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Engineering tissues for *in vitro* applications

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Engineered tissues can be employed for studies on the fundamental mechanisms of embryology and adult physiology and for investigating the evolution of disease processes. They also provide platforms to evaluate the behavior of new chemical entities in drug development. The recent development of three specific technologies has greatly facilitated the engineering of tissues for *in vitro* applications: the microfabrication tools that serve to both define the cellular microenvironment and enable parallelization of cell-based assays; synthetic, tunable hydrogels to create three-dimensional microenvironments; and bioreactors to control nutrient transport and fluid shear stress. Furthermore, convergence of these tools is providing investigators with the opportunity to construct and study tissues *in vitro* with unprecedented levels of sophistication.

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Introduction

Engineering of tissues for therapeutic applications is one of the potential paths to replacing damaged tissues in 'regenerative medicine'; however, it has been recently recognized that engineering of tissues for *in vitro* applications also holds tremendous potential value. *In vivo*, the responses of individual cells are regulated by spatiotemporal cues that reside in the local microenvironment such as the extracellular matrix (ECM), neighboring cells, soluble factors and physical forces, all presented in a three-dimensional context. Upon isolation from their *in vivo* milieu, a multitude of cell types display phenotypic instability [1,2]; therefore, successful recapitulation of high-fidelity tissue models *in vitro* will require an understanding of just how cells respond to such microenvironmental stimuli towards defining structure/function relationships for tissues. A variety of novel tools have been developed recently that will aid in this effort including microfabrication-based tools to specify cell–substrate interactions [3,4•], tunable synthetic hydrogels for the creation of three-dimensional tissues [5••], and controlled bioreactors for subjecting tissues to flow [6]. Here, we will provide selected examples of recent efforts to use such tools for engineering highly functional tissues. We will also discuss arenas in which these tissues are finding utility.

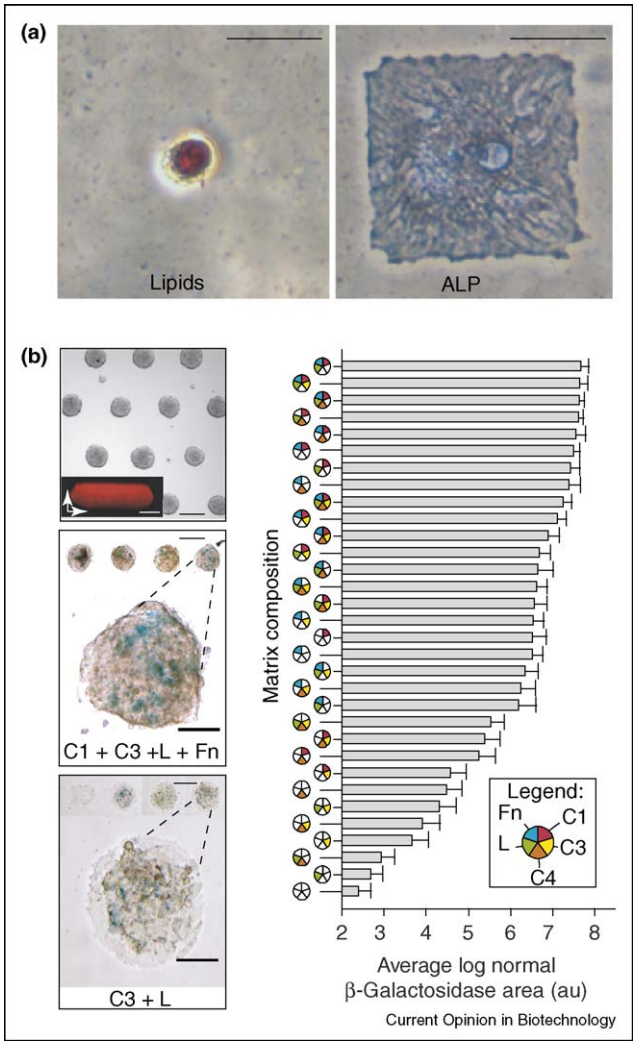
Microfabricated two-dimensional tissues: controlled cellular microenvironments and cellular microarrays

Conventional monolayer cultures are generated by randomly seeding cells onto homogenous substrates. Through the use of selective surface modification, microfabrication tools are now used to fabricate heterogeneous surfaces that offer control over cell–ECM and cell–cell interactions with micrometer precision [3]. A variety of such 'micropatterning' techniques have been developed and reviewed elsewhere [7,8]. Briefly, photolithography is utilized to pattern photoresist (light-sensitive polymer) onto a silicon or glass wafer. The wafer can be processed further to create cellular micropatterns or polydimethylsiloxane (PDMS), a biocompatible silicone rubber, can be cast on the wafer to yield a stamp (termed soft lithography [9]). PDMS stamps can be used for the microfluidic delivery of biological agents (i.e. proteins, cells) or used in microcontact printing of organic molecules (i.e. ECM proteins) onto substrates.

The use of microfabrication tools to investigate biological phenomena in different model systems has been an active area of research [7,10]. Pioneering work by Chen *et al.* [11,12••] demonstrated that the extent of spreading across a rigid substrate provided a physical cue not only for regulating cell fates, such as proliferation versus apoptosis in endothelial cells, but for the differentiation of adult stem cells as well (Figure 1a). Such ability to direct cell fate and function through the surface patterning of biomaterials has broad implications for the construction of engineered tissues.

In addition to the ability to probe the behavior of adherent single cells, micropatterning has proven to be an enabling tool in specifying the interaction between neighboring cells — both homotypic (same cell type) and heterotypic (between different cell types). For example, homotypic interactions between two neighboring cells were precisely investigated using microfabricated 'bowtie' wells that decouple cell–cell contact from cell

Figure 1



Microfabrication tools to direct stem cell fate. **(a)** The degree of cell spreading of human mesenchymal stem cells controls their differentiation down the adipogenic (fat) or osteogenic (bone) lineage. In particular, cells rounded up on small adhesive islands showed accumulation of lipids (fat cells), whereas cells well-spread on large islands contained active alkaline phosphatase (ALP; marker of osteoblasts). Scale bars represent 50 μm. (Figure adapted from [12] with permission.) **(b)** Extracellular matrix (ECM) microarray used to investigate the differentiation of embryonic stem (ES) cells toward an early hepatic fate (as indicated by expression of a β-galactosidase (β-gal) reporter fused to the fetal liver-specific gene, Ankrd17). Top panel: cells cultured with leukemia inhibitory factor (LIF) showed three-dimensional features (inset is x–z confocal section, ~77 μm thickness) reminiscent of embryoid bodies. When cultured with retinoic acid (RA), the cells grew as a relatively thin sheet (~25 μm thick, images not shown). Scale bar represents 250 μm (50 μm for inset). Middle and lower panels: Micrographs showing varying levels of β-gal reporter activity in ES cells cultured on different ECM combinations. The presence of collagen I, collagen III, laminin and fibronectin (C1 + C3 + L + Fn) collectively induced noticeably more β-gal reporter expression in ES cells than the mixture of collagen III and laminin. Scale bars represent 250 μm (50 μm for magnified views). Bar graph: Hierarchical depiction of 'blue' image area for each of the ECM mixtures. Error bars represent SEM (n = 32). (Figure adapted from [23] with permission).

spreading [13]. Similarly, the role of mechanical tension was explored in micropatterned, multicellular sheets of cells using microfabricated arrays of 'posts' as sensors [14••]. Microfabrication tools have also been utilized to evaluate the relative roles of homotypic and heterotypic interactions, thought to be important in the liver, between hepatocytes and non-parenchymal cells [15,16].

The cohort of tools described above primarily explores 'static' interactions through specification of cell adhesion; however, *in vivo*, cells are exposed to stimuli that change with time. The ability to dynamically modify cell-surface and cell–cell interactions during culture has been a recent area of investigation. For example, a thermally responsive material poly(*N*-isopropyl acrylamide) (pNIPA), which switches from cell adhesive (hydrophobic) at 37 °C to cell repulsive (hydrophilic) at 20 °C, has been exploited to harvest spheroids by releasing patterned multicellular islands [17]. This strategy has been enhanced further by fabricating a microheater array underneath a pNIPA layer to locally regulate cell adhesion [18]. In addition to thermal actuation of surface interaction, mild electrical stimuli have been utilized to alter surface wetting properties [19]. In another instance, Jiang *et al.* [20] used electrochemical desorption of a patterned self-assembled monolayer (SAM) to initiate adsorption of serum proteins and subsequent cell migration. Recently, Yeo *et al.* [21] have achieved dynamic, molecular-level control of a surface by modifying SAMs to present electrically active esters that can both release and bind ligands (i.e. cell adhesion peptides) upon electrochemical treatment. To allow for even more versatility in experimental design, our group recently developed a mechanically actuated dynamic substrate that is particularly useful for investigating interactions between two cell populations over time (E Hui and SN Bhatia, unpublished).

The utility of microfabrication in tissue engineering extends far beyond the ability to perturb the cellular microenvironment. Another major strength of this repertoire of tools is the ability to miniaturize and parallelize cell-based assays. Just as cDNA microarrays revolutionized genomics, cell microarrays formed by robotic spotting offer the potential for large-scale, systematic screening of cellular phenotypes [22]. Cells are either printed directly onto a planar surface or seeded onto spotted biomolecules (i.e. ECM proteins [23•], polymers [24•] or short interfering RNA [25]). For biomolecule microarrays, a non-adhesive background surface (i.e. polyacrylamide) can be utilized to prevent cell migration from spotted regions and thereby maintain pattern fidelity. For instance, Anderson *et al.* [24•] generated an array of synthetic polymers by depositing a variety of commercially available acrylate monomers that were subsequently photopolymerized onto a polyhydroxyethyl methacrylate-coated glass slide. The substrates were seeded with human embryonic stem cells and analyzed

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for differentiation markers utilizing immunofluorescence across 1700 cell–material interactions. Rather than investigate novel materials, Flaim *et al.* [23•] probed the interaction of cells with native ECM molecules in combinatorial mixtures. The role of integrin crosstalk was probed in the phenotypic stability of primary hepatocytes together with differentiation of embryonic stem cells towards an early hepatic fate [23•] (Figure 1b). Recently, Soen *et al.* [26•] have extended the ECM array to include mixtures of immobilized morphogens and other signaling proteins such as Notch ligands and Wnt factors. The continued development and use of such multisignal microarrays is likely to provide important clues as to the microenvironmental regulation of cell-fate processes.

Despite the utility of microfabricated 'two-dimensional' model systems, many cell types require a three-dimensional microenvironment to accurately reflect their *in vivo* counterparts (e.g. tumor cells, chondrocytes) [27]. Therefore, in the next section, we discuss strategies for engineering customizable three-dimensional microenvironments for tissue constructs.

Synthetic three-dimensional microenvironments

Considerable research has focused on mimicking the biochemical composition, fibrillar structure and viscoelastic gel characteristics of the natural tissue matrix [28,29••]. Both naturally derived and synthetic biomaterials have been extensively explored as three-dimensional scaffolds [5••]. Of the many synthetic biomaterials being explored, hydrogels in particular have been widely adopted for three-dimensional cell culture because their high water content and mechanical properties resemble those of native tissues [28,30•,31,32]. Furthermore, many hydrogels can be polymerized in the presence of cells, thereby ensuring a uniform cellular distribution throughout the three-dimensional network. For example, photopolymerizable poly(ethylene glycol) (PEG)-based hydrogels are of great interest for tissue engineering owing to their biocompatibility, hydrophilicity, and their ability to be customized by varying chain length (to control microporosity and thus mechanical properties) or by chemically adding biological molecules [29••]. PEG-based hydrogels have been used for the encapsulation of a diverse array of cell types such as chondrocytes [33], vascular smooth muscle cells [34], osteoblasts [35] and mesenchymal stem cells [36].

Pioneered by Hubbell and coworkers, PEG-based hydrogels can be chemically customized using different types of biologically functional building blocks to enable bidirectional signaling with cells [5••]. Recently, Pratt *et al.* [29••] demonstrated the design of a PEG scaffold that can modulate cell behavior through bound adhesion ligands and growth factors, and can be remodelled using cell-associated proteases. In another study, Raeber *et al.* [37•] used protease-sensitive, cell-adhesive PEG hydrogels with defined microporosity to parse the roles of individual chemical and mechanical factors that contribute to the three-dimensional migration of dermal fibroblasts.

In addition to the homogenous incorporation of proteins into hydrogels, concentration gradients can also be created to study effects on cellular fates such as migration. For instance, DeLong *et al.* [38] used a gradient marker with photopolymerization to create a simple, linear basic fibroblast growth factor (bFGF) concentration gradient in PEG hydrogels. Both smooth muscle cell proliferation and migration were increased substantially when bFGF was present in the gels. As has been demonstrated in other arenas, microfabrication techniques can again extend the capabilities of this approach by enabling the generation of more complex microfluidic gradients [39•].

Early experience with PEG hydrogels was limited to relatively robust cell systems such as fibroblasts [40], immortalized cell lines [41] and chondrocytes [33]; however, recent experience has extended the utility of the PEG platform to notoriously finicky cell types such as hepatocytes. For example, we have observed that this platform can be tuned to accommodate the survival of hepatocytes through alterations in pore size, photoinitiator composition, inclusion of adhesive ligands, and incorporation of stromal cells (VL Tsang, AA Chen, LM Cho *et al.*, unpublished). In addition to enabling the three-dimensional culture of cells from a growing list of normal cell types, PEG-based hydrogels have also found utility in the encapsulation of cells from diseased tissues towards studying pathophysiological processes. For instance, Wang *et al.* [42•] encapsulated osteoblasts from a mouse model of Apert syndrome, a disease characterized by malformations of the skull, limbs and viscera. The transgenic defect in FGF receptor resulted in alterations in immunostaining for osteoblast markers after three weeks in PEG hydrogel culture, which is consistent with altered cartilage and bone development *in vivo* (Figure 2a).

In addition to the encapsulation of somatic cells, several groups have explored the encapsulation of progenitor and stem cells in PEG hydrogels [36,43]. As tissue formation, homeostasis and regeneration are all dependent on the differentiation of stem cells down specific lineages, three-dimensional hydrogels that mimic natural three-dimensional stem cell microenvironments could prove important towards understanding and controlling stem cell differentiation [44,5••]. For instance, Mahoney *et al.* [45] encapsulated neural precursor cells into PEG hydrogels containing hydrolytically degradable lactide units. These precursor cells proliferated and subsequently differentiated into both functional neurons and glia. In another set of studies, Williams *et al.* [44] showed that bone marrow-derived mesenchymal stem cells (MSCs) encapsulated into a non-adhesive PEG hydrogel underwent

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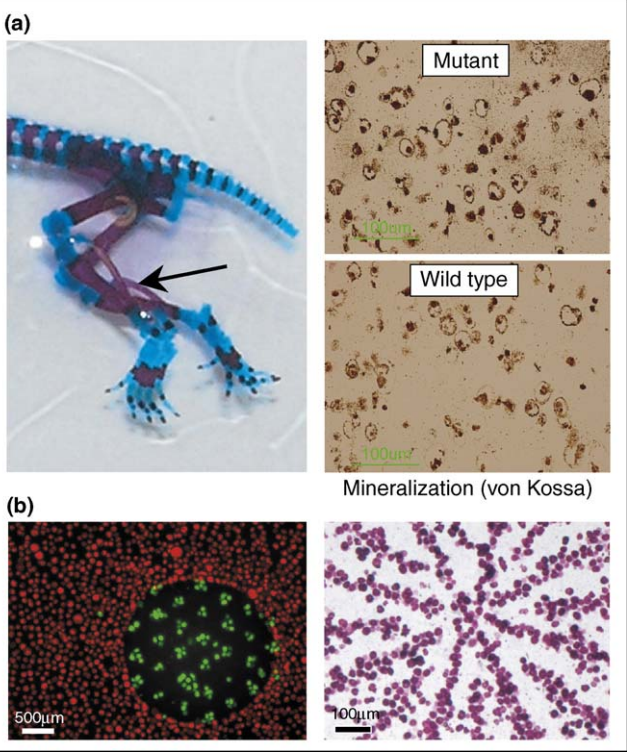
Figure 2



PEG hydrogels for engineering three-dimensional cellular microenvironments. **(a)** Skeleton of Fgfr2$^{S252W/+}$ (fibroblast growth factor receptor 2) mutant mouse (transgenic model of Apert syndrome) with bone stained purple (Alizarin Red Stain) and cartilage stained blue (Alcian Blue). Osteoblasts were first isolated from the middle shaft of long bones (see arrow) of either wild-type mice or mice with a mutation in the Fgfr2 receptor, and then encapsulated in PEG hydrogels and cultured in osteogenic medium for three weeks to allow for osteogenesis. As compared with wild-type controls, mutant cultures displayed increased mineralization (von Kossa staining) — images courtesy of Jennifer Elisseeff, Johns Hopkins University. **(b)** Micropatterning of hydrogel structures at various length scales using photo- and electropatterning techniques. Hydrogel microstructure with electropatterned green-labeled cells surrounded by a field of randomly distributed red-labeled cells. Green-labeled cells mixed with pre-polymer solution were first patterned in clusters using dielectrophoresis (electropatterning) and then encapsulated within larger hydrogel structures using UV irradiation through a photomask (photopatterning). Red-labeled cells mixed in pre-polymer solution were then introduced into the chamber and the entire assembly was exposed to UV light to create a rectangular hydrogel slab containing the different cells. (Figure adapted from [4] with permission). Primary rat hepatocytes arranged in cords and stained for intracellular glycogen, thereby showing versatility of electropatterning in the cell type and micropattern geometry. (Figure adapted from [52] with permission).

chondrogenesis more efficiently in the presence of exogenous transforming growth factor-β, an observation unlikely to be made in 'two-dimensional' culture.

Collective experience in therapeutic tissue engineering has led to the recognition that the architecture of three-dimensional scaffolds is crucial to the function of engineered tissues. Specifically, the shape of a tissue can alter nutrient transport, interactions between cell populations, and the distribution of mechanical force. In recent years, rapid prototyping technologies have been applied towards the fabrication of three-dimensional scaffolds with tunable micro- and macroscale features [46,47]. Here, too, the PEG hydrogel platform can be adapted to incorporate this capability [48–50]. Patterned photomasks can localize the ultraviolet exposure of the pre-polymer solution and thereby dictate the structure of the resultant hydrogel [41,51]. This photopatterning process can be repeated using additional photomasks to generate multi-layered constructs with different cell types. To specify the interactions of cells within a three-dimensional hydrogel context, Albrecht *et al.* [52] recently developed a method termed 'electropatterning' that utilizes mild electric field gradients to position cells within the material before photoencapsulation. Using electropatterning, the authors demonstrated that the microscale organization of chondrocytes can regulate matrix biosynthesis. They have further combined photopatterning with electropatterning to achieve hierarchical control over tissue structure in hydrogels over a length scale ranging from microns to centimeters [4•] (Figure 2b).

Clearly, hydrogel platforms that are inert, modifiable and photopolymerizable — such as those based on PEG — offer a wide array of possibilities for forming tissues *ex vivo*. Nonetheless, these three-dimensional tissues cultured under static conditions still lack key physiological stimuli that can be introduced through the incorporation of bioreactor technology. In the next section, we discuss strategies for culturing engineered tissues in bioreactors with a focus on microfabricated bioreactors that enable the production of miniaturized arrays of perfused tissues.

Bioreactors for *in vitro* applications

Bioreactors are devices in which the biological and/or biochemical processes develop under closely monitored and tightly controlled environmental and operating conditions (i.e. pH, temperature, pressure, nutrient supply and waste removal, and shear stress) [6]. A plethora of different bioreactor designs have been described in the literature for the culture of two-dimensional and three-dimensional tissue constructs [53]. Here, our focus is primarily on small-scale bioreactors that have been developed recently for *in vitro* applications.

In vivo, the fluidic microenvironment around cells within a given tissue is often highly varied and therefore controllable variations in the fluidic environment are an important component of fabricating tissues *ex vivo*. Fluid flow in bioreactors can be used to generate or mitigate gradients of stimuli and thereby impact tissue function. For example, standing gradients in oxygen tension exist in virtually every tissue of the body. Simple gradients have been generated by progressive depletion of a

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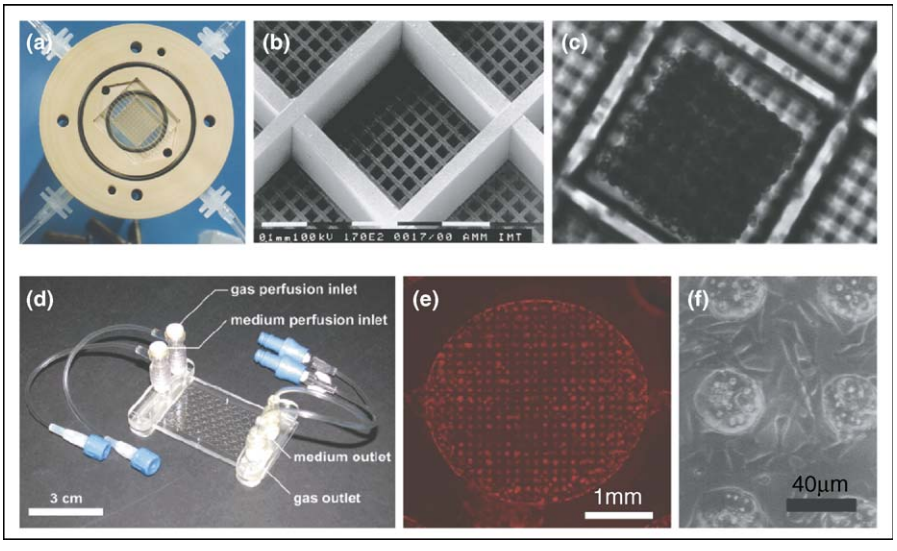
substrate such as oxygen in a parallel-plate reactor resulting in compartmentalized tissue (liver) function that exhibited several hallmarks of hepatic tissue *in vivo* [54]. More complex patterns in the soluble environment often exploit the lack of convective mixing in microfluidic networks [55] to explore such diverse phenomena as neutrophil chemotaxis [56] and intracellular mitochondrial trafficking [57]. In another design, two laminar streams were separated by a compartment containing shallow microgrooves, which allowed only the passage of neural axons [58]. With this device, axons and cell bodies of neurons could be subjected to distinct soluble environments enabling the study of this elongated cell type in more controlled ways.

In contrast to the examples above, bioreactors can also be used to facilitate nutrient delivery. Indeed, limitations in diffusive nutrient transport are especially problematic in the fabrication of three-dimensional tissues. Convective transport around and through an engineered tissue at the proper flow rate can dissipate gradients of nutrients and maintain tissue mass. In a novel strategy, Yu *et al.* [59] mixed microspheres of different density to vary flow velocity around and through scaffolds in rotating wall bioreactors. Compared with static three-dimensional controls, culturing rat primary calvarial cells under dynamic flow conditions led to more uniform distribution of cells in the scaffold interior, enhanced phenotypic protein expression and improved mineralized matrix synthesis. Bioreactors have also been utilized to facilitate perfusion of immobilized three-dimensional cellular aggregates. For example, a planar polymer scaffold with 900 microcontainers containing laser-drilled pores for fluid perfusion has been reported for the culture of three-dimensional hepatic aggregates (Figure 3a–c) [60•,61]. In another platform, an array of microchannels created using deep reaction ion etching of silicon wafers is used to culture hepatic aggregates that adhere to the channel walls [62]. Given the high oxygen uptake rate of hepatocytes, it is likely that perfusion in both these platforms helps to limit necrosis in the core of the hepatic aggregates. In an alternative approach, our group has subjected three-dimensional hybrid hepatic tissues created by photopatterning PEG-encapsulated hepatocytes to perfusion in a bioreactor to improve the viability and liver-specific functions of hepatocytes (VL Tsang, AA Chen, LM Cho *et al.*, unpublished). On the other hand, McGuigan *et al.* [63•] have recently utilized several collagen gel rods, which contain encapsulated cells and are surrounded by endothelial cells, to assemble a micro-vascularized tissue construct that can be perfused with culture medium or whole blood.

In addition to allowing convective transport of nutrients, bioreactors can also enable culture of tissues under continuous flow conditions for promoting reduced handling

Figure 3



Microfabricated bioreactors for the culture of two-dimensional and three-dimensional tissue constructs. **(a–c)** Planar polymer scaffold with 900 microcontainers for the culture of uniformly sized three-dimensional cellular aggregates. The bottom faces of the microcontainers are modified to contain laser-drilled pores for fluid perfusion through the tissue. (a) Open bioreactor housing with inserted microstructure for application in perfusion or superperfusion mode. The microcontainers are arranged in the middle 1 cm^2 surface. (b) Scanning electron micrograph of a single microcontainer (300 × 300 × 300 μm^3). (c) Primary rat hepatocytes organized in three-dimensional cuboids inside microcontainers. (Figure adapted from [60•] with permission.) **(d–f)** Non-addressable array (8 × 8) of microfluidic wells for creating micropatterned co-cultures of primary hepatocytes and 3T3 fibroblasts. Two microfluidic networks are used to independently perfuse the co-cultures with culture medium and oxygen. (d) Photograph of device, (e) micrograph of fluorescently labeled hepatocyte clusters, and (f) phase-contrast micrograph of micropatterned co-cultures. (Figure adapted from [66] with permission.)

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and increased automation. A notable advance in the field is the use of refreshable Braille displays to power integrated pumps and valves through local deformations of channel networks within elastomeric devices (i.e. PDMS-based) [64]. The use of such Braille displays has spurred the development of handheld microfluidic devices that can circulate medium continuously for weeks through cultures [65••]. In another example, Kane *et al.* [66] developed an 8×8 element non-addressable array of microfluidic wells capable of supporting micropatterned hepatocyte-fibroblast co-cultures. Two microfluidic networks independently perfuse the co-cultures with culture medium and oxygen. Results indicated that these co-cultures remained functional (albumin and urea secretion) for 32 days (Figure 3d–f).

Although much work has been done to develop culture models of single tissues, integration of multiple tissue models towards development of the so called 'human-on-a-chip' is a relatively new concept [67]. *In vivo*, interactions between multiple organ systems are important for the maintenance of homeostasis, and such interactions can also mediate the toxicity of pharmaceuticals. Recently, Viravaidya *et al.* [68•] have developed a multi-chamber microbioreactor to study bioaccumulation, distribution and toxicity in different tissue compartments connected via circulating fluid. The tissue compartments in this device are currently modeled by simple immortalized cell lines, but clearly the ability to study the interaction of high-fidelity tissue models in a microfluidic network would be quite powerful.

Overall, a variety of large-scale bioreactors have been developed for fabricating two-dimensional and three-dimensional tissues for clinical applications. Although microfluidics has spurred the development of miniaturized two-dimensional cultures for *in vitro* use, development of small-scale three-dimensional bioreactors is a relatively new direction in tissue engineering. We anticipate that in the future, two-dimensional and three-dimensional models of human and animal tissues with controlled soluble factor microenvironments will play an important role in drug screening as well as in evaluating mechanisms underlying biomedical phenomena.

Conclusions

The *ex vivo* engineering of high-fidelity tissues is being facilitated by three specific technologies: microfabrication tools to precisely control cellular microenvironments and create miniaturized cell-based assays for screening applications; synthetic tunable hydrogels to create three-dimensional scaffolds that interact with cells in a bidirectional manner; and bioreactors to culture tissues under flow conditions towards controlling nutrient transport, enabling continuous culture and subjecting cells to mechanical forces (i.e. shear stress). In the future, we anticipate that highly functional tissue units might be used in a modular manner to build complex tissues with multiple cell types and hierarchical structural features. These tissues will require an integrated vascular supply to achieve proper functional mass for clinical applications. For *in vitro* applications, such as screening in drug development, the integration of multiple tissues into a 'human-on-a-chip' platform could serve to better define the effects of endogenous and exogenous compounds on patients.

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