supported 3D cell cultures in microfluidic devices have employed native extracellular matrix proteins as the basis of hydrogel scaffolding such as collagen, fibrin, hyaluronic acid (HA), matrigel, fibronectin, agarose, poly(ethylene glycol) diacrylate (PEGDA), and a mixture of both, as summarized in Table 1. For instance, the combination of different hydrogel types composed of collagen and HA has allowed the study of endothelial cell sprouting and migration in response to a VEGF (a growth factor) gradient controlled by microfluidic channels during *in vitro* mimicking of sprouting angiogenesis [66]. Such a composite hydrogel showed an improvement in the adhesion, migration and proliferation of human umbilical vein endothelial cells (HUVECs) [66].

Further efforts have been embarked to optimize the structural characteristics of hydrogel matrices and to create methods for producing more reproducible parameters such as pore size, fiber thickness, matrix gradients and cell seeding capabilities [16] [12] [19] [66]. Sung *et al* showed that polymerization of collagen matrix inside microchannels could be controlled by varying the pH and pre-incubation temperatures to yield thinner or thicker collagen fibers [16]. In the same study it was found that human mammary fibroblast (HMF) cells cultured in thicker collagen fibers presented more actin stress fibers and enhanced cell viability, noting that well-controlled conditions for collagen matrix polymerization were necessary for reproducible HMF cell culture.

In addition, the microfluidic technology enables the production of various shapes, and dimensions of scaffold to meet different needs in 3D cell culture. The work of Golden *et al* has detailed the fabrication of collagen and fibrin microfluidic gels with channel networks inside, which allows for rapid mass transport in bulk gels [12]. This was achieved by micromolding of gelatin meshes in PDMS microfluidic networks, followed by encapsulation in collagen, matrigel and fibrinogen gel precursors, and subsequent flushing of gelatin meshes by heating and washing. Regarding the scaffold shape, Hwang *et al* developed a method to produce poly (lactic-co-glycolic acid) (PLGA) microfiber-shaped scaffolds within a PDMS microfluidic chip for tissue engineering [67].

Lastly, laminar flow in microfluidic channels has been used to create multiple hydrogel layers for 3D cell culture. Kunze *et al* presented a 3D neural culture in a multi-layered agarose-alginate scaffold formed by four parallel inlet channels in a PDMS microfluidic

device. Thus, microfluidic technology created physiologically 'realistic' neural cell layers, because cortical neurons were organized in six different cell layers in their native state [56].

Gel-free 3D cell culture

Although the use of hydrogels as scaffold for 3D cell cultures has been used for many applications and affords many advantages, cell-laden hydrogel cultures do possess some limitations. Natural hydrogels vary in their properties and composition, and the limited transport of nutrients and oxygen across thick and dense hydrogels can impede the viability of cells cultured within them. Furthermore, the use of hydrogels to culture certain types of cells that proliferate under high cell densities and low ECM ratios, such as multi-cellular tumor spheroids (MCTS), might not be the most suitable approach [10]. As such, several strategies in microfluidics have been considered to successfully create 3D culture that does not rely on gels as an embedding material.

The use of the inter-cellular polymeric linker, polyethylenimine-hydrazide (PEI-hy), has been reported for the growth of a human adenocarcinoma cell line [10]. By modifying the sialic acid groups on cell-surface glycoproteins with sodium periodate ($NaIO_4$), the modified aldehydes were able to react with hydrazides on the inter-cellular linker resulting in cell aggregation on contact without the use of hydrogels.

Micro-wells have also been used to create 3D culture that does not depend on hydrogels [11]. By allowing cells to be perfused from the bottom of a polycarbonate mesh micro-well bottom, 3D cultures were regulated by the amount of medium that was able to flow upward through the culture wells, leading to a maximum culture thickness of 300 μm [11].

The microfluidic technology also enables gel-free culture of spheroids, an *in vitro* tumor model. In another study, the gravity-driven introduction and incubation of a suspension of PC-3 growth media and ascorbic acid into the microfluidic device prior to cell seeding, allowed for uniform seeding and ascorbate-induced expression of osteoblast-specific markers that were responsible for the mineralization of the matrix [5]. This design allowed the gel- free formation of uniformly-sized spheroids in a 2-layered PDMS device to model 3D metastatic prostate cancer (Figure 4), with co-cultures of cell types that naturally surround the bone microenvironment *in vivo*. A modified hanging drop method for culturing tumor spheroids inside PDMS microbubbles has also been reported [4]. For this scheme, a method for the fabrication of PDMS microbubbles in the size range of 200-500 μm was developed using gas expansion molding (GEM). Cell capture inside the microbubbles was achieved by incubation of PDMS surface with recombinant E-selectin/Fc Chimera in PBS prior to cell seeding, resulting in E-selectin interactions between E-selectin counter ligands on cell surfaces and pre-treated PDMS devices. Colo 205 spheroids cultured inside the PDMS microbubble array were able to remain viable for up to 5 days and were employed in further Doxorubicin toxicity studies [4].

New techniques for 3D cultures that use a combination of sorting and in situ assembling techniques have also been accounted [63]. Schütte *et al* reported the use of dielectrophoresis (DEP) for cell sorting and assembly of sinusoid-like 3D co-cultures of human primary hepatocytes and endothelial cells [63]. Only living cells were effectively guided by dielectrophoretic forces into cell-assembly gaps preconditioned with collagen, thereby ensuring only the most viable cell candidates were assembled into sinusoid-like cultures.

Lastly, although micro-pillar bounded microchannels have been used for cell capture and cell culture with the aid of hydrogels [60] [61] [18] [21], the incorporation of polyelectrolyte complex coacervation has allowed cell immobilization without the use of hydrogels. Some work have reported the use of polyelectrolyte complex coacervation or fibronectin coating

prior to cell seeding in combination with micropillar cell capture and immobilization to culture a myriad of cell types in 3D [60] [14] [17] [62] [20] [21]. For instance, work by Choi *et al* reported the use of micropillars to capture, immobilize and culture neurosphere aggregates derived from human adipose tissue-derived stem cells (hATSCs) by pre-treatment with fibronectin [20].

Applications

Utilizing the microfluidic technology discussed above, numerous microfluidic systems have been used in different cell-based and tissue-based applications. Most applications are centered on cell biology, drug discovery, toxicological studies, and tissue engineering. A summary of applications for individual cell types is listed in Figure 5.

Cell type & related cell-based applications

Different cell-based applications based on microfluidic 3D cell culture are listed and categorized by cell type in Table 2. Details are described in the following section.

Stem cells

Stem cells can now be artificially grown and transformed (differentiated) into specialized cell types with characteristics consistent with cells of various tissues, such as muscles or nerves, through cell culture. Embryonic cell lines and autologous embryonic stem cells generated through therapeutic cloning have also been proposed as promising candidates for future therapies [73]. Researchers believe that stem cells hold the key to combat incurable diseases to date.

Conventional static cell cultures are not amenable to precise control over stem cell differentiation. On the contrary, microfluidics has significant advantages in the control of complex stimuli for stem cell differentiation. Yu *et al* developed a 3D microfluidic channel-based cell culture system (3D-μFCCS) that allowed cells to be perfusion-cultured in 3D, and applied this system to the study of the differentiation of rat bone marrow mesenchymal stem cells (BMSCs) *in vitro* [60]. This 3D-μFCCS system showed that microfluidics provided a more conducive microenvironment for BMSC differentiation than a 2D cell culture. Vunjak-Novakovic *et al* developed a co-culture system to study cell–cell interactions between human mescenchymal stem cells (MSCs) and HUVECs in a spatially controlled three-dimensional fibrin hydrogel model [55]. MSCs showed strong distance-dependent migration toward HUVECs, and supported the formation of stable vascular networks, providing a simple model to study the cell-cell communication relevant to engineered vascularized tissues. In a different study, Putnam *et al* described another 3D microfluidic device as a model system to study the molecular regulation of perivascular stem cell niches [18] (Figure 6). MSCs formed pericytic associations with the endothelial cells (ECs) but promoted capillary morphogenesis with distinct kinetics, due to the interaction between α6β1 integrin receptor of MSCs and EC-deposited laminin. Recently, Kang *et al* developed another gel-free 3D-μFCCS to study Wnt5a-mediating neurogenesis of human adipose tissue-derived stem cells (hATSCs). The low oxygen microenvironment provided by the 3D-μFCCS activated the Wnt5A/β-catenin signaling pathway in hATSCs and resulted in self renewal and trans-differentiation of hATSCs into neurons [20].

Cancer cells

Cancer is a wide-reaching cause of chronic and terminal human diseases. Cancer progression consists of cells invading local tissues and metastasizing to other parts of the body via blood stream or lymphatic system. Microfluidics, as one of the most promising

platforms to address the inherent complexity of cellular systems, presents great advantages in discovering the intricacies of cell-to-cell variability, drug-cell interactions and its relevance to cancer therapy. Liu *et al* designed a microfluidic 3D cell co-culture device to investigate the effect of Carcinoma-associated fibroblasts (CAFs) on cancer cell invasion in 3D matrix [81]. It was observed that CAFs promoted salivary gland adenoid cystic carcinoma (ACC) cell invasion in 3D matrix in a spheroid fashion, indicating that CAFs play a critical role in cancer invasion. Beebe *et al* reported a simple microfluidic 3D compartmentalized system to study the transition from ductal carcinoma in situ (DCIS) to invasive ductal carcinoma (IDC), a critical step in breast cancer progression [57]. Human mammary fibroblasts (HMFs) were co-cultured with epithelial cells (MCF-DCIS) in this system to promote a transition from DCIS to IDC *in vitro*.

In addition, anti-cancer drug screening in 3D microfluidic platforms has also attracted significant attention. Agastin *et al* [4] and Lee *et al* [3] developed different microfluidic array systems to culture tumor cells for drug screening and toxicity testing. Although multi-cellular tumor spheroids (MCTSs) have been established as a 3D physiologically relevant tumor model for drug testing in cancer research, it is difficult to control the MCTS testing parameters and the entire process is time-consuming and expensive. In the work from Agastin *et al*, a PDMS microbubble array was developed to overcome these limitations in tumor spheroid culture. The toxicity of the anti-cancer drug, doxorubicin, on Colo 205 cells in spheroids was tested to validate this system for drug screening purposes. Colo 205 spheroids cultured in flow showed a threefold increase in resistance to doxorubicin, compared to Colo 205 monolayer cells cultured under static conditions [4].

Liver cells

For cell-based assays, there has been very active research on liver cell culture facilitated by microfluidics due to an increasing demand for valuable *in vitro* tools for hepatoxicity assays that meet the challenge of maintaining hepatocyte phenotypes *in vitro*. Liver culture models can provide a basis for understanding liver diseases and drug metabolism. Yu *et al* developed a microfluidic 3D hepatocyte chip (3D HepaTox Chip) for *in vitro* drug toxicity testing, and demonstrated *in vitro* hepatotoxicity testing with the potential to predict *in vivo* toxicity [14,60]. Leclerc *et al* established a method and a protocol for performing a toxicogenomic analysis of HepG2/C3A cultivated in a microfluidic biochip. The research results provided new insight into the use of microfluidic biochips as new tools in toxicity and mechanistic research in pharmaceutical drug studies and predictive toxicity investigations [74] [21]. Some other applications of liver cell culture using microfluidics will be discussed in the following section of tissue-based applications, due to their proximity to liver ‘tissues’.

Neural cells

Neural cells are important in the studies of signal transduction and neurological disorders (e.g., Alzheimer's disease), and have potential applications as drug testing biosensors due to their specific binding profiles with drugs and toxins. To study electrophysiological properties of neural cells, Wheeler *et al* have demonstrated the design, fabrication, packaging, characterization, and functionality of an electrically and fluidically active 3D micro-electrode array (3D MEA) for neuronal cell cultures [6]. The 3D MEA consists of a stack of individually patterned thin films that form a cell chamber conducive to maintaining and recording the electrical activity (through Au electrodes) of a 3D network of rat cortical neurons. Cells have been shown to survive in the 3D MEA for four weeks, and the influence of the drug tetrotodoxin on the activity of the culture was tested as well [6]. Kanagasabapathi *et al* developed another planar micro-electrode array structure integrated

with a dual-compartment neurofluidic system, with inter-connecting microchannels to connect neurons from their respective compartments. Cross-correlation based techniques showed that the two neuronal populations were functionally connected with each compartment [78]. Furthermore, rat primary cortical neurons were cultured successfully for up to three weeks in the micropatterned multi-layered scaffold [71]; and it was also observed that B27--a well-known media supplement to stimulate axonal growth of neurons--concentration gradient enhanced neurite outgrowth.

Brain slices are well-established models for a wide spectrum of *in vitro* investigations in neuroscience. Nevertheless, thick brain slice cultures suffer necrosis. Potter *et al* reported an interstitial microfluidic perfusion technique to culture thick (up to 700 μm) organotypic brain slices. They designed a micro-perfusion chamber to facilitate laminar, interstitial perfusion of oxygenated nutrient medium throughout the tissue [76]. The method gave approximately 84% viability of functionally active tissue after 5 days *in vitro*, meanwhile preserving *in vivo* cytoarchitecture.

Cardiac Cells

Cardiac cells may become the most important cells for new discoveries in patient cardiac treatment, for improvements patient heart function and for the general treatment of heart diseases. Vunjak-Novakovic *et al* designed a simple and practical system that coupled a microfluidic platform with an array of micro-bioreactors to explore the cultivation of primary neonatal rat cardiac myocytes. Coating the chamber surfaces with collagen enabled myocytes to form spatially uniform layers. Cell viability, morphology and phenotype were well maintained after four days of cultivation. Spontaneous macroscopic contractions demonstrated the myocytes were functionally active throughout the culture period [64]. Wan *et al* developed a different PDMS microfluidic device to investigate the differentiation of murine embryonic stem cells into cardiomyocytes [68]. Their study demonstrated that the microfluidic system contributed to the enhanced cardiomyogenic differentiation compared with conventional well-plates.

Other cells

Other cells [12,16,18,19,54,59,61,79,80], such as human dermal fibroblasts, human mammary fibroblasts (HMFs), human dermal microvascular endothelial cells (HDMECs), A549 cells--human lung epithelial cell line, and so on, have also been studied in microfluidic 3D culture, as listed in Table 2.

Organ-on-a-chip: engineered tissue-based applications

Tissue engineering is an exciting field focused on developing tissues or organ substitutes that restore, maintain, or improve tissue function or a whole organ. Microtechnologies are emerging as powerful tools for tissue engineering. Recently, organ-on-a-chip systems are attracting significant attention [82].

Vukasinovic *et al* developed a microfluidic perfusion platform that allows reproducible culturing of tissue equivalents within dynamically controlled environments. This platform had potential applications in regenerative medicine [70]. Gottwald *et al* described a chip-based platform for the *in vitro* generation of tissues in three-dimensional organization [11]. Primary and established cell lines (Hep G2, C3A, primary rat hepatocytes, mouse embryonic cells P19, and embryonic stem cells of line R1) have been successfully cultivated and analyzed for functional properties. Furthermore, Whitesides and his co-workers combined the advantages of microfluidic systems with the versatility of modular tissue engineering [83], and developed a chip-based system to create an artificial “tissue construct” [84].

PDMS was used to mold and assemble different pieces (modules) of cell-containing gels in a microfluidic channel for 3D cell culture. This system allows for the construction of multiple cell type 'modular tissue' at high cell density (10^8 –10^9 cells / cm^3). Based on similar concept of modular assembly, other work has been reported to pattern cells for 3D cell culture [85] [86] [87].

Due to the importance of liver, several microsystems have reported culturing of liver tissues for functionality and heptoxicity tests. Stelzle *et al* developed an organ-like liver 3D co-culture of hepatocytes and endothelial cells based microfluidic system for liver toxicity testing and liver sinusoids assembly [63]. Additionally, Hattersley *et al* described a microfluidic approach to study the viability and functionality of rat explant liver tissue. Their work successfully exploited the benefits of the microfluidic environment to create pseudo *in vivo* conditions *in vitro* and applied it to an investigation of ethanol hepatoxicity [75].

An Organ-on-a-Chip is a 3D microfluidic cell culture system that simulates the activities, mechanics and physiological responses of entire organs or/and organ systems. It constitutes the subject matter of significant biomedical engineering research. The convergence of Lab-on-Chips and cell biology has permitted the study of human physiology in an organ-specific context, introducing a novel model of *in vitro* multicellular human organisms. The Ingber group at Wyss Institute reported a biomimetic microsystem that reconstitutes the critical functional alveolar-capillary interface of the human lung–'Lung-on-a-Chip' [88]. The issue of tissue interface of the alveolus is replicated in the microdevice by co-culturing human alveolar epithelial cells and pulmonary capillary endothelial cells on the opposite sides of a thin, flexible, porous and ECM-coated PDMS membrane. It reproduces complex integrated organ-level responses to bacteria and inflammatory cytokines induced into the alveolar space. The nanotoxicology from silica nanoparticles were also studied using this system and it revealed that cyclic mechanical strain accentuates toxic and inflammatory responses of the lung to nanoparticles. Lung-on-a-chip system provides a low-cost alternative to animal and clinical studies for drug screening and toxicology applications. As shown in Figure 7, Yu *et al* developed a multi-channel 3D-μFCCS with compartmentalized microenvironments to mimic four human organs: C3A (liver), A549 (lung), HK-2 (kidney) and HPA (fat), and the result demonstrated the compartmental isolation between different cell types similar to the *in vivo* situation [62].

Future Perspective

Although microfluidic 3D cell culture provides great potential for biomedical applications and tissue engineering, it also faces many challenges. For example, access to cultivated cells in microsystems is tough and sampling from microsystems for further assays is often complicated. One great challenge for microfluidic 3D cell culture devices might be development of methods and devices dedicated to *in vivo*-like cell metabolism and functions study, and drug discovery. Biology laboratories usually lack microfabrication instrumentation, commercialization of such mature and easy-to-use microfluidic devices to make them available to biologists is also a great challenge. Biologists usually expect high-throughput assay tools that can provide high reproducibility, but microfluidics often meet technical problems to meet such needs from biologist, though parallel assays could be possible.

In the near future, we expect new research in microfluidic 3D cell culture to extend in two directions. The first one should be the formulation of cost-effective and easy-to-use microfluidic 3D cell culture systems. Although numerous 3D cell culture systems have been developed since the 1990s, this technique is still not a widely-utilized practice primarily for

two reasons and the aforementioned challenges: 1) the high cost of currently required fabrication protocol; 2) degree of complication in executing its practice. The second direction should be the integration of complicated microsystems that can closely mimic *in vivo* conditions. Furthermore, it is expected that advances in microfluidics cell culturing technique will propel the new developments and discoveries in tissue engineering. As such, the concept of "organ-on-a-chip" and "human-on-a- chip" will attract more attention in the near future. Some of such systems could be used as an inexpensive alternative to animal model and clinical testing for drug discovery and development.

Acknowledgments

Financial support from the University of Texas at El Paso and UT System for the STARS award is gratefully acknowledged. XiuJun Li is an Assistant Professor at the University of Texas at El Paso, and interested in microfluidics for biomedical assays and tissue engineering.

Key Terms

Carcinoma-associated fibroblast (CAF)	a key determinant in the malignant progression of cancer that represents an important target for cancer therapies.
PDMS	polydimethylsiloxane, a polymeric substrate for microfluidic devices.
Soft lithography	one technique for fabricating elastomeric material-based devices using molds. It is commonly used in PDMS microdevice fabrication.
Organ-on-chip	microsystems with integrated 3D cell cultures that can mimic physiological and mechanical activities and functions of organs *in vivo*.
Photolithography	one essential process in microfabrication. It uses light (usually UV light) to transfer a geometric pattern from a photomask to a photoresist layer on the substrate.
α-SMA	alpha smooth muscle actin. It is the predominate isoform in thin filaments that form part of the contractile machinery in smooth muscle.
Angiogenesis	the physiological process involving the growth of new blood vessels. It is a normal and vital process in growth and development, as well as in wound healing. However, it is also a fundamental step in the transition of tumors from a dormant state to a malignant one.

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**. Demonstration of "Lung-on-a-chip".

Executive summary

Introduction

- Traditional 2D monolayer cell culture cannot accurately recapitulate the structure, function and physiology of living tissues.
- 3D cell culture gained momentum when converging with the microfluidic technology. The microfluidic 3D cell culture provides a powerful tool to mimic highly complex and dynamic *in vivo* environment.

Microfluidic platforms for 3D cell culture

- Microfluidic perfusion 3D cell culture can continuously provide nutrients and oxygen for cell proliferation, thus diminishing the effect of medium-starved necrotic regions.
- Although different glass-based, polymeric material-based, and paper-based microfluidic platforms have been developed for 3D cell culture, PDMS-based microdevices are the dominant form of microfluidic 3D cell culture platforms, due to its ease of fabrication process and low cost.

Supporting matrices for 3D cell culture in microfluidic devices

- Supporting matrices (e.g. collagen gel) used in traditional 3D cell cultures are adapted in many applications of microfluidic 3D cell culture. Meanwhile, several microfluidic strategies have been reported to create gel-free 3D cell cultures, which overcome some limitations in gel-supported systems, such as limited oxygen and nutrient transport across thick and dense hydrogels.

Applications

- Multiple cell types (e.g., stem cells) have been successfully cultivated or co-cultured in microfluidic systems in many biologically applications, such as cell biology, drug discovery, and tissue engineering.
- • Microfluidic 3D cell culture has great potential in tissue-based applications, such as the emerging organ-on-a-chip system. Organ-on-a-chip could provide a low-cost alternative to animal model for drug discovery and development.

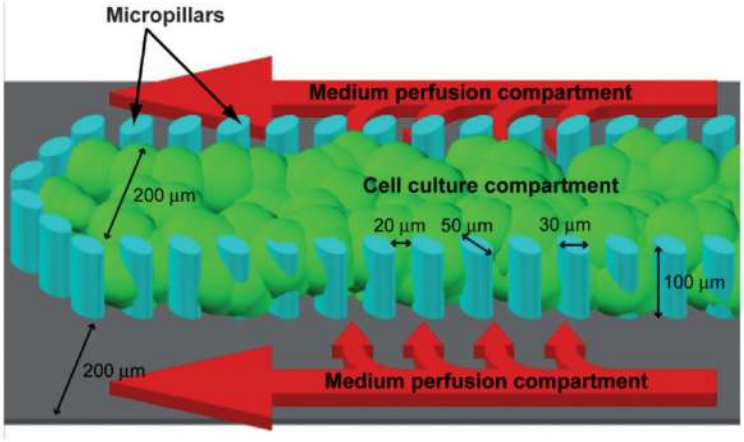

Figure 1.

A microfluidic perfusion 3D cell culture system using micropillars to separate cells with perfusion medium. Cells are perfused with cell culture medium through gaps between micropillars [14]. Reprinted with permission from the Royal Society of Chemistry.

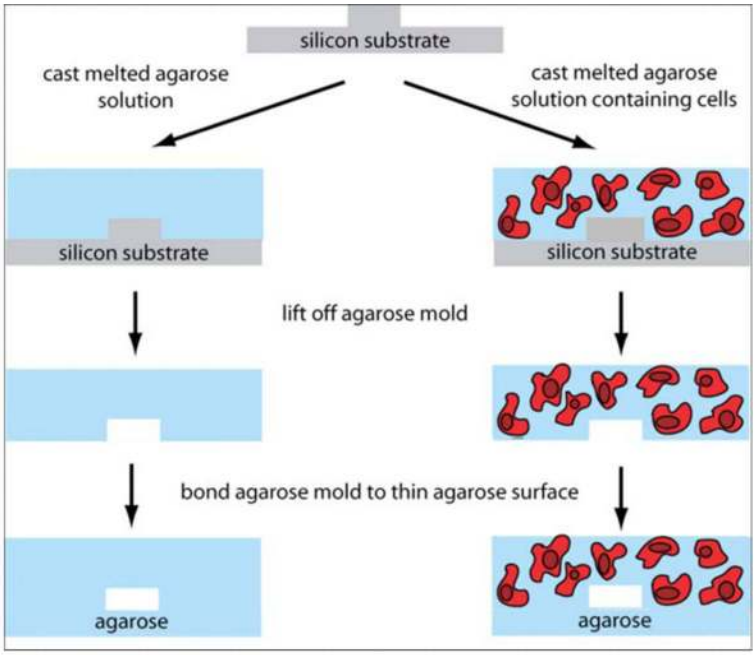

Figure 2.
Schematic of the fabrication of cell-laden agarose-based microfluidic devices without (left) and with (right) embedded cells. Standard soft lithography technique was used to mold molten agarose on a SU-8 patterned silicon wafer. Two agarose substrates were then thermally bonded together [47]. Reprinted with permission from the Royal Society of Chemistry.

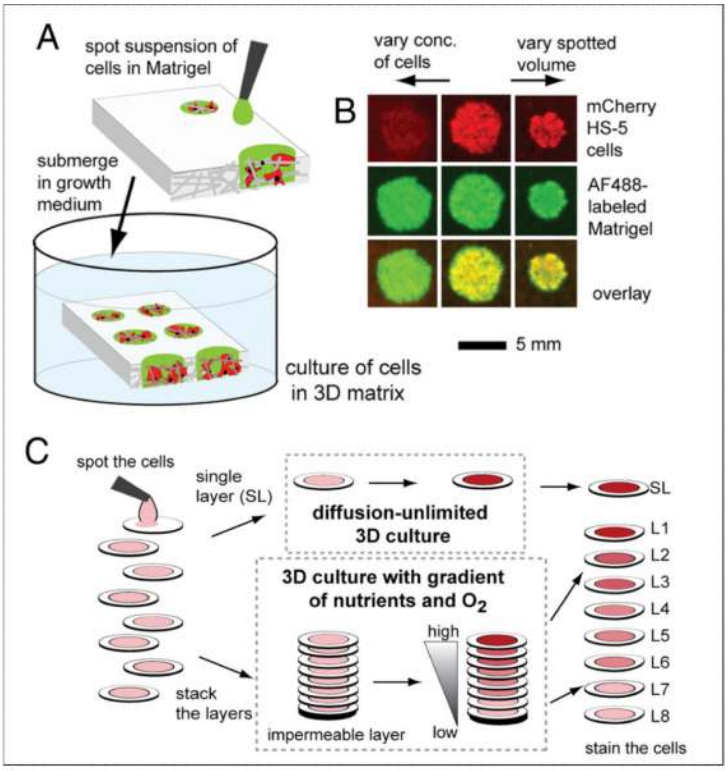

Figure 3.

Paper-based 3D cell culture. (A) Schematic of the generation of paper-based 3D cell culture on a single layer of paper. Chromatography or filter paper is permeated with Matrigel or other hydrogel precursor and cell suspension by spotting, producing cell-laden hydrogels of thickness equal to the paper substrate. (B) Fluorescence images of cells cultured in paper. (C) Schematic illustration of stacking and destacking of multiple layers of paper impregnated with cells for the study of gradient-dependent 3D cell culture [40]. Reprinted with permission from National Academy of Sciences.

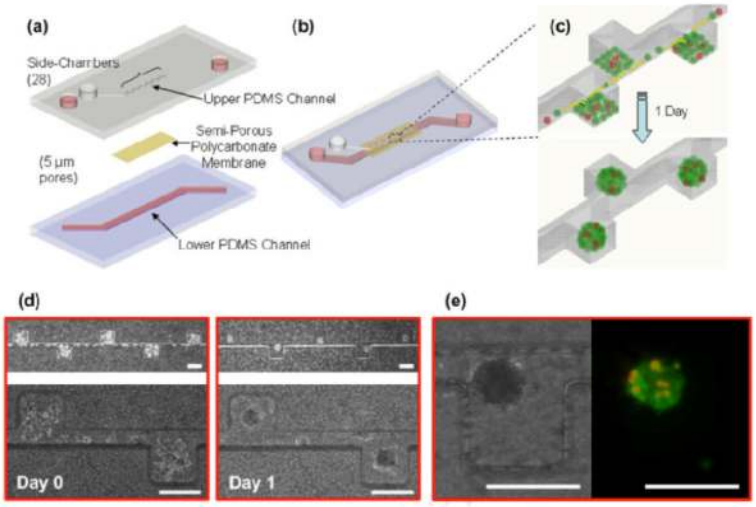

Figure 4.

(a-c) Schematic of microfluidic spheroid formation in a two-layer microfluidic PDMS device. Top layer consisted of dead end channels with 28 side-chambers for cell capture and spheroid culture formation, while the bottom layer allowed the flow of medium through the channel. (d-e) Actual time-lapse images of PC-3^{DsRed} co-culture spheroid formation within microchannels [5]. Reprinted with permission from *Biomaterials*.

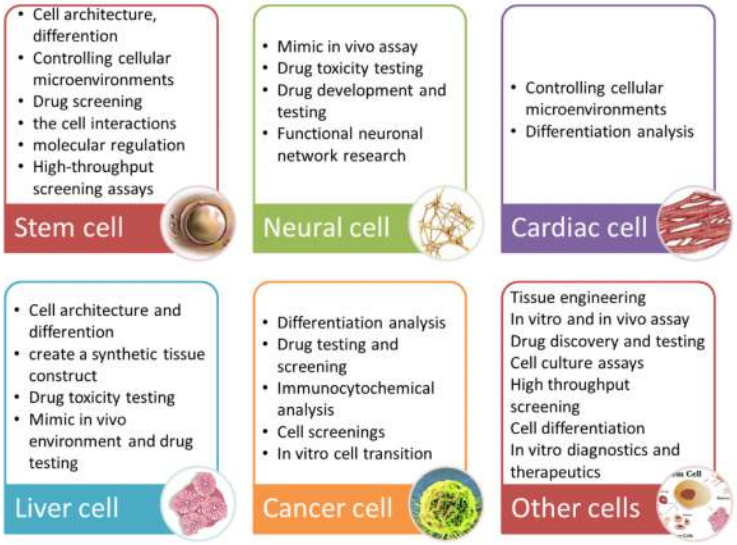

Figure 5.
Summarized applications of microfluidic 3D cell culture according to cell type

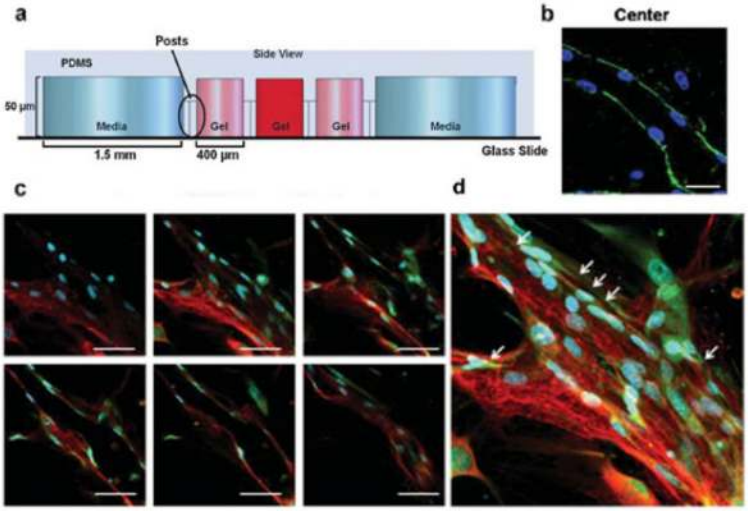

Figure 6.

Perivascular association between HUVEC and MSC in co-culture (b-d) inside a micropillar-bound microchannel (a). Formation of capillary networks inside microfluidic channel was observed by confocal microscope imaging. Green indicates presence of laminin in (b); MSCs stained for α-SMA staining (green) lie in close proximity to the newly deposited basement membrane (laminnin, red) in (c-d), and all nuclei were stained with DAPI, blue [18]. Reprinted with permission from *Biotechnology and Bioengineering*.

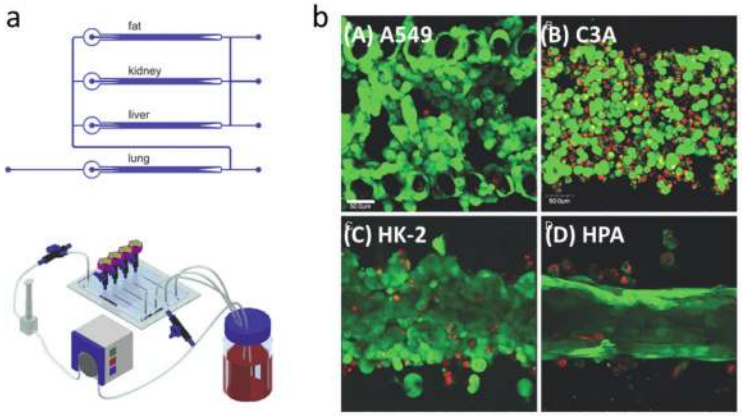

Figure 7.
3D microfluidic cell culture system for mimicking four human organs [62]. (a) Microfluidic system set-up. (b) Evaluation of cell viability. Reprinted with permission from the Royal Society of Chemistry.

Table 1

3D cell culture supporting systems in microfluidics

3D Technique	Cell Line	Year	Reference
Gel-supported			
Collagen	Human dermal microvascular endothelial cells (HDMECs)	2007	[12]
	Human adult dermal microvascular endothelial cells (HMVEC-ad)	2008	[61]
	Human mammary fibroblasts (HMF)	2009	[16] [57]
	Human mammary epithelial cell line	2011	[59]
	MCF-DCIS		[57]
	Murine embryonic stem cell line CGR8	2011	
	Human breast carcinoma cell line T47D	2011	[68]
	Mouse melanoma cell line EMT6	2011	[59]
	Mouse mammary carcinoma cell line EMT6	2011	[58]
Fibrin	Human dermal fibroblasts	2007	[12]
	Human mesenchymal stem cells (MSCs)	2009	[55]
	Human umbilical vein endothelial cells (HUVECs)	2010	[18]
Hyaluronic acid (HA)	Murine C3H myoblast cell line C2C12	2007	[64]
	Non-differentiated (hESC) human embryonic stem cell lines H9 and H14		
Matrigel	BAC1.2F5 and LADMAC cell lines	2008	[69]
	Mouse embryonic fibroblast cell line NIH 3T3	2008	[54]
	Mouse embryonic osteoblastic cell line MC3T3-E1	2008	[13]
	Primary embryonic rat cortical neurons E-18	2009	[70]
	Primary P1 astrocytes	2009	[70]
	Human breast adenocarcinoma cell line MCF-7	2011	[3]
	Non-neoplastic human mammary epithelial cell line MCF-10A	2011	[3]
	Human breast adenocarcinoma cell line MDA-MB-231	2011	[65]
Fibronectin	Hepatocellular carcinoma cell line HepG2	2011	[21]
	Hepatocyte cell line C3A	2011	[21]
Agarose	Murine hepatocyte cell line AML12	2007	[47]
poly(ethylene glycol) diacrylate (PEGDA)	Mouse embryonic fibroblast cell line NIH 3T3	2010	[19]
Gel-mixtures	*Agarose-Alginate Gel Mixture*		
	Primary embryonic rat cortical neurons E-19	2010	[71] [56]
	Semi-interpenetrating network of HA and Collagen		
	Human umbilical vein endothelial cells	2011	[66]

3D Technique	Cell Line	Year	Reference
	(HUVECs)		
Gel-free			
Micro-wells	Hepatocellular carcinoma cell line HepG2	2007	[11]
	Primary rat Hepatocytes	2007	[11]
	Rat bone marrow mesenchymal stem cells (BMSCs)	2007	[11]
Inter-cellular polymeric linkers	*polyethyleneimine*-hydrazide (PEI-hy)		
	Human lung adenocarcinoma cell line A549	2008	[10]
	Hepatocyte cell line C3A	2008	[10]
	Bone marrow mesenchymal stem cells (BMSCs)	2008	[10]
Other	*Ascorbic acid-promoted mineralization of ECM*		
	Human prostate adenocarcinoma cell line PC-3	2009	[5]
	Magnetic-field-assisted electrostatic self-assembly		
	Human breast adenocarcinoma cell line MCF-7	2010	[72]
	Modified hanging-drop method in PDMS microbubbles		
	Human colon adenocarcinoma cell line Colo 205	2011	[4]
	Human breast adenocarcinoma cell line MDA-MB-231	2011	[4]
	Dielectrophoresis sorting and guided-assembly		
	Cryopreserved human Hepatocytes and endothelial cells	2011	[63]
	Fibronectin coating and micropillar immobilization		
	Human adipose tissue-derived stem cells (hATSCs)	2011	[20]

Table 2

Cell types and applications of microfluidic 3D cell culture

Cell type	Cell line	Application	Reference
Stem Cells	Rat Bone marrow mesenchymal stem cells (BMSCs)	Cell architecture, differentiation and drug screening	[10,60]
	Mouse embryonic stem (ES) cells of line R1	Differentiation analysis	[11]
	Human embryonic stem cells (hESCs) H9 and H13	Controlling cellular microenvironments	[64]
	Mouse osteoblastic cell line (MC3T3-E1)	Drug screening	[13]
	Human mesenchymal stem cell (MSC)	Study the cell interactions	[55]
	Human mesenchymal stem cells	study the molecular regulation	[18]
	Human adipose tissue-derived stem cells (hATSCs)	High-throughput screening assays	[20,59]
Cancer Cells	Human Carcinoma Cell lines, MCF7 human breast cancer cells	Cell architecture and differentiation	[3,60]
	Human Carcinoma cell lines	Differentiation analysis	[11]
	PC-3 prostate cancer cells	Drug testing	[5]
	Human breast adenocarcinoma cell line	Immunocytochemical analysis	[72]
	Colon cancer cell line and breast cancer cell line	Drug screening	[4]
	Human mammary epithelial cells	*In vitro* cell transition	[3]
	Human mammary fibroblast cells	Drug screening	[57]
	Human breast carcinoma T47D cell line	Cell screenings	[59]
	Mouse melanoma (B16.F10) and mouse mammary carcinoma (EMT6)	Control of bacterial, population and cancer treatment	[58]
	Human breast carcinoma cell line MDA-MB 231	Cell architecture, paper-based assays	[4,40,65]
	Mouse mammary carcinoma cell line EMT6	E. Coli proliferation *in vitro* tumor	[58]
Liver Cells	HepG2 human hepatocytes	Cell architecture and differentiation	[21,60,74]
	Primary rat hepatocytes	Cell architecture and differentiation	[60]
	AML-12 murine hepatocytes	Create a synthetic tissue construct	[47]
	Wistar rats Hepatocytes	Drug toxicity testing	[14,17]
	C3A liver cells	Mimic *in vivo* environment and drug testing	[10,21,62]
	Explant liver tissue from Wistar rat	Investigation of ethanol hepatoxicity	[75]
	HepG2 and C3a human liver hepatocellular carcinoma cell lines	Toxicity testing	[11,74]
	Cyropreserved human hepatocytes and endothelial cells	Drug toxicity testing	[63]
Cardiac Cells	C2C12 mouse myoblast cell line	Controlling cellular microenvironments	[64]
	primary rat cardiac myocytes from neonatal rat hearts	Controlling cellular microenvironments	[64]
	Embryonic stem cells differentiated into cardiomyocytes	Differentiation analysis	[68]
Neural Cells	Primary rat E-18 cortical neurons and P1 astrocytes	Mimic *in vivo* assay	[70]
	Thick brain slices	Mimic *in vivo* assay	[76]
	Embryonic day 18 rat cortical neurons	Drug toxicity testing	[6]
	Dissociated cortical neurons of embryonic rats	Drug development and testing	[71,77]
	Primary neuron dissociated cells, day-18 embryonic rat	Functional neuronal network research	[78]

Cell type	Cell line	Application	Reference
Other Cells	Human dermal fibroblasts and human dermal microvascular endothelial cells (HDMECs)	Tissue engineering	[12]
	Human adult dermal microvascular endothelial cells (HMVEC-ad)	*In vitro* assay	[61]
	A549 cells- human lung epithelial cell line	Mimic *in vivo* environment and drug testing	[10,62]
	NIH 3T3 mouse embryonic fibroblast cells and Madin-Darby Canine Kidney Epithelial Cells (MDCK Line)	Tissue engineering	[19,54,79]
	Human Foreskin Fibroblast (HFF)	Cell culture assays	[16,57,80]
	Human mammary fibroblasts (HMFs)	High throughput 3D cell screening	[16,59]
	Human umbilical vein endothelial cells (HUVEC)	Cell differentiation	[18,40,55,66]
	NIH 3T3 fibrobroblasts	*In vitro* diagnostics and therapeutics and tissue engineering and drug discovery	[19,79]