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Review Article

Recent Advances of Biologically Inspired 3D Microfluidic Hydrogel Cell Culture Systems

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Abstract

The application of hydrogels as a matrix for 3-dimensional cell cultures has become an indispensable tool in tissue engineering, biotechnology and biomedical research due to the improved functionality and viability of the *in vitro* biological system. The combination of 3-dimensional hydrogel cell cultures with microchip technology further allows (i) spatial and temporal control of cell growth, (ii) application of defined mechanical (e.g., shear, strain, stretch) and chemical (e.g., gradients) stimuli, as well as (iii) monitoring of dynamic cellular responses using integrated sensing strategies. The main advantage of hydrogels for microfluidic cell cultures, however, is their mimicry of extracellular matrix structures including adequate porosity for cellular organization, biocompatibility, and representative stiffness, all key parameters that promote native-like tissue function. This review focuses primarily on recent advances in biologically inspired microfluidic systems that are based on 3-dimensional hydrogel cell cultures and discusses advantages and current challenges, various applications of microfluidic hydrogel cell cultures and future perspectives.

Application of Microfluidics in Life Sciences

Microfluidics can be defined as the study of fluid and air flows in microchannels and was initially introduced to facilitate liquid handling and sample preparations. Early work dates back to 1969 with Lew's work on a theoretical solution for mimicking blood and air flow in a microcirculatory system of the lung [1]. In this precursor stage of microfluidics the aim was to create a biomimetic system, which facilitated the study of biological pathways *in vitro*. It was not until the 1990s that the field of microfluidics emerged from miniaturization efforts and Micro-Electro-Mechanical Systems (MEMS) as an enabling technology platform for dispensing systems, analytical separations, chemical reactions, and bioanalysis applications. Since then, microfluidics has evolved into an established technology ranging from medical solutions (e.g., microfluidic inhalers), *in vitro* diagnostics (e.g., point of care) and production applications (e.g., microreaction technologies) [2]. More recent research applications include microchips for genomics, proteomics and cell-based assays.

These microfluidic cell cultures are considered potential candidates to provide next generation cell analysis systems. Starting from single cell analysis using miniaturized flow cytometers [3] a variety of microfluidic devices have been developed for cell studies to investigate cell transport and cultivation in the absence and presence of concentration and temperature gradients or shear force conditions. The main benefit of microfluidic systems for cell culture analysis is that they can perform a number of crucial liquid handling steps including cell loading, nutrient supply and waste removal under physiologically relevant shear force conditions, all while offering real time microscopy [4]. Microfluidics also enables precise regulation of soluble factors including drug candidates, growth factors at specific solution concentrations and gradients, thus providing robust and reproducible measurement conditions. An alternative application of microfluidics for cell analysis is micropatterning to (a) optimize control of cellular behavior [5], (b) allow cell migration [6], (c) spatially resolve co-cultures systems [7] and (d) define cell repulsive and adhesive areas [8].

Despite recent achievements of microfluidic 2D cell culture systems [9], they still do not address the fact that *in vivo* cells coexist in 3D communities that are influenced by spatial orientation of cells and cell-to-cell contact within the extracellular matrix [10]. It has been repeatedly demonstrated that the presence of a 3D matrix promotes many biologically relevant functions otherwise not observed in 2D monolayer cell cultures [11]. Consequently a transition from 2D to 3D cell cultures 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 [12]; these non-microfluidic advances are further discussed elsewhere [13]. It has been shown that the chemical composition of the ECM is a crucial factor regarding cell shape, differentiation and interaction [14]. For example, 3D cell culture is particularly interesting for investigation of tissue cells normally situated within a dependent, functional Extracellular Matrix (ECM), such as chondrocytes [15]. While

cartilage ECM can be mimicked with biocompatible hydrogel, such as hyaluronic acid [16], with variable porosity and mechanical stiffness, 3D cultured chondrocytes also display a more native morphology and secrete ECM components. Moreover, when biodegradable scaffolds are used for 3D chondrocyte culture, native-like articular cartilage replaces the degraded hydrogel in a time dependent fashion [17].

In recent years, bioMEMS applications for hydrogels have been reviewed elsewhere [18] and a variety of microfluidic 3D cell culture platforms have been developed for recreating highly complex and well-controlled 3D microenvironments that mimic the biological niche [19]. In particular, culturing cells in hydrogels has shown to be useful in helping cells retain their native tissue-specific functions by mimicking the *in vivo* 3D tissue environment [20]. The combination of 3D-hydrogel cell cultures with microfluidics offers several advantages including (1) appropriate microscale dimensions that are comparable to *in vivo* microstructures; (2) establishment of chemical gradients to create dynamic 3D microenvironments; and (3) creation of reproducible medium-matrix biointerfaces, culminating in unprecedented temporal and spatial cellular control [21]. Fabrication of microfluidic devices [22] and microfluidics dedicated to cell culture have been reviewed previously [23]. Consequently the present review introduces recent advances and future applications of microfluidic 3D hydrogel cell cultures in the following sections.

3D-Hydrogels for Microfluidic Cell Culture Applications

Advantages and disadvantages of hydrogels for cell culture applications

Hydrogels are three-dimensional networks composed of various natural and synthetic polymers that retain water by swelling up to a percentage of 90%, thus mimicking the naturally surrounding of the extracellular matrix [24]. Hydrogels can be classified in two basic categories based on the origin of their composing polymer such as natural and synthetic monomers [25]. Natural or biological hydrogels used for microfluidic cell culture applications include agarose [26], chitosan [27], alginate [28], Hyaluronic Acid (HA) [29], collagen [30], dextran [31], fibrin [32], Matrigel [33], laminin [33], and silk fibroin [27]. In turn, synthetic hydrogels have been used in combination with microfluidics and are based on Poly-Ethylene Glycol (PEG) [34], Poly (Ethylene Glycol) Diacrylate (PEG-DA) [35], 2-Hydroxyethyl Methacrylate (2-HEMA) [36], Poly-2-Hydroxyethyl Methacrylate (PHEMA) [37], Poly-L-Lactic Acid (PLLA) [38], Poly-Lactic-co-Gly-colic Acid (PLGA) [39], Poly-Glycerol Sebacate (PGS) [40] and PuraMatrix™ [41], which is a fully synthetic peptide-based polymer. Among these, the most extensively employed hydrogel for microfluidic cell culture applications is PEG and compounds thereof.

Important functions of hydrogels in microfluidic devices are the establishment of cellular barriers, the encapsulation of cells and/or drugs and their distribution, as well as the production of scaffolds and wound healing matrices. Table 1 lists commonly used hydrogels for microfluidic 3D cell cultures based on the function of the matrix. This broad bandwidth of hydrogel functions is also reflected in their importance in current and future biomedical applications. The main advantage of hydrogels for microfluidic cell cultures, however, is their mimicry of extracellular matrix structures including adequate porosity for cellular organization, biocompatibility, representative stiffness and influence on cellular fate [42,43], all key parameters that promote native-like tissue function. As an example, due to their relative ease of use [44], matrix density can be readily adjusted by the degree of polymerization, which has been shown to significantly impact cell fate due to apparent diffusion restrictions and increased mechanical stiffness [45]. In another study, synthetic hydrogels were combined with biopolymers to mimic the different zones of *in vivo* cartilage using stem cells [46,47]. Moreover, the development of so-called "smart hydrogels" has allowed for time-dependent release of bioactive compounds to trigger cell responses [48,49]. Furthermore, hydrogel barriers have also been used to create chemical gradients [50]. A detailed review on fabrication of advanced hydrogels for ECM mimicry is covered elsewhere [51].

Function	Hydrogel (Name / origin*)		Composites (cells, drugs, proteins)	References
Barrier				
	Dextran	N	Breast carcinoma, endothelial cells	Zervantonakis IK et al., [52]
	Collagen	N	Primary human kidney proximal tubular epithelial cells; cisplatin	Jang KJ et al., [53]
Cell Delivery				
	PEG-DA	S	Hepatocytes	Li CY et al., [54]
	PEG	S	Leukemia suspension cells; cervical cancer cells	Patel et al., [55]
	Dextran-chitosan	N	Fibroblasts	Oh J et al., [31]
	PEG	S	Mouse myoblast cells; placenta-derived human mesenchymal stem cells; ESC	Allazetta S et al., [51]
	PGS-PEG	S	Rabbit bone marrow derived mesenchymal stem cells	Wu Y et al., [40]
Drug Delivery/ Screening				
	HA	N	Bone metastatic prostatecancer cells; camptothecin; docetaxel; rapamicin	Gurski LA et al., [56]
	Alginate	N	Vitamin B12	Bal D et al., [57]
	Collagen	N	Skeletal muscle cells	Shimizu K et al., [30]
	PHEMA	S	Poly(l-histidine)	Tarameshlou M et al., [58]
	PLLA	S	Paclitaxel	He T et al., [38]
	PuraMatrix TM	S	Breast cancer; non-small cell lung cancer; microvascular endothelial cells	Dereli-Korkut Z et al., [41]
	PEG-DA	S	Hepatocytes	Li CY et al., [54]
	PLGA	S	Paclitaxel; BSA; cadmium sulfide nanoparticles	Heslinga MJ et al., [39]
	Agarose-chitosan	N	5-fluorouracil	Zamora-Mora V et al., [26]

| Encapsulation				
	PEG	S	Leukemia suspension cells; cervical cancer cells	Patel et al., [55]
	Alginate	S	Hybridoma cells; mouse breast cancer cells	Akbari S et al., [59]
	PLLA	N	Paclitaxel	He T et al., [38]
	Dextran-chitosan	N	Fibroblasts	Oh J et al., [31]
	HA	N	Mesenchymal stem cells	Bian L et al., [29]
	Fibrin	N	Chondrocytes; mesenchymal stem cells	Huipeng MA et al., [60]
	Collagen	N	Skeletal muscle cells	Shimizu K et al., [30]
	PHEMA	S	Poly(l-histidine)	Johnson RP et al., [37]
	Fibrin	N	Human umbilical vein endothelial cells; human lung fibroblasts	Whisler JA et al., [61]
	PGS-PEG	S	Rabbit bone marrow derived mesenchymal stem cells	Wu Y et al., [40]
	PLGA	S	Paclitaxel; BSA; cadmium sulfide nanoparticles	Heslinga MJ et al., [39]
	Matrigel	N	Adult murine pancreatic cells	Jina L et al., [33]
Entrapment				
	HA	N	Mesenchymal stem cells	Bian L et al., [29]
	Alginate	N	Breast cancer cells; doxorubicin	Yu L et al., [62]
Implant				
	2-HEMA	S	Silicon	Schwerdt HN et al., [36]
	PVA	S		Bian L et al., [29]
	PGS-PEG	S	Rabbit bone marrow derived mesenchymal stem cells	Yu L et al., [62]
Scaffold				
	Fibrin	N	Human umbilical vein endothelial cells	Park YK et al., [32]
	PEG	S	Mouse embryo fibroblasts	Guarnieri D et al., [34]
	Fibrin-Collagen	N	Human umbilical vein endothelial cells	Park YK et al., [32]
	Collagen	N	Human umbilical vein endothelial cells	Park YK et al., [32]
	HA	N	Glioma cells; chondrogenic MSC	Lee KH et al., [63] and Toh et al., [16]
	Chitosan-Silk	N	Hepatocytes	He et al., [27]
	Laminin	N	Adult murine pancreatic cells	Jina L et al., [33]

Table 1: Overview of frequently used hydrogels for 3D-microfluidic cell cultures.

Despite their many advantages, a number of drawbacks using hydrogels for microfluidic cell culture applications still exist and are associated with biodegradability, limited reproducibility and lack of standardization. For instance, in order to inhibit rapid degradation the addition of supplements, such as Aprotinin, throughout culture life may be required to maintain biodegradable hydrogel structures as ECM [64]. Additional technical limitations include bubble formation and inherent difficulties with introducing cell laden hydrogels in microfluidic channels prior to polymerization. Lastly, the optimum length of culture time for 3D cell populations has yet to be established for microfluidic devices [65].

Relevance of Hydrogels for Microfluidic 3D Cell Culture Systems

Since the Extracellular Matrix (ECM) is part of the natural microenvironment that influences cell organization, behavior and fate, it plays a key role for the development of advanced *in vitro* 3D cell-based assays [51]. For instance, substrate mechanics can also impact elements of cytoskeletal signaling; by varying mechanic properties of the applied substrate, MSC can be directed toward tendon or bone differentiation [66]. Furthermore, the interactions between stem cells and the ECM is known to generate signals relevant for cell proliferation, stimulation, differentiation and apoptosis, thus ultimately influencing tissue formation, repair and healing processes. When these communications fail, degenerative and autoimmune diseases, cancer, and diabetes can develop [67] or progress pathologically *in vivo*. Similarly, cell-cell interactions including signaling between same cell type and co-cultures significantly influence tissue organization, remodeling and stem cell differentiation [68,69]. Consequently microfluidic 3D cell culture systems have been used to study cell-matrix interactions as well as paracrine signaling in co-cultures of stem cells [70]. A recent example using a microfluidic channel network containing several interconnected chambers investigated the interaction between different cell types and diverse tissues and organ structures such as blood vessels [71]. Additionally, micropatterned cells have been used in cancer research to assess cell migration and invasive capacity of co-cultures in different hydrogels including collagen type I, Matrigel and fibrin [72]. Results of a similar study (Figure 1) showed that tissue function was significantly enhanced when hepatocytes were mixed with non-parenchymal cells in varying hydrogel layers with differing stiffness [73]. Another example of micropatterning, figure 2 displays neural cells in hydrogel for researching neuronal network formation [74]. Overall, hydrogels used in 3D cell culture settings mimic the extracellular matrix including chemo- and mechanotransduction events, thus allowing the investigation of cell-cell interaction as well as cell-matrix interactions. Although natural hydrogels are inherently biocompatible and usually biodegradable, synthetic hydrogels offer ease of use and decreased back ground noise when employing proteomic analyses and other
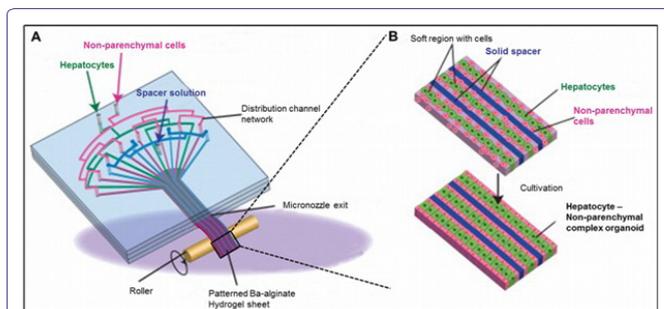
Figure 1: Schematic showing the preparation procedure of the stripe-patterned heterogeneous hydrogel sheet (A) and formation of heterotypic micro-organoids in the soft/solid hydrogel sheet (B). Reproduced with permission of Elsevier [73].
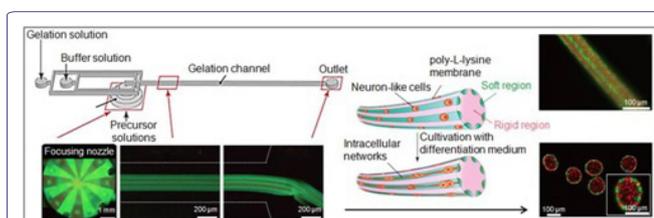
Figure 2: Patterned hydrogel microfibers in microchannels.

biologic assays [75]. These simulated ECM techniques are expected to 'bridge the gap' between monolayer cell culture and intensive animal trials [76].

Microfluidic system incorporating micronozzle array structures for producing patterned, complex hydrogel microfibers (Schematic &fluorescence micrographs) composed of a rigid and cell-encapsulating soft regions for guiding cell proliferation and forming intercellular networks. Inset (right) shows a bundle of hydrogel microfibers. Adapted from Kitagawa Y, Naganuma Y, Yajima Y, et al., [74]. Patterned hydrogel microfibers prepared using multilayered microfluidic devices for guiding network formation of neural cells. *Biofabrication*, 6: 035011. http://iopscience.iop.org/1758-5090/6/3/035011) © IOP Publishing. Reproduced with permission. All rights reserved.

Manipulation and Sensing of Microfluidic 3D Hydrogel Cultures

3D Hydrogel cell culture treatment using shear forces, stretching and patterning

One of the main benefits of applying hydrogel for microfluidic cell cultures is the ability to establish of chemical and biological gradients in the hydrogel. For instance, the combination of different hydrogel types composed of collagen and hyaluronic acid has shown an improvement in the adhesion, migration and proliferation of Human Umbilical Vein Endothelial Cells (HUVECs) in response to a Vascular Endothelial Growth Factor (VEGF) gradient controlled by microfluidic channels during *in vitro* mimicking of sprouting angiogenesis [77]. As illustrated in figure 3, microfluidics can be used to stimulate 3D hydrogel cultures thus simulating different cellular *in vivo* situations including shear stress, strain and stretch, compression, gravity and intracellular architecture [78]. In other words, pulsatile or constant shear force at different flow rates and biaxial or uniaxial stretching can be applied to mimic more complex biological niches. It is important to note that any changes of the ECM

composition or stimulation will influence not only the interactions between cells and ECM, but also mediate cell to cell communication and signal transduction, thus guiding biological responses [79]. For instance, shear flow can provoke a direct tension force on the cellular cytoskeleton, provisioning the opening of stretch-sensitive ion channels and allowing ion flux inside the cell, thus counteracting mechanical deformations in the membrane. Laminar flow dynamic effects specific to vascular endothelial cells have been extensively reviewed elsewhere [80]. Studies in bone on a chip measured calcium dynamics at different flow rates showing that the Ca^{+2} response of osteoblasts was enhanced in high shear-stress conditions [81]. Also the application of shear forces (Figure 3d) have been shown to modify the morphology of endothelial cells into a more elongated shape [82], which in turn induced different cellular connections and changes in the cytoskeleton [78]. Other examples of shear force guided cell behavior involve flow directed axon guidance of neurons [82] and
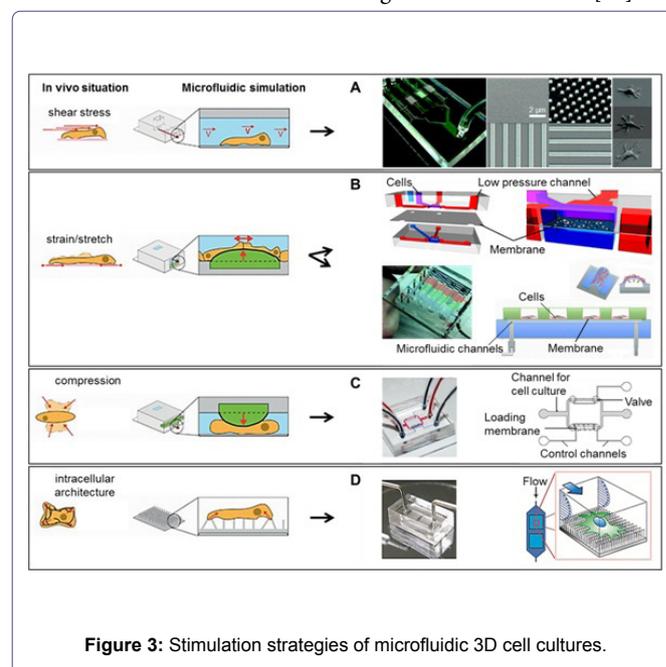
Figure 3: Stimulation strategies of microfluidic 3D cell cultures.

gene regulation [22,84]. Finally, a microfluidic device was developed to investigate how mechanical stress affects protein uptake by renal tubular epithelial cells [85].

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A. Shear stress induced cell stimulation and images showing the fabricated microfluidic channel having four branch channels with patterned substrate containing a flat surface, 400 nm pillars, 400 nm perpendicular lines, and 400 nm parallel lines (SEM images). Reproduced in part from Ref 96 with permission of The Royal Society of Chemistry

B. Micro-scaled cell stretching devices using multi-layer PDMS-based device in combination with pneumatic actuation of a flexible membrane. Reproduced in part from Ref 161 with permission of Springer; reproduced in part from Ref 190 with permission of The Royal Society of Chemistry

C. Microfluidic device for compressive cell stimulation that mimics the mechanical strains in blood vessels. Reprinted in part from Kim

YC, et al., [86].Microfluidic biomechanical device for compressive cell stimulation and lysis. Sensors and Actuators B: Chemical, 128: 108-116. Copyright (2007), with permission from Elsevier

D. Elastomeric microposts integrated into microfluidic for flow-mediated mechanotransduction analysis of single HUVECs plated on the PDMS micropost arrays coated with different adhesive patterns of fibronectin (left: uniform coating, right: an array of circles). The PDMS microposts were labeled with DiI (red), while HUVECs were stained for the nucleus (blue) and actin microfilaments (green). Scale bar, 50 μm. Reproduced in part from Lam RH, et al., [82] Elastomeric microposts integrated into microfluidics for flow-mediated endothelial mechanotransduction analysis. Lab Chip; 12: 1865-1873, with permission of The Royal Society of Chemistry

For some organs such as the heart, lungs or arteries, there is a repetition in stretching and straining movements that can also be mimicked in microfluidic devices by integrating flexible membranes [87] or by combining sold and fluid mechanical stress [88]. A prominent microfluidic device developed as a tool that can be used with any kind of cells under mechanical stimulation and optical imaging was developed by Huang [89]. A similar approach shown in figure 3b studied how fibroblasts change their orientation according to the direction of the stretching [90]. In addition to stretching, compression is also an important stimulation for simulating the environment for cells forming bones and cartilage [91-94]. Also, stem cells have been found to differentiate on a chip under straining stimulation simulating the vascular system [95], as illustrated in figure 3b. In order to study cellular response to compression (Figure 3c), a chip that produces cellular lysis was designed to facilitate on chip cell-based analysis [86]. Compression studies in a chip with individual leukemic cells showed that extracellular calcium uptake was upregulated in stimulated cells [96]. Furthermore, mechanical compression has been used to investigate axonal degeneration after compression trauma [97] and for some diseases such as osteoarthritis [98]. Moreover, it seems that compression forces provoke shear stress of interstitial fluid in bone, thus upregulating osteogenesis [99]. Fluid dynamics, both 2D and 3D, in skeletal tissue engineering are reviewed in depth elsewhere [100].

Another cell manipulation method employed in microfluidics is micropatterning to study cell-cell and cell-matrix interactions to provide a deeper understanding in regard to bottom up tissue engineering [101]. In particular, this technique has been used for cell sorting and enrichment on-a-chip by using a diversity of nanostructures (Figure 3a) which provoked adhesion alterations between different cells [102]. Moreover, micro scale cell patterning is considered a promising field in regenerative medicine because it addresses the limitations of macro scale tissue engineering [103]. These traditional, top down engineering approaches in which cells are seeded onto bioscaffolds lack functional histostructural tissue integrity, result in suboptimal *in vitro* and *in vivo* performance. In turn, micro scale cell patterning is advantageous due to its inherent high resolution, creating cell based and cell derived tissue that mimics the native organ. Microfluidic cell patterning has been most commonly described using dielectrophoretic sorting and 3D printing; protein gradients have also been reported using microfluidic patterning [104] Hydrogel based microtissues have been used to assemble functional organoid conglomerates [105,106] and microvascular networks [107]. Despite its potential, microfluidic

bottom up techniques are still susceptible to microfluidic complications, particularly those affecting long term cell culture handling including shear stresses, medium evaporation, and limited capacity for cellular growth [108].

Cell-Based Biosensing Assays for Hydrogel Cultures

In recent years, a variety of biosensing techniques based on optical, mechanical, electrical and magnetic methods have been integrated into microfluidic devices for measuring and analyzing cellular behavior [109-114]. Despite these advances, the above mentioned sensing systems are predominantly applied for 2D cell cultures, while microfluidic 3D hydrogel systems are still limited to optical microscopy. Consequently optical methods such as standard microscopically supported monitoring are state of the art when working with three-dimensional structures. In figure 4b, Nguyen et al., used classical fluorescence microscopy to monitor the sprouting and neovascularization behavior of endothelial cells from an artificial vessel into a collagen matrix [115]. Son and co-workers used time lap se fluorescence microscopy for detection of cell-secreted proteases,
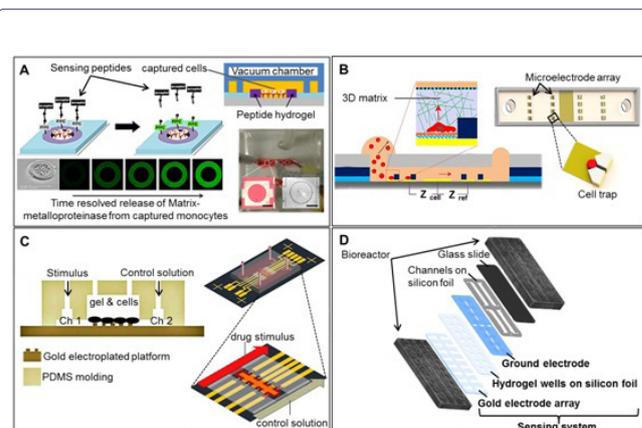
Figure 4: Sensing strategies in microfluidic 3D cell cultures.

A) Detection of MMP9protease release from Lymphoma cells bound within hydrogel rings using FRET. Protease molecules diffused into the gel and cleaved FRET peptide, resulting in fluorescence signal loss. Scale bar: 500 μm. Adapted with permission from Son KJ et al., [116] Micropatterned Sensing Hydrogels Integrated with Reconfigurable Microfluidcs for Detecting Protease Release from Cells. Anal Chem. 85, 11893-11901. Copyright (2013) American Chemical Society

B) Microfluidic chip with integrated electrical cell-impedance sensors for monitoring single cancer cell migration. Cross-section of the device including schematic of the sensor chip consisting of microelectrode arrays, cell capture arrays, and microfluidic channel with inlet and outlet, a V-shaped structure for single cell trapping. Reprinted in part with permission from Nguyen TA et al., [118]. Microfluidic Chip with Integrated Electrical Cell-Impedance Sensing for Monitoring Single Cancer Cell Migration in Three-Dimensional Matrixes. Anal Chem; 85, 11068-11076. Copyright 2013 American Chemical Society

C) Hydrogel-based diffusion chip containing impedance sensors for cell viability assay and drug toxicity screening. Schematic: diffusion from condition channel to control channel through the hydrogel chamber. Adapted from Tran TB [120] Hydrogel-based diffusion chip with Electrical Cell-substrate Impedance Sensing (ECIS) integration for cell viability assay and drug toxicity screening. Biosensors and Bioelectronics; 50: 453-459, Copyright (2013), with permission from Elsevier

D) Impedimetric biosensor array consisting of 16 gold electrodes to study neuronal differentiation and signaling processes. Reprinted from Valero T et al., [119] Studies on neuronal differntation and signalling processes with a novel impedimetric biosensor. Biosensors and Bioelectronics; 26, 1407-1413., Copyright (2010), with permission from Elsevier

responsible for the degradation of extra cellular matrix structures [116]. They designed a FRET (Fluorescence Resonance Energy Transfer) pair bound to a peptide, cleavable by the Metalloproteinase (MMP) 9, and incorporated them into a hydrogel-ring, as demonstrated in figure 4a. Lymphoma cells were then captured via antibodies inside of the ring and afterwards triggered for the production and release of MMP9. The released proteins diffused into the hydrogel where they activated the FRET pair and therefore gave rise to time resolved measurement using fluorescence microscopy. Alternatively, Xu et al., produced 50nl collagen droplets with a layer thickness of approximately 20-40μm containing encapsulated cells by using common printing techniques [117]. The resulting optical properties enabled the researchers to include a lens-less charge-coupled imaging system to investigate small disturbance in cell alignments caused by external stimuli.

One electroanalytical technique that has been used in 3D-hydrogel microfluidic devices is called Electrical Cell-Substrate Impedance Systems (ECIS), which detects alterations in electric properties of the cell-loaded hydrogel. For instance, Nguyen et al., developed a microfluidic chip for the investigation of single cell migration through a 3D matrix, which could be linked to further investigation on the initial steps of the invasion-metastasis cascade of cancer [118]. The described device shown in figure 4b included eight double microelectrodes which were separated by a large counter electrode and placed into a microfluidic channel. The working electrodes were lined with a V-shaped capture structure for hydrodynamic single cell capture. The results of this study showed, after establishment of the chemoattractant gradient, for the MDA-MB-231 cells, a rapid variation of the impedance magnitude of about 10Ω/s, while within the controls no significant impedance value changes could be observed, for either the less-metastatic MCF-7 cells or the electrodes without any captured cells. Other application for the use of electrical cell-impedance sensing were shown by Valero et al., which used this technique to investigate the differentiation and signaling processes of neuronal differentiation in three dimensional matrixes (see figure 4d and by Tran and co-workers, in which a cell based assay system was established for drug toxicity and anticancer drug studies [119,120], as seen in figure 4c.

An alternative sensing approach involves the wide variety of physical and mechanical properties of hydrogels, which offers researchers the ability to employ temperature, pH, and ionic strength responsive hydrogels as sensors. Furthermore, biochemical compounds such as peptides or proteins are often incorporated into hydrogels which lend even more possibilities for use as components of sensor structures. For example, in a previously published review Liu 2011 explains the integration of DNA aptamers and the subsequent increasing opportunities to use such hydrogels for on-chip analysis: smart hydrogels incorporating DNA aptamers are further described by Xiong et al., [121,122].

Applications of Microfluidic 3D Cell Culture Systems

Regenerative medicine and tissue engineering applications

In the last decade regenerative medicine has become an important part in clinical and pharmaceutical drug screening applications [123]. Table 2 summarizes the work done in this area. It is envisioned that microfluidic 3D cell culture systems may provide a deeper understanding of the parameters that influence tissue regeneration, healing and repair. The creation of a functional organ structure requires cells in culture that are provided with supportive structures, binding sites, nutrients, physical gradients and molecules, similar to those available to the specific *in vivo* tissue [65]. To provide such an appropriate microenvironment, different parameters such as ECM composition, 3D geometry, stiffness of the scaffold, cell density, nutrient supply, biomolecular gradient, mechanical stimuli or shear forces need to be regulated [124]. It has been shown that gradients of growth factors and other molecules, such as chemokines and cytokines that are naturally found in tissues, can be established in hydrogels [125] to achieve a more accurate microenvironment, as shown in figure 5a. While under physiological conditions, these gradients are relevant for cell migration, proliferation, homeostasis and angiogenesis [124] in pathological states these gradients also control inflammation, wound healing and cancer growth [126]. Knowledge of these processes is of prime importance for the understanding of underlying mechanisms which could perpetuate tissue dysfunction versus healing [124].

Targeted Tissue	Selected Cultures	Hydrogel	Technique	Function	Reference
Kidney	Madin Darby canine kidney cells	Geltrex/agarose	On-demand microgel formation	3D kidney epithelialization	Eydelnant et al., [127]
Liver	Primary rat hepatocytes and Swiss 3T3 cells	Alginate	Hydrogel microfiber formation	Liver specific function	Yamada et al., [128]
Liver	Human hepatoma and Swiss 3T3 cells	Alginate	Variable matrix stiffness	Enhanced tissue function	Kobayashi et al., [73]
Liver	HepG2 cells	Agarose	Quantum dot cytotoxicity	Drug diffusion from vasculature to liver	Wu et al., [129]
Pancreas	Mouse insulinoma 6 Beta cells	PEG	Cadherin staining	Cell aggregation	Bernard et al., [130]
Neurite	Dissociated cortical neurons of embryonic rats	Alginate/agarose	Differential interference contrast microscopy	Neurite density	Kunze et al., [131]
Neurite	Dissociated cortical neurons of embryonic rats		Hyperphosphorylation	Alzheimer-like neurodegeneration	Kunze et al., [132]
Neurite	Dissociated cortical neurons of embryonic rats	Alginate/agarose	Multi-layer scaffolding	Physiologic cell layering	Kunze et al., [133]
Neurite	Neuron like PC12 cells	Alginate microfibers	Tissue scaffold synthesis	Intercellular networking	Kitigawa et al., [74]
Neurite	Neuro2a cells	Collagen & collagen/laminin	Eletrical impedance spectroscopy	Assess neural differentiation in 3D	Valero et al., [119]
Neurite	Embryonic rat hippocampal & DRG neurons		Atomic force microscopy	Compressive cell stimulation	Magdesian et al., [97]

Neurite	Mouse ESC	Gelatin	Spatiotemporally controlled neuronal commitment	Biomolecular dose & timing, stem cell fate	Cosson et al., [108]
Cardiovascular	Porcine aortic valvularinterstital& endothelial cells	Gelatin-methacrylate	Physiologic spatial arrangement of co-culture	Shear stress regulated paracrine interactions	Chen et al., [134]
Cardiovascular	Human mesenchymal stem cells		Hydrodynamic actuation	Cyclic circumferential strain	Zhou et al., [95]
Vascular	HUVECs	Type I Collagen	Physicochemical regulation	Angiogenesis analysis	Verbridge et al., [135]
Vascular	HUVECs +/- stromal fibroblasts	HA–dextran, agarose with collagen	Soft-lithographic and micro-molding	Structural & biochemical patterning	Jiang et al., [136]
Vascular	Endothelial cells	Rat tail type I collagen	3D biomimesis	Neovascularization	Nguyen et al., [115]
Vascular	HUVECs	Degradable PEG	Pre-vascularized PEG scaffolding	Mass transfer, angiogenic quantification	Cuchiara et al., [137]
	HL60 (leukemic cells); Jurkat lymphocytes		Deflectable membrane mechanical stimulation	Cell mechanosensation	Xu et al., [96]
Skin	Epidermal and dermal cells	Type I Collagen	3D cell layering	Cellular function analysis	Morimoto et al., [138]
Skin	Normal human dermal fibroblast cells (NHDF)		Magneto-resistive real time monitoring	Label free detection of cellular phagocytosis	Shoshi et al., [112,113]
	Primary rat bladder SMC	Collagen	Label free cell based bio-sensing	Morphological cellular responses	Xu et al., [117]
	U-937 lymphoma cells	PEG/PEG-DA	FRET based signal transduction	Protease detection	Son et al., [116]
Cancer	MDA-MB-231 & MCF-7 cancer cells	Matrigel	Electric cell-substrate impedance sensing	Cancer cell migration	Nguyen et al., [118]
	HeLa carcinoma & human dermal fibroblasts		High resolution oxygen imaging	Oxygen distribution	Ungerböck et al., [114]
Stem cells	Mesenchymal stem cells	Polyacrylamide	Traction force microscopy	Cell migration	Vincent et al., [139]
Bone marrow	MC3T3-E1 osteoblasts, murine HSPCs	Collagen (variable densities)	Gradient hydrogel generation	Hematopoesis	Mahadik et al., [125]
Stem cells	Human mesenchymal stem cells	PEG	3D Co-culture	Cell signaling studies	Hamilton et al., [70]
Neurite	Retinal ganglion axons		Optofluidic control	Shear force cell stimulation	Gu et al., [83]
	Human fibroblasts		Anisotropic biaxial stretching	Cell mechanobiology	Tremblay et al., [90]
	Mammary gland epithelial cells		Deformable mechanical PDMS membrane	Compressive cell stimulation and lysis	Kim et al., [86]
Cancer	Cervical cancer cells & mouse embryonic fibroblasts	Agarose	Electric cell-substrate impedance sensing	Cell viability assay, drug toxicity screening	Tran et al., [120]
Cancer	HUVECs, NHDF, varied tumor cells, T cells		Optical light scattering and impedance sensing	Metastasis	Charwat et al., [109]

Table 2: Regenerative medicine and tissue engineering applications recently reported using microfluidic techniques.

A prominent example of microfluidic 3D cultures for tissue engineering applications involves on chip vascularization to study sprouting angiogenesis and progression of tube formation [140-142]. Different approaches using hydrogels have been developed to produce vascular formation on a chip. During one such study a lumen inside the microvessel was created to improve the 3D structure of the endothelial monolayer and medium supply using a mixture of collagen type I and Matrigel In this example, only collagen type I was used and channels were lithographically imprinted before seeding endothelial cells [135]. Another study used PDMS and prevascularized PEG hydrogel as part of the chip on which HUVEC were seeded, angiogenesis was quantified and mass transfer studied over a period of 48 and 96 h [137]. Gradients have also been applied to investigate sprouting angiogenesis in 3D hydrogels [141,143]. In a completely different approach, microgrooves were stamped in PDMS and Human Umbilical Vein Cells (HUVEC) and fibroblasts were co-cultured in order to study sprouting and capillary network formation [136]. More in-depth vascular specific microfluidic applications are covered elsewhere [144]. Another important vascular organ structure includes cerebral capillaries due to their special characteristics imposed by the brain. The Blood Brain Barrier (BBB), formed specifically by astrocytes and pericytes, is composed of strongly connected endothelial cells which protect the brain from excess permeability [71]. The state of the art for 3D cultures mimicking BBB involves seeding of endothelial cells that are separated via a membrane from astrocytes and pericytes to study barrier function, uptake and transfer of drugs [145], however as far as we know there is not work accomplished by using microfluidics systems.

Other important on chip hydrogel-based tissue models constitute the heart, intestine, liver and kidney to elucidate possible side effects of novel drug candidates and to study tissue failure and repair. One strategy used to study tissue repair after myocardial infarct involved the injection of cells directly into fibrin [146] and PEG modified with fibrinogen hydrogels [147]. A model of heart valves has also been studied using a multilayer chip with compartmentalized cell cultures (valvular interstitial cells and valvular endothelial cells) separated by a membrane that allows cell interaction [134], illustrated in figure 5c. Microscale and microfluidic 3D models of intestinal villi have been reported to investigate oral drug absorption kinetics [148,149]. The liver on a chip is also an interesting organ model, not only to study hepatic diseases, but also as it metabolizes the vast majority of all oral

drugs and filters toxins from circulating blood [150]. Consequently, liver tissues on a chip have been used as a drug screening model, precluding animal experimentation and human pre-clinical trials [71]. In depth, liver specific microfluidic reviews are available [150,151]. To study the process of diffusion from the vasculature to the liver, hepatocytes located in agarose at different distances from a channel mimicking a blood vessel were used to detect cytotoxic effects of quantum dots [129]. It was shown that microfluidic 3D culture of hepatocytes in a microdevice maintained functionally over a period of 4 weeks, while 3D structures in Matrigel, collagen type I, gelatin and alginate enhanced cell viability and slowed dedifferentiation [152]. Existing microchip devices for liver regeneration research are already commercially available from HepaChip [153], including a perfused multiwell plate [154,155]. Another attractive approach is the co-culture of hepatocytes with feeder cells which could be maintained for up to 3 months in alginate and showed enhanced hepatic functions such as albumin secretion and urea synthesis [128]. In a similar approach, a 5 day model for kidney epithelialization was used to test a new microdevice in which digital surface controlled the size, shape, and location of the hydrophilic sites for hydrogel adherence. Cell viability was measured during this assay designed to study medium exchange technique [127], as seen in figure 5b. In a separate study, fluid shear stress, hormone and osmotic gradients were used in a microfluidic device to study renal tubular cells [156].

Additional microfluidic hydrogel-based microsystems have been developed to study the pancreas, skin and neural activities; also, microfluidic artificial lung technology has recently been optimized to more closely resemble human gaseous exchange [157]. A technique of controlling the size of multiple wells in a microchip was designed and tested with pancreatic b-Cells. The viability of these cells when forming aggregates rose from 20% to 90%, when seeded in PEG-based hydrogels that promoted cell aggregation. Results of these experiments showed that in the aggregates, cells were connecting themselves via E-cadherin junctions and they maintained their natural function of insulin secretion [130]. Skin is probably the most developed engineered tissue clinically, as skin grafts are already used in severe burn patients and to enhance wound healing. However, engineered skin can also be used on a chip to study skin related diseases as well as to test drugs and cosmetics. One innovative approach uses collagen beads with microsized skin on a chip to perform high-throughput assays. Here, keratinocytes and fibroblasts are cultured and form a compartmentalized structure which has been validated as a useful skin model [138]. Neurons, in turn, are complex cells subject to degenerative diseases and paralytic conditions; hypoxic effects on microfluidic stem cell neuronal differentiation have been reported [158]. The propagation of diseases related to tau protein was studied on a chip by seeding healthy cortical neurons in two channels separated by a network of neurites. Subsequently, Alzheimer disease was induced in one of the neurons' channels achieving both states, healthy and ill, in the same chip [132]. Since neurite density is critical for neuron viability, the number and length of neurites were investigated in 3D in a microdevice using 4 to 6 layers of hydrogel. Static and perfusion studies were also performed, showing that neurite density was significantly increased in the presence of microfluidic perfusion [131]. Finally, a microfluidic system (see figure 5d) was developed as a model in the study of neurons and neurite network formation [133].

A variety of stem cells have been used in 3D cultures inside hydrogels in microfluidic devices to study proliferation and differentiation capacities for tissue engineering applications. The most
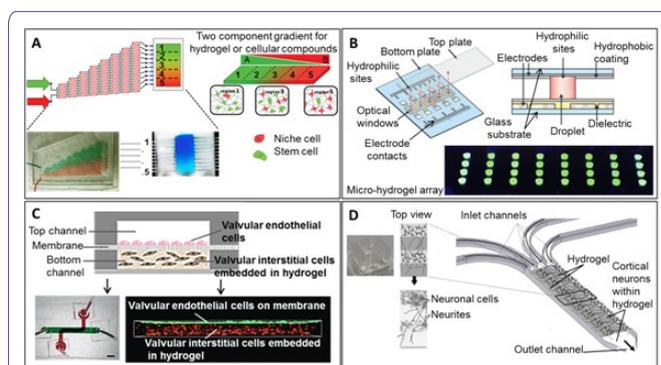
Figure 5: Devices used for regenerative medicine.

A) Microfluidic hydrogel gradient generator capable of modulating hematopoietic stem cell culture environment using two-component opposing gradient hydrogel with overlapping patterns of matrix and cell content within discrete hydrogel regions. Reproduced in part with permission from Mahadik BP et al., [125]. Microfluidic generation of gradient hydrogels to modulate hematopoietic stem cell culture environment. Advanced healthcare materials; 3: 449-458. Copyright 2014 John Wiley and Sons

B) Schematic of device: a bottom plate with patterned electrodes and a top plate bearing patterned hydrophilic sites for formation of individual microgels for reagent delivery/analysis. Reprinted by permission from Macmillan Publishers Ltd: Nature Communications; 5: 3555, Copyright 2014 (Eydelnant 2014) [127]

C) 3D microfluidic system of valvular endothelial cells separated from 3D hydrogel-laden interstitial cells via membrane. Perspective of a 3D confocal reconstruction demonstrating the compartmentalization and spatial arrangement of fluorescently-labelled cells in the bilayer membrane after 3 days of co-culture. Reproduced in part from Ref 20 with permission of The Royal Society of Chemistry

D) Micropatterning neural cell cultures in 3D with a multi-layered scaffold using a PDMS microdevice. 3D schematic of system: top view image demonstrates cell growth via optical microscopy. Reprinted from Kunze A et al., [133]. Micropatterning neural cell cultures in 3D with a multi-layered scaffold. Biomaterials; 32: 2088-2098, Copyright (2011), with permission from Elsevier

common cell type employed are Mesenchymal Cells (hMSCs) as they can be easily obtained from the patient and their migration potential and differentiation in the body is great. Migration studies of hMSCs in fibrin gels of different stiffness were studied mimicking different pathological states in the ECM showing that actin and microtubules are both responsible for migration [139]. Vascularization studies using hMSCs harvested from three different anatomic locations were performed in the presence of HUVEC. MSCs from the bone marrow were shown to migrate farther and to be the most supportive to HUVEC undergoing tubule formation. Moreover, cell characterization after 2 weeks of culture showed that cells were dedifferentiating into pericytes [159]. A less often used cell type is Embryonic Stem Cells (ESC), known to be extremely pluripotent. To date, human and murine ESC have been used in combination with microfluidics [160-164].

Tumor Research and Anticancer Drug Delivery Research

Cancer research is performed on many different levels, such as genomics, proteomics and therapeutic studies of candidate drugs, which are often tested in various animal models. Once more, the integration of microfluidics in this field of biological and medical research shows countless advantages, the most prominent being the replacement for animal studies and thereby lowering the immense costs, in both monetary and in animal lives [150]. In addition,

accuracy of animal trials in predicting efficacy or toxicity in humans is unreliable, making microfluidic applications all the more promising [165]. This research is still in its infancy but promising developments have evolved over the past years, including rapid prototyped multilevel microfluidics [166]. Automation has been reported for high throughput microfluidic cancer research, and validated using an established breast carcinoma co-culture [167]. Tumors are able to create a self-protective microenvironment, which hinders the chemotherapeutic regimen from effective toxicity. By integrating cancer research in the previously described 3D microfluidic cell culture setups, various strategies have been tested for drug development and encapsulation thereof, in order to circumvent this critical effect. Furthermore studying the parameters of successful drug delivery to the site of the tumor is crucial for the development of new therapeutics. By integration of 3D cell cultures, mimicking the tumor environment in a microfluidic setup, perfusion, uptake and distribution of diverse drugs in the tumorous tissue can be monitored [168] and evaluated simultaneously. Albanese et al., created a 'tumor-on-a-chip' system in order to investigate nanoparticle uptake of tumor spheroids under physiological conditions. Their setup allows real-time monitoring by confocal microscopy of drug accumulation at the tumor sites. Their findings propose that the tumor-on-a-chip system provides a powerful tool for the screening of nanoparticles to reach an optimal design prior to *in vivo* studies [169]. Another study proposed a micro-perfusion 3D cell culture for chemosensitivity assays, using Epirubicin against colorectal adenocarcinoma cells. This study displayed greater resistance to cytotoxicity in 3D cancer cells compared to those cultured and treated in monolayer [170] similar findings were reported with doxorubicin in another study [171].

Especially in the long-term therapy of cancer, controlled and continuous drug administration is essential for a successful therapeutic effect. In the case of drug carriers for protein-based formulations, certain stability has to be achieved in order to avoid early drug decomposition in the organism and lower decreased cytotoxic effects. Pessi et al., developed double emulsion droplets to incorporate protein in PVA, polycaprolactone and PEG via microfluidic technology. These polymeric microcapsules showed high encapsulation efficiency (up to 84%) of the protein and a drug release of 30% within the following 168hrs [172]. Also with regard to finding optimal drug concentrations, microfluidic setups have become a promising strategy. Jastrzebska et al., designed a microfluidic device in which a concentration gradient generator is incorporated to investigate the response of normal and cancer cells to the exposure of two different anti-cancer drugs [173]. Not only were they able to define the most effective drug concentration but also developed a system that generally allows the analysis of cell morphology and cytotoxicity of diverse cells and/or drugs simultaneously within very well defined parameters. A micro total bioassay system was employed to study pharmacodynamics of an oral chemotherapy agent against breast cancer, incorporating liver, duodenum, and stomach [174]. In a final chemotherapeutic example, nephrotoxicity of anticancer drugs was investigated in blood-renal barrier chip [175]. In turn, various research groups concentrate on the better understanding of cancer cell migration, metastasis and its prevention [176]. For instance, the Irimia group focuses on cell migration and developed a microfluidic device for cell migration assays, which have been successfully used for cancer cell migration studies [177]. Using advanced microfluidic systems, precise measurements of directionality and persistence during migration were studied, which are crucial parameters in the development of cancer metastasis.

Future Perspectives and Current Challenges

Despite recent advances in microfluidic 3D hydrogel cell cultures for biomedical research, some concerns remain and involve the availability of biocompatible materials and the question of whether or not microfluidic cell cultures can survive long enough to produce clinically relevant results [65]. Biochemical assays may be of limited value due to the inherent small number of cells in microdevices [178]. Another problem associated with microfluidic 3D hydrogel cultures constitutes reproducibility and device validation. Consequently, fully automated and integrated cell culture systems are needed to increase reproducibility, which is also a core request of regulatory agencies. In more sophisticated microfluidic devices, integrated microvalves and micropumps will allow the computer-controlled application of active compounds and the regulation of concentration gradients, presence of adequate shear forces and compression profiles [179]. Next generation microfluidic 3D-hydrogel cell cultures will further integrate available multilevel sensing strategies to provide high content analysis. Applicable analytics for 3D microfluidic cell cultures have yet to be exhausted and currently include both on chip and off chip analyses such as impedance biosensing [180], magnetic assays [112], immune assays, FACS, metabolomics [181,182] and proteomics via Mass Spectrometry, ELISA, fluorescence microscopy, detection of differentiation markers [183] and micro total analysis systems, previously reviewed in depth elsewhere [184]. Furthermore, pharmacologic studies of individual target tissues [185] using organ-on-a-chip technology [186] will be further supplemented by multiple organoid cell cultures [187]. In addition to toxicology screening efforts, microfluidic 3D-hydrogel models are expected to become an enabling tool for medicine to investigate various diseases such as axonal damage [179,188], alveolar epithelial injuries [189] and osteoarthritis. Finally, stem cell research is expected to benefit significantly from Lab-on-a-chip advances in automation and high throughput screening [190].

Conclusion

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, Matrigel, fibronectin, agarose, poly-ethylene glycol diacrylate, and a mixture of both. Although 3D hydrogels have been shown to exhibit significant technological improvements compared to the monolayer culture, the broad practical application of such methods has not yet been achieved. Common limitations of all 3D-gel based microfluidic cell cultures include the creation of a reproducible medium-gel interface, direct medium perfusion and the inability to microstructure the 3D-hydrogel. An additional limitation of all existing microfluidic 3D cell culture systems is the lack of integrated monitoring functions to follow specific cell reactions in a non-invasive manner and over long periods of time.

Acknowledgement

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