Bioengineering Methods for Analysis of Cells In Vitro

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Annu. Rev. Cell Dev. Biol. 2012. 28:385–410

The *Annual Review of Cell and Developmental Biology* is online at cellbio.annualreviews.org

This article's doi:
10.1146/annurev-cellbio-101011-155709

1081-0706/12/1110-0385$20.00

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Keywords

microfabrication, biomaterial, high-throughput, extracellular matrix, mechanobiology

Abstract

Efforts in the interdisciplinary field of bioengineering have led to innovative methods for investigating the complexities of cell responses in vitro. These approaches have emphasized the reduction of complex multicomponent cellular microenvironments into distinct individual signals as a means to both (*a*) better construct mimics of in vivo microenvironments and (*b*) better deconstruct microenvironments to study them. Microtechnology tools, together with advances in biomaterials, have been fundamental to this progress by enabling the tightly controlled presentation of environmental cues and the improved systematic analysis of cellular perturbations. In this review, we describe bioengineering approaches for controlling and measuring cell-environmental interactions in vitro, including strategies for high-throughput analysis. We also describe the mechanistic insights gained by the use of these novel tools, with associated applications ranging from fundamental biological studies, in vitro modeling of in vivo processes, and cell-based therapies.

Contents

INTRODUCTION

Since the earliest days of cell culture, investigators have strived to establish improved in vitro models that capture the most relevant aspects of in vivo physiology. Through this iterative process, in vitro culture platforms have been adapted continually to better recapitulate in vivo contexts and deconstruct complex mechanisms underlying cell and tissue processes. In particular, it is increasingly appreciated that cellular fate and function are regulated by the integration of both chemical and physical signals present within cellular microenvironments. Such environmental cues are presented to cells in the form of soluble factors, including growth factors and hormones, or as insoluble stimuli, such as cell-cell interactions and extracellular matrix (ECM) components. The importance of microenvironmental regulation in cell function is emphasized by the many cell types that display rapid phenotypic instability following isolation from their in vivo environment (Anderson et al. 1970, DeLeve et al. 2004, Guguen-Guillouzo & Guillouzo 1983, Guidry 1996, Lacorre et al. 2004, Russ et al. 2009). Over the past two decades, multidisciplinary efforts in cell biology and bioengineering have led to highly functional in vitro culture platforms that enable the controlled presentation of microenvironmental signals. In this review, we discuss recent work toward the development of bioengineering methods for manipulating and measuring cell-environmental signal interactions as well as the role of these interactions in dictating cell function. We focus specifically on approaches at the interface between cell biology, microfabrication technologies, and in vitro tissue engineering applications, although additional parallel advances in computational analysis and imaging tools have been critical for progress in the field. We refer the reader to several recent reviews on these topics (Deisboeck et al. 2011, Huang et al. 2009, Kirouac & Zandstra 2006, Peltier & Schaffer 2010, Pepperkok & Ellenberg 2006, Wessels et al. 2010).

Tissues are hierarchical and contain microarchitectural features that can be studied at many length scales. These include the subcellular/cellular scale (1–10 μm), which influences cell function; the multicellular scale (10–100 μm), which dictates the type and degree of intercellular interactions; and the tissue scale (100–1000 μm), which corresponds to the functional units of tissues (**Figure 1**). Here, we discuss seminal studies and current methods that address each of these levels of tissue hierarchy. We first focus on 2D platforms leveraging microfabrication tools that are designed to decouple complex spatiotemporal cues, including cell-cell interactions and other combinatorial signals. We then discuss the importance of mechanical properties of the microenvironment and approaches for probing the mechanical regulation of cellular function.

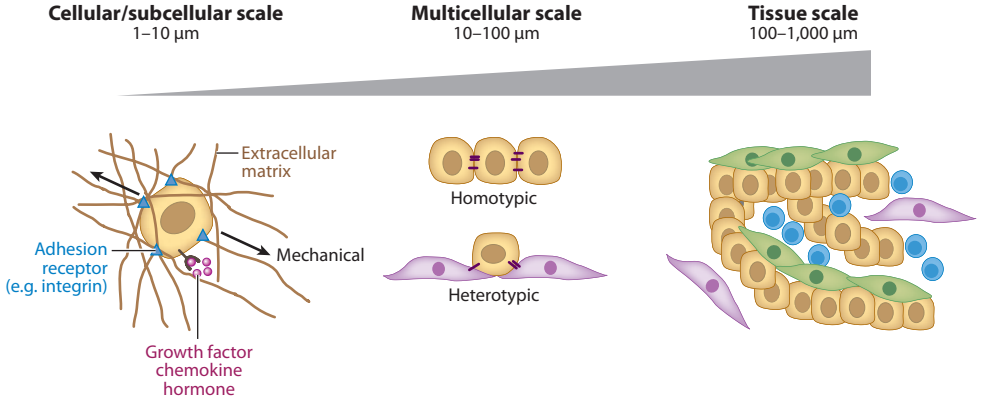

Figure 1

Hierarchical structural features in tissues. At the cellular/subcellular scale, soluble factor signaling, adhesive interactions with extracellular matrix and adjacent cell membrane proteins, and mechanical stimuli regulate cell functions. At the multicellular scale, the degree of homotypic and heterotypic cell-cell interactions can greatly influence cell fate and function. At the tissue subunit scale, the combinatorial effects of multicellular interactions, extracellular matrix environments, and the collective 3D architecture dictate tissue functions. A range of bioengineering methods has been developed to examine structure-function relationships at each of these length scales.

Next, we discuss the challenges in the development and assessment of 3D culture models as well as recent advances in the engineering of biomaterials, which provide improved control of biological signal presentation and tissue architecture. We also describe the utility of flow-based systems for both scaling up and scaling down in vitro cultures. Finally, throughout the review we briefly highlight key biological insights gained by the application of these novel tools, and we conclude with an overview of current challenges and future directions.

MICROFABRICATED TWO-DIMENSIONAL PLATFORMS AND ARRAYS

Controlled Cell-Matrix and Cell-Cell Interactions

A broad range of microfabrication approaches have been applied toward the tightly controlled analysis of cell-matrix and cell-cell interactions (**Figure 2***a*,***b***). In standard 2D monolayer cultures, homogeneous surfaces are used as a substrate for the adhesion of randomly seeded cells. Initial groundbreaking efforts integrating tools commonly utilized in the semiconductor industry for circuit fabrication were focused on methods to control the surface positioning of ECM proteins with micrometer-scale resolution in a process termed micropatterning (Kane et al. 1999, Singhvi et al. 1994). Such patterning can be achieved by exposing photosensitive materials to UV radiation through a patterned mask, in a method called photolithography, or through the transfer of molecules to a surface using a biocompatible silicone rubber, polydimethylsiloxane (PDMS), in a method referred to as soft lithography. In soft lithography, PDMS components are molded with microscale resolution from a silicon master template fabricated previously with photolithography techniques, and then they are used as a stamp to pattern molecules or hydrogels, such as agarose, polyethylene glycol (PEG), or polyacrylamide, on a target surface. Micropatterned substrates have facilitated extensive investigations of cell-ECM interactions and the influence of cell shape on cell function by enabling the independent modulation of cell-ECM contact and cell spreading (Chen

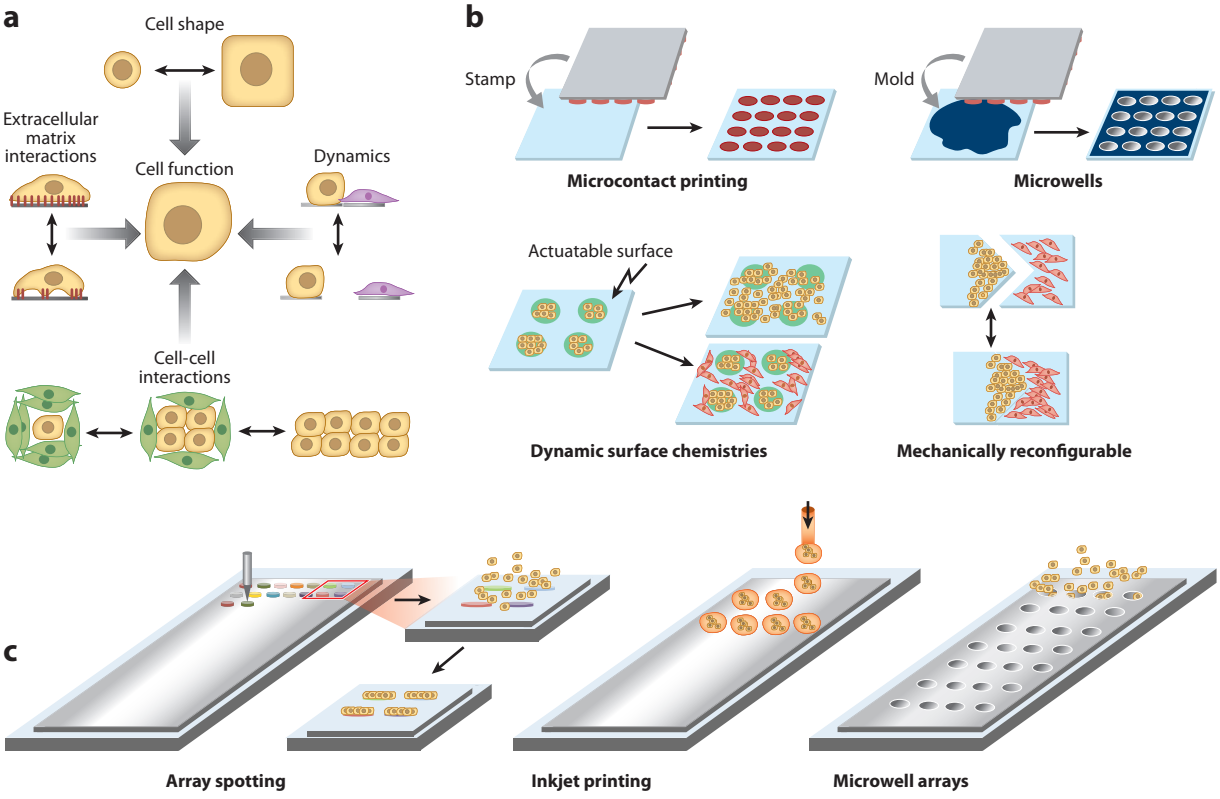

Figure 2

Microsystems for the systematic analysis of cell-cell and cell-matrix interactions. (*a*) Microfabrication tools that enable the 2D patterning of extracellular matrix (ECM) molecules have been developed to deconstruct the effects of cell shape and ECM interactions on cell functions. In addition, microfabricated substrates have been utilized to precisely control cell-cell interactions in vitro as well as to assess the influence of homotypic and heterotypic interactions and the dynamics of these processes. (*b*) Soft-lithography techniques, incorporating patterned polydimethylsiloxane (PDMS) substrates, can be used to generate micropatterned 2D surfaces through a microcontact printing (i.e., stamping) process as well as 3D microwell structures through the molded polymerization of hydrogels (*top*). Dynamic systems, such as actuatable surface chemistries and reconfigurable devices, are utilized for establishing patterned cocultures of two or more cell types as well as for investigating the dynamics of cell-cell and cell-matrix interactions (*bottom*). (*c*) Cellular microarrays can enable the high-throughput analysis of cell-environmental interactions, drug screening, and clonal heterogeneity. Various approaches, including the spotting of biomolecules, printing of cells within 3D hydrogels, and microwell fabrication, have been developed and employed for high-throughput studies.

et al. 1997). Subsequent studies building on this approach have explored the association of cell geometry and intracellular mechanics with cellular fates. For example, cell shape and the degree of cell spreading have been demonstrated to regulate cytoskeletal tension and Rho GTPase signaling in mesenchymal stem cells, which acts to influence their lineage commitment (Gao et al. 2010, Kilian et al. 2010, McBeath et al. 2004, Wang et al. 2011b). Patterning of adhesive domains with subcellular resolution has provided insights into the processes of focal adhesion formation and integrin activation (Arnold et al. 2004, Goffin et al. 2006). Microfabricated substrates have also demonstrated the effect of cell shape on the orientation of cell divisions (Minc et al. 2011, Thery et al. 2005).

Micropatterning tools also have been utilized widely for the precise 2D positioning of cells as an approach to control the degree of cell-cell interactions, both homotypic

(same cell type) and heterotypic (different cell types) interactions. For example, Nelson & Chen (2002) used micropatterning ECM islands with a specified range of diameters as an approach to modulate adherent cells' homotypic interactions with neighboring cells and to systematically explore effects on cell function. In recent work, Tseng et al. (2012) used micropatterned fibronectin surfaces to demonstrate the influence of ECM interactions on the positioning of intercellular junctions as a result of mechanical effects. Numerous studies have examined interactions with the mechanical properties of the cell microenvironment, which we discuss in detail below. As an approach to decoupling the influence of cell-cell contact from that of cell-spreading effects, Nelson & Chen (2002, 2003) used bowtie-shaped agarose microwells to investigate homotypic interactions between two adjacent cells, which revealed mechanisms underlying processes of cell proliferation and apoptosis. Furthermore, microwell systems, together with micropatterned ECM islands or modified suspension culture methods, have been utilized widely for the formation of embryonic stem (ES) cell aggregates (embryoid bodies) with controlled diameters, in a series of studies that has highlighted the role of embryoid body size and cell-cell interactions in ES cell differentiation (Bauwens et al. 2008; Carpenedo et al. 2007; Hong et al. 2010; Khademhosseini et al. 2006a,b; Moeller et al. 2008; Mohr et al. 2006; Niebruegge et al. 2009; Peerani et al. 2007).

In addition to single–cell type systems, microtechnology approaches have been exploited in the analysis of interactions between distinct cell lineages. For instance, micropatterned cocultures of hepatocytes and stromal cells, fabricated with either photolithographic or soft lithographic methods, have been used to assess the relative roles of homotypic and heterotypic interactions in phenotypic stabilization in vitro, which has demonstrated that a balance of these different interaction signals supports optimal hepatocyte function (Bhatia et al. 1999, Khetani & Bhatia 2008). By promoting the long-term in vitro stabilization of hepatocytes, particularly human hepatocytes, such an approach has enabled studies investigating drug metabolism, hepatocellular toxicity, and hepatotropic pathogens (Khetani & Bhatia 2008, Ploss et al. 2010). Notably, the selective adhesion of hepatocytes to the patterned ECM is a key determinant for the fabrication of micropatterned hepatocyte cultures. Overall, the generation of patterned 2D cocultures of two or more cell types requires clear selective adhesion characteristics of the cells of interest or alternative strategies for directly positioning the cells or for the sequential modification of surfaces. For example, dielectrophoresis methods, which are based on the induction of dipole moments in cells within nonuniform electric fields, have been used to position cells for patterned cocultures (Suzuki et al. 2008). Additionally, microfluidic systems, discussed in more detail later in this review, have shown utility for the simultaneous patterning of multiple cell types with defined 2D positioning and intercellular boundaries (Chiu et al. 2000, Khademhosseini et al. 2005, Torisawa et al. 2009). Stencils containing micrometer-scale holes of modular dimensions, typically fabricated using PDMS or parylene, have also been demonstrated as an effective patterning tool, as they form a physical barrier that restricts cell interaction and adhesion to specific, defined substrate regions (Cho et al. 2010, Folch et al. 2000, Ostuni et al. 2000, Wright et al. 2008). To modulate surface properties sequentially, many approaches have been developed on the basis of electrical (Chan et al. 2008, Fan et al. 2008, Kaji et al. 2004, Li et al. 2007b, Shah et al. 2009, Yousaf et al. 2001), light (Dillmore et al. 2004; Kikuchi et al. 2008, 2009; Ohmuro-Matsuyama & Tatsu 2008; Petersen et al. 2008), or thermal actuation (Tsuda et al. 2006, Yamato et al. 2001). Although each of these systems has unique design parameters and capabilities, generally the active regulation of surface hydrophobicity or adhesion peptide/protein presentation patterns is used to facilitate the sequential addition of multiple cell types in a spatially controlled manner.

Actuatable systems have been utilized not only as a method for initiating patterned cocultures but also as an approach for the temporal modulation of cell-matrix and cell-cell interactions and, therefore, investigations into the dynamics of cellular responses. Examples include the electrochemical modification of self-assembled monolayers, which has been employed as a method to initiate protein adsorption and to switch a nonadhesive region surrounding previously adherent cells into an adhesion-competent region for migration studies (Jiang et al. 2005, Yousaf et al. 2001). A self-assembled monolayer–based approach also has been utilized for the electrochemical release of adhesive peptides from a substrate, which can act as a trigger for cell detachment and temporally controlled removal of cells from the culture (Yeo et al. 2003). In another approach, Okano et al. (1995) used heating of thermally responsive polymers, such as poly(N-isopropylacrylamide), to release cell sheets or spheroids from a culture surface, and Cheng et al. (2004) explored strategies incorporating localized heating with microfabricated systems for improved spatial control. Mechanically actuated substrates have also been developed for the analysis of dynamic processes. Specifically, Hui & Bhatia (2007) modified microfabricated silicon devices consisting of two interlocking components to mediate cell attachment, and each was seeded independently with a cell type of interest. As part of the device design, the components can be positioned into and out of two distinct configurations, one in which cells make contact and one in which they are separated by an 80-μm gap. This platform has been utilized for probing the dynamics of cell-cell contact and short-range paracrine signals in hepatocyte–stromal cell interactions (Hui & Bhatia 2007, March et al. 2009). Collectively, the development of microfabrication technologies with progressively improved spatial and temporal control within in vitro culture platforms should continue to provide novel strategies for deconstructing complex cell-cell interaction mechanisms.

Cellular Microarrays

In addition to the precise control of environmental signals, microtechnology tools can be adapted for high-throughput parallel analysis (**Figure 2*c***). These high-throughput approaches can facilitate the systematic screening of cellular responses with substantially improved statistical power, together with the capability to explore a range of combinations of signals or perturbations inaccessible with other techniques. Cellular microarrays represent one such high-throughput approach, in which live cells are printed directly into an array pattern or seeded onto printed spots of biomolecules. Printed cell arrays, typically consisting of cells encapsulated within hydrogel droplets, have been explored as platforms for investigating the effects of biomaterial properties (Jongpaiboonkit et al. 2008), cell-cell interactions (Fernandes et al. 2010, Xu et al. 2011), and small molecules (Kwon et al. 2011, Lee et al. 2008). For example, a dual-array system, in which cellular microarrays are treated with a complementary stamp array containing combinations of drug metabolism enzymes (cytochrome P450 isoforms) and their metabolites, has shown utility for examining cytotoxicity profiles (Lee et al. 2008). In other strategies, arrayed spots of biomolecules generated with either contact printing or various piezoelectric (inkjet)-based methods have formed the foundation for a broad spectrum of investigations. In these systems, the spots typically include adhesive components to retain cells, in addition to combinations of other factors to stimulate cells or elements for detecting and measuring cellular processes. In particular, microarrays of ECM molecules have revealed substantial effects of combinatorial ECM presentation on cellular functions. Initial experiments in this area demonstrated the capabilities of an ECM microarray approach by focusing on the influence of ECM combinations on hepatocyte adhesion and survival, the early differentiation of ES cells, and notable synergistic or antagonistic effects of ECM components (Flaim et al. 2005, 2008). Subsequent studies have employed

arrayed ECM proteins for investigating a range of cell types (Brafman et al. 2009a,b; Huang et al. 2010; Mei et al. 2008; Woodrow et al. 2009), and efforts to expand the throughput of ECM microarrays should continue to provide insights into the underlying role of ECM in processes such as stem cell differentiation and tumor metastasis.

In addition to ECM molecules, printed arrays containing combinations of growth factors or cell-surface ligands have provided clues into how cells, particularly progenitor cell types, respond to complex extracellular signals. For example, the effect of the Notch ligand, Jagged-1, on the differentiation of neural (Soen et al. 2006) or mammary (LaBarge et al. 2009) progenitor cells was shown to be dependent on the context of the combinatorial stimuli, specifically, the presence or absence of Wnt or ECM proteins, respectively. Cellular microarrays based on spotted biomaterial libraries have also been explored and have provided key information regarding the effect of polymer backbone chemistries and end-group functionalization on pluripotent and multipotent stem cell proliferation and differentiation (Anderson et al. 2004, Benoit et al. 2008, Mei et al. 2010, Saha et al. 2011, Unadkat et al. 2011, Zhang et al. 2009). The extensive empirical data obtained from high-throughput material screens can offer an understanding of cell-material interactions that is difficult to predict a priori. In parallel with the advances in presenting extracellular signals within microarray formats, the development of lentiviral microarrays has been applied toward the miniaturization of RNA interference screens (Wheeler et al. 2005) and is being explored currently as a tool for high-throughput functional genomics. Finally, for some applications, cellular microarrays have been developed to include detection schemes as a way to monitor cellular processes within an array format. For example, antibody or aptamer-based approaches have been integrated for detecting the secretion of specific proteins (Chen et al. 2005, Ge et al. 2010, Liu et al. 2011, Tuleuova & Revzin 2010).

Arrays of microwells have also been utilized widely for the high-throughput analysis of cellular functions. Specifically, microwell systems have found great utility in the assessment of the clonal heterogeneity of stem and progenitor cells as well as in studies focused on suspension cell types such as lymphocytes and hematopoietic stem cells, for which the 3D structure of the microwell maintains the seeded cell and progeny within the array (Charnley et al. 2009). Microwell arrays have been fabricated through direct etching of silicon or glass, by photopolymerization methods, and/or through soft-lithography-based molding of hydrogel materials. In one application, Wood et al. (2010) used microwell arrays fabricated in agarose for trapping single cells and performing high-throughput "comet" assays for DNA damage. Based on the material properties of the microwell platform, microwells can be functionalized with biomolecules, and in an approach analogous to the printed array systems described above, the effect of extracellular niche signals can be examined (Gobaa et al. 2011). Lutolf et al. (2009) used such an approach to investigate in vitro self-renewal of hematopoietic stem cells as well as the regulation of this process by recombinant protein signals, including Wnt3a and N-cadherin. A microwell approach, together with a cytokine detection method termed microengraving (Han et al. 2010), also has revealed functional heterogeneity within lymphocyte populations, such as the unrelated capacities for cytolysis and IFN-γ secretion in individual CD8+ T cells (Varadarajan et al. 2011). Similarly, microwell arrays have been utilized for examining individual B cell stimulation responses and for the detection of antigen-specific antibody-secreting cells (Jin et al. 2009, Yamamura et al. 2005). Other techniques, such as PCR, have been incorporated into microwell systems for integrated genomic analysis (Lindström et al. 2009). Microwells, hydrodynamic traps, and various channel configurations also have been incorporated into microfluidic devices as a means to create cellular arrays in which soluble stimuli can be controlled precisely (Di Carlo et al. 2006,

King et al. 2008, Lecault et al. 2011, McKenna et al. 2011). Further, the coupling of cellular array approaches with time-lapse imaging systems has highlighted important dynamic properties, such as proliferation kinetics and gene expression, for individual or small groups of cells (Albrecht et al. 2010, King et al. 2007, Lutolf et al. 2009). Overall, cellular microarray platforms can provide unprecedented throughput for systematically deconstructing the multicomponent signals regulating cellular function.

Manipulation and Measurement of the Mechanical Environment

Several landmark studies have demonstrated that the mechanics of an in vitro environment dictate cell function (Discher et al. 2005, Engler et al. 2006, Wang et al. 1993). Hence, such culture substrates require computable if not tunable mechanical properties. The property reported most often is the stiffness of the 2D substrate. Increasing substrate stiffness appears to increase the activation of integrins upon their binding, the degree to which cells spread and flatten against the substrate, and the activation of myosin-dependent stress (Friedland et al. 2009, Fu et al. 2010). Changes in cell attachment and morphology in response to substrate stiffness can be observed visually, but measuring the stress generated by cells requires additional manipulation of the substrate. Most methods for measuring stress use a constitutive equation to calculate stress fields from substrate deformation, though biosensors are becoming an increasingly abundant option. The following section details multiple platforms developed not only to alter the mechanical properties of the extracellular environment but also to measure the intracellular generation of mechanical stress and transmitted force (**Figure 3**).

Traditionally, cells are grown on either glass or polystyrene, which have fixed elastic moduli greater than most in vivo tissue microenvironments, excluding bone. Depending on the concentration of the acrylamide and bisacrylamide cross-linker, the stiffness of a polyacrylamide substrate can be tuned to values between tens of pascals and hundreds of kilopascals (Wang & Pelham 1998, Yeung et al. 2005), which covers the range of in vivo soft tissue elastic moduli from brain (0.2–1 kPa) to fibrotic tissue (20–60 kPa) (Discher et al. 2009). Cells also can be seeded on polymerized gels of native proteins including fibrin, collagen, and Matrigel (Hong & Stegemann 2008, Kubota et al. 1988, Stegemann & Nerem 2003). Though these proteins have the advantage of being present in vivo, their multiple caveats include nonlinear elastic and viscoelastic properties, large pore sizes susceptible to migration, and difficulty with tuning stiffness values. Cross-linked hyaluronan, a native proteoglycan, overcomes these disadvantages with tunable elasticity, pore size, and

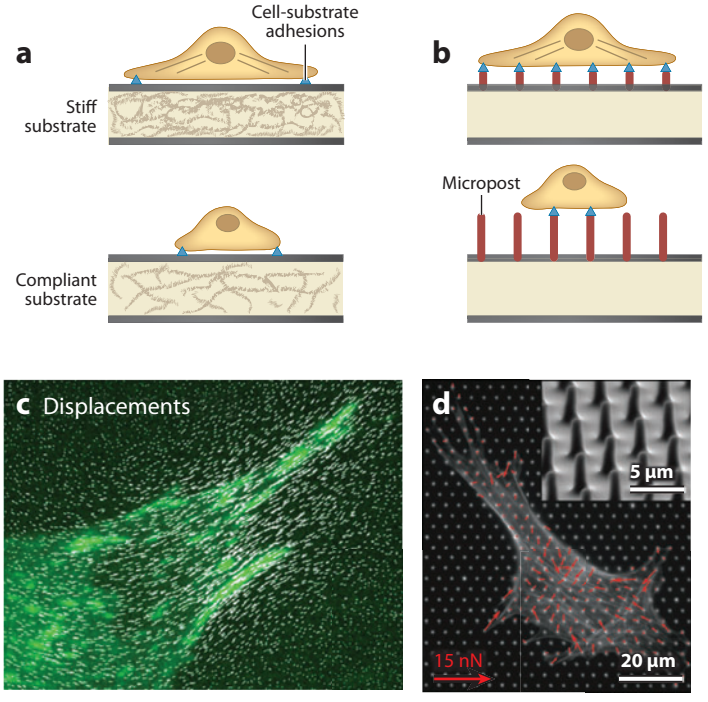

Figure 3

The mechanical microenvironment. The mechanical environment of 2D substrates can be manipulated either (*a*) by changing the material properties of the substrate or (*b*) by varying the height of microposts within microfabricated systems. Both methods allow for direct measurement of forces exerted by attached cells. For example, (*c*) beads can be implanted inside substrates for traction force microscopy, and (*d*) the deflection of labeled microposts can also yield analogous data on cellular forces. Figures adapted with permission from Sabass et al. (2008) and Yang et al. (2007).

stiffness ranging from 10 to 650 Pa (Burdick & Prestwich 2011). Hyaluronan also can be formulated to be degradable, similar to synthetic substrates of PEG (Mann & West 2002) and dextran (Levesque & Shoichet 2007). PDMS is a synthetic substrate with an elastic modulus that can be tuned between 5 kPa and 2 MPa (Prager-Khoutorsky et al. 2011). Unlike the other substrates mentioned, PDMS does not require hydration and is convenient for soft lithography techniques.

In addition to changing the native stiffness of the material itself, one can also alter the rigidity of the substrate by modulating the geometry through the application of microtechnology approaches. Specifically, by molding PDMS to form arrays of slender vertical posts, it has been demonstrated that the dimensions of the posts (e.g., height) can be used to vary substrate rigidity (Fu et al. 2010, Ghibaudo et al. 2011, Schoen et al. 2010, Yang et al. 2011). Using this system, studies have demonstrated changes in cell spreading, focal adhesion assembly, and stem cell differentiation, as have been reported previously to occur on polyacrylamide gels of different stiffness (Tserepi et al. 2005). An important distinction between this geometric approach and the cross-linking approach to manipulating stiffness is that the latter also impacts nanoscale structure, gel hydration, permeability to growth factors, and ligand presentation. The former impacts only micrometer-scale rigidity sensed between neighboring adhesions, which suggests that this microscale rigidity is what cells transduce to impact cell function.

Micropillar systems also have the advantage of allowing explicit measurement of the stress exerted by cells. The deflection of the pillars correlates directly to stress applied by the cell at the cell-substrate adhesions (Li et al. 2007a). In a similar manner, polyacrylamide substrates can be embedded with fluorescent beads to illuminate cell forces through a method called traction force microscopy (Lee et al. 1994). Because polyacrylamide is an elastic solid, the stress exerted by the cell can be derived from the displacement of the beads. However, mathematical models are required to relate the deformation field of the beads to the applied stress (Wang & Lin 2007). Initial studies utilized a Boussinesq solution (Lo et al. 2000), which relates the deformation of an infinite medium to a point load. However, because the polyacrylamide gels are not infinite, corrections to the model have been formulated. In addition to analytical models, finite element modeling has also been utilized to calculate stress from a given displacement field (Yang et al. 2006).

Biosensors present an alternative to measuring stress from observed substrate deformation. These sensors link fluorescence resonance energy transfer probes to the sides of an elastic linking molecule so that the emitted fluorescence can be correlated with applied force. Unlike micropost arrays and traction force microscopy, biosensors can illuminate the mechanics of subcellular structures. Grashoff et al. (2010) applied this concept to vinculin to understand its role in focal adhesion structure and cycling. An elastic domain derived from spider silk was bounded by two fluorescent probes and inserted between the head and tail domains of the molecule, which bind to different elements of the focal adhesion. This strategy allowed the authors to distinguish between vinculin recruitment and force transmission, which were determined to be controlled independently during cell adhesion. Stabley et al. (2012) also applied this method to cell-surface receptors, specifically EGF receptors, which undergo endocytosis once activated by a ligand. Rather than spider silk, the authors used PEG monomers of different lengths as the elastic linker because its force-displacement relationship could be predicted with a wormlike chain model. One end of the PEG molecule was bound to biotin, to allow for attachment to the substrate surface, and the other to an EGFR ligand labeled with a fluorescent probe. This method plausibly can be applied to other cell-surface receptors to measure applied force at specific adhesion sites.

ENGINEERED THREE-DIMENSIONAL CULTURE ENVIRONMENTS

Biological Signal Presentation Within Natural and Synthetic Biomaterials

In vivo, the myriad of microenvironmental cues with which cells interact are presented within a 3D context. This 3D architecture of tissues establishes another dimension of interactions with ECM and other cell types that is not recapitulated in standard 2D model systems with flat substrates, and it has been shown to affect adhesion-receptor and growth factor–receptor signaling (Fischbach et al. 2009, Hsiong et al. 2008, Roskelley et al. 1994, Wang et al. 1998), among other pathways. Three-dimensional tissue architectures can also prompt the formation of soluble or matrix-bound gradients and can exhibit unique mechanical characteristics that influence cell function (DuFort et al. 2011). Consequently, to complement approaches utilizing strictly 2D cultures, substantial work has been focused on developing improved 3D in vitro culture platforms (**Figure 4**). At the center of these efforts is the considerable research aimed at the development of 3D scaffolds that mimic the ECM of a specific tissue. Both natural/biologically derived and synthetic biomaterials have been explored extensively for these purposes. The most common natural ECM scaffolds utilized for in vitro applications include collagen, fibrin, Matrigel, alginate, and hyaluronic acid. Beginning with work by Bissell and colleagues, collagen gel scaffolds, as well as Matrigel and collagen/Matrigel composite materials, have been demonstrated to be ideal platforms for investigating mammary cell proliferation and morphogenesis, and they have formed the foundation for much of the current knowledge on the 3D regulation of mammary cell function and the importance of matrix mechanics in these processes (Debnath et al. 2003, Gudjonsson et al. 2002, Nelson et al. 2006, Paszek et al. 2005, Petersen et al. 1992, Streuli & Bissell 1990). Furthermore, 3D culture systems have been explored for the functional stabilization of primary cell types in vitro. For example, encapsulation within alginate is utilized commonly for maintaining the rounded morphology and function of primary chondrocytes (Hauselmann et al. 1992, Mok et al. 1994). Similarly, "double gel" configurations, in which primary hepatocytes are sandwiched between two layers of collagen gel, have been shown to promote stabilized hepatocyte morphology and functions for approximately one week in culture (Dunn et al. 1989, Guillouzo 1998). In addition to scaffolds containing one or a few reconstituted ECM components, the past several years have seen a resurgence in approaches incorporating ECM preparations from native tissues to better recapitulate the complexities of in vivo ECM environments, for example, in the liver (LeCluyse et al. 1996, Lin et al. 2004b, Rojkind et al. 1980, Sellaro et al. 2010). In particular, decellularization strategies have been developed for organs such as the lung (Petersen et al. 2010), liver (Uygun et al. 2010, Zhou et al. 2011), and heart (Ott et al. 2008) in which ECM components and microarchitecture are maintained. Although these systems have been pursued primarily for eventual transplantation contexts, they also exhibit great potential for in vitro studies. In addition, experiments focused on identifying changes in matrix composition that underlie normal and pathogenic processes in vivo (Naba et al. 2011) should continue to reveal important parameters for the design of the most appropriate ECM scaffold systems for in vitro analysis.

To develop more controlled and reproducible 3D environments, synthetic biomaterial scaffolds have been explored widely. In particular, hydrogels, which exhibit high water content and tissue-like mechanical characteristics, have been utilized extensively for both in vitro and in vivo tissue-engineering applications (Peppas et al. 2000). For example, PEG-based hydrogel systems exhibit many desirable properties including a resistance to the nonspecific binding of biological molecules owing to their hydrophilicity as well as the capacity for polymerization in the presence of cells, which provides the capability for

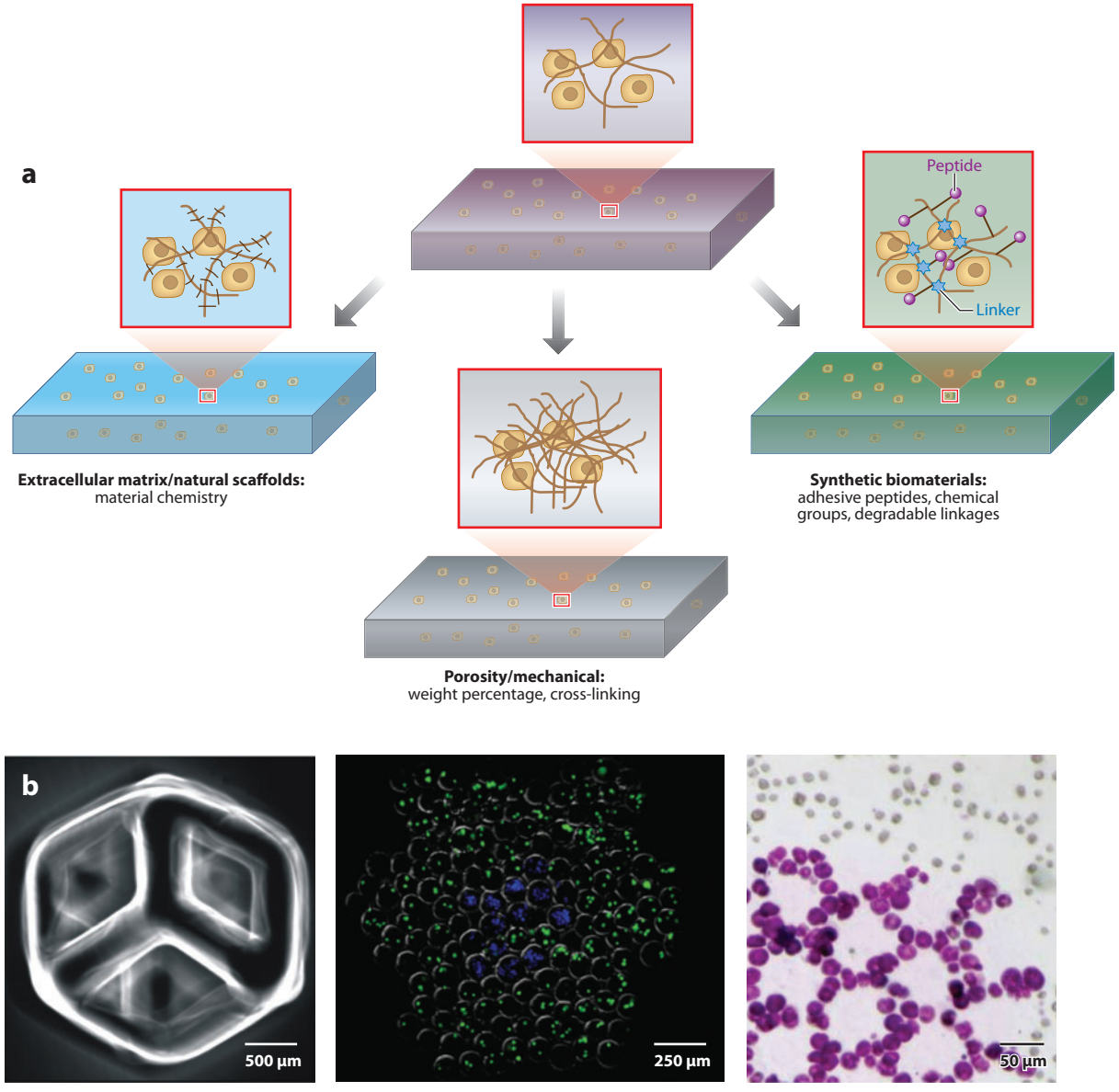

Figure 4

Engineered 3D culture environments. (*a*) A broad range of natural extracellular matrix (ECM) and synthetic biomaterial scaffolds have been developed and utilized extensively to examine cellular function within a 3D context. Material chemistry as well as the porosity and mechanical properties of the scaffolds can greatly affect cell survival, signaling, and differentiation. Synthetic biomaterials, such as polymer hydrogel systems, are highly tailorable through the incorporation of adhesive peptides, protease-sensitive sites for degradation, and other bioactive functional groups. (*b*) The 3D architecture of cell-biomaterial scaffolds can be controlled with patterning and assembly methods. Liu Tsang et al. (2007) used hydrogel photopatterning to fabricate a multilayer branched construct (*left*). Li et al. (2011) utilized DNA-directed assembly of microscale PEG hydrogels to generate larger multicellular structures containing two cell types, differentially labeled green and blue in this image (*middle*). Underhill et al. (2007) organized hepatocytes (*purple*, hepatocyte-specific glycogen stain) within PEG hydrogels with dielectrophoresis and then subsequently surrounded them with dielectrophoresis-patterned fibroblasts by additive photopatterning. Reproduced by permission of the Royal Society of Chemistry and Elsevier.

complete encapsulation with uniform cellular distribution. In addition, PEG-based hydrogels are highly tunable. Monomer chain length and branching configurations can be adapted to influence porosity and mechanical properties, and bioactive elements can be coencapsulated or conjugated to the polymer network to add biological functionality. Specifically, the addition of peptides such as RGD (Asp-Gly-Arg)-containing sequences, and the integration of matrix metalloproteinase-sensitive sequences, have been employed as methods to incorporate adhesive and cell-mediated degradation properties, respectively (Lutolf & Hubbell 2005). Other chemical modifications, such as the addition of heparin molecules or small functional groups, have been shown to modulate the retention of cell-secreted growth factors and ECM and therefore to influence encapsulated cell function (Benoit et al. 2007, 2008; Lin et al. 2009; Varghese et al. 2008). As in 2D scaffolds, cell-cell interactions have been demonstrated to regulate cell functions within 3D scaffolds. For example, homotypic and heterotypic interactions can influence liver cell survival and function in 3D contexts (Chen et al. 2011, Eschbach et al. 2005, Harada et al. 2003, Moghe et al. 1997, Sudo et al. 2009, Thomas et al. 2006, Underhill et al. 2007). Consequently, in addition to efforts focused on the controlled integration of adhesive ligands to provide ECM-like properties, studies have explored the material presentation of cell surface molecules, such as ephrin receptors and ligands (Lin & Anseth 2011, Moon et al. 2007), to mimic cell-cell interactions. Additional schemes for tailoring the biological properties of 3D constructs include the incorporation of releasable factors by the coencapsulation of nano/microparticles with modular release kinetics (Bian et al. 2011, Park et al. 2005) or through the conjugation of bioactive factors with cleavable linkages (Salvay & Shea 2006). Furthermore, a series of recent studies by Anseth and coworkers demonstrates newly developing approaches for the dynamic manipulation of hydrogel factor presentation and degradation based on photoactuation chemistries (Adzima et al. 2011; DeForest & Anseth 2011, 2012; DeForest et al. 2009; Kloxin et al. 2009). Such strategies, in combination with methods for fabricating complex 3D architectures (discussed in detail below), continue to encourage the development of improved 3D platforms by enabling the incorporation of progressively diverse biological signals for cell-material interactions and by increasing the range of tunable parameters for experimental manipulation.

Similar to 2D platforms, there are various methods to modulate the mechanical environment of cells seeded in 3D constructs. A simple means of altering the substrate stiffness is to release the construct from the walls of its culture platform, essentially unfettering the cells and allowing for substrate compaction (Bell et al. 1979, Harris et al. 1981). Initially, released substrates have lower static stiffness compared with constrained controls, but the mechanical environment changes as the substrate compacts (Ferrenq et al. 1997). An alternative to this approach is to tether 3D substrates to walls of varying compliance, which allows for precise control of the mechanical environment (Legant et al. 2009). Matrix microstructure can also be tuned to alter the stiffness of native protein hydrogels and synthetic constructs, beyond simply changing concentrations or polymerization kinetics. Examples include increasing substrate stiffness by glycosylating Matrigel (Kuzuya et al. 1996) and photocross-linking PEG constructs (Stahl et al. 2010). Finally, to measure the 3D mechanical environment, 3D traction-force microscopy has been developed to provide insight into the forces exerted by the cells on the surrounding matrix (Franck et al. 2011, Legant et al. 2010, Maskarinec et al. 2009). However, these methods are limited to ordered, elastic microstructures and cannot capture the applied forces in collagen and other viscoelastic networks.

Fabrication and Patterning of Complex Architectures

To provide improved resolution in the 3D structure of engineered in vitro tissues, an array

of patterning and assembly strategies have been developed (**Figure 4**). These advanced fabrication methods can aid in tuning the macro- and microscale structure of 3D constructs to optimize in vitro cell function and can also serve as tools for investigating the role of 3D structure (e.g., soluble-factor or matrix gradients, 3D cell-cell interactions) in cellular processes. Historically, a range of rapid prototyping technologies has been applied toward the generation of porous biomaterial scaffolds for therapeutic tissue engineering (Tsang & Bhatia 2004), and substantial work continues to be directed toward this area, with an emphasis on improved 3D resolution (Lewis 2006). In the past decade, many of these principles for controlling 3D structure have been adapted for hydrogel applications. For instance, photopatterning methods, in which the cell/prepolymer (e.g., PEG diacrylate) solution is exposed to UV light through a photomask, have been utilized to create constructs with a spectrum of shapes and sizes as well as multilayer hydrogels with different cell types and modular architectures (Beebe et al. 2000, Hahn et al. 2006, Liu Tsang et al. 2007, Liu & Bhatia 2002, Revzin et al. 2001, Underhill et al. 2007). Laser-based stereolithography techniques also have been applied to the fabrication of multilayer PEG hydrogels, including composite systems incorporating tethered ECM molecules such as collagen (Chan et al. 2010, 2012). Microscale patterning of 3D hydrogels has been shown to improve the viability of encapsulated cells by mitigating the nutrient-delivery limitations present in a bulk gel configuration (Liu Tsang et al. 2007). Furthermore, multilayer constructs designed to examine cell-cell interactions have provided insights into the role of cell communication in 3D structural heterogeneity, such as the zonal organization of cartilage (Sharma et al. 2007). Many studies have also demonstrated the utility of fluidic devices for generating hydrogel gradients and have employed this tactic for investigating gradients of adhesive ligands, macromolecules, or drugs (DeLong et al. 2005a, b; Kim et al. 2010; Ostrovidov et al. 2012). Finally, in contrast to these top-down–defined fabrication strategies, recent approaches have suggested that 3D structures could be assembled in a bottom-up manner from individual microscale components, leveraging concepts of self-assembly processes that occur in many contexts (Whitesides & Grzybowski 2002). For example, Li et al. (2011) showed that DNA-templated assembly of cell-laden, 100-μm-diameter hydrogels was an effective method for the patterning of larger multicomponent structures. Numerous hydrogel printing technologies also have been proposed as platforms for the assembly of 3D tissue constructs (Fedorovich et al. 2011, Jakab et al. 2010). Specifically, inkjet-based approaches, laser-mediated printing, and mechanical extrusion methods have been explored and optimized for the deposition of hydrogel/cell subunits to build larger constructs with predefined 3D geometries (Gruene et al. 2011, Jakab et al. 2004, Nishiyama et al. 2009) in a process commonly referred to as organ printing. In addition, the fabrication of multitiered 3D structures has been achieved through layering of cell sheets released from temperature-responsive polymer surfaces (described above) (Ohashi et al. 2007) and through the stacking of paper-supported hydrogel layers (Derda et al. 2009).

As a complement to strategies that specify overall scaffold size and geometry, investigators also have explored methods to dictate the structure of 3D gel systems at the cellular scale. For example, dielectrophoresis-based patterning can be utilized to position cells within a hydrogel prior to photoencapsulation (Albrecht et al. 2005, 2006). By using this approach, Albrecht et al. (2006) demonstrated the role of cell-cell interactions in regulating chondrocyte matrix synthesis. For the in vitro formation of endothelial tubular structures, PDMS substrates containing microfabricated grooves have been used to mold collagen or fibrin gels containing endothelial cells (Chrobak et al. 2006, Raghavan et al. 2010a). Molded microgel structures also have demonstrated utility for deciphering cell responses to soluble stimulation in 3D, specifically, the

signaling effects of growth factors involved in kidney tubulogenesis (Raghavan et al. 2010b). Furthermore, the patterning of mammary epithelial cells within collagen gels, using a microwell strategy, has formed the foundation for a series of studies examining the mechanisms underlying branching morphogenesis as well as the role of tissue mechanics and local autocrine gradients in this process (Gjorevski & Nelson 2010, Nelson et al. 2006, Pavlovich et al. 2011).

BIOREACTORS AND MICROFLUIDICS

Bioreactors are devices that are designed to precisely control the in vitro culture environments of cells and tissues by regulating the exchange of nutrients as well as the presentation of both chemical (e.g., soluble stimuli) and physical (e.g., mechanical, electrical) signals. These parameters can act to condition in vitro tissue models to specific environments, simulating in vivo contexts, and can serve a defining role in cell fate and function. Accordingly, a diverse range of bioreactors has been developed toward the optimization of engineered tissue platforms, particularly for 3D scaffold systems that can exhibit demanding control requirements (Burdick & Vunjak-Novakovic 2009). In addition, microengineered bioreactor approaches represent the focus of extensive recent work aimed at recapitulating the full scope of hierarchical in vivo interactions (cell-cell, tissue-tissue, organ-organ) in on-chip formats (Esch et al. 2011, Huh et al. 2011). Here, we focus primarily on progress in two areas: (*a*) the development of microscale fluidic platforms for the in vitro analysis of cells within continuous flow and gradient contexts and (*b*) challenges and strategies for scaling up in vitro culture systems (**Figure 5**).

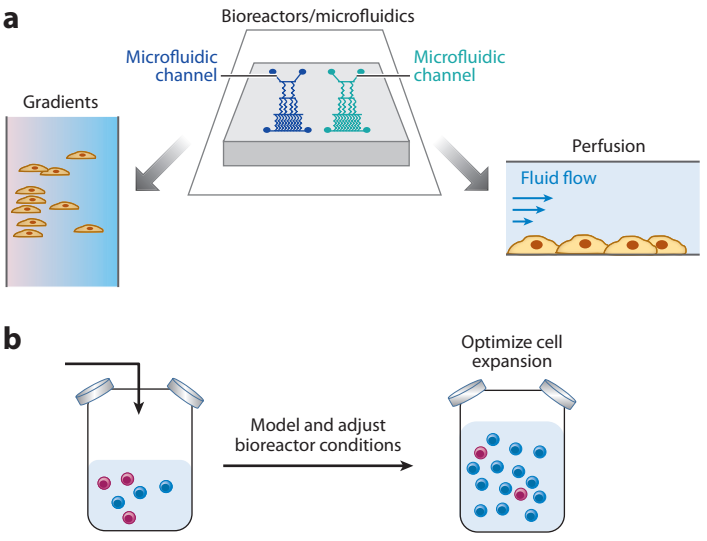

Figure 5

In vitro bioreactors and microfluidic platforms. (*a*) In vitro flow-based systems enable the investigation of cellular responses to hydrodynamic shear forces as well as the assessment of the collective effects of perfusion on cell viability and proliferation. In addition, microfluidic platforms can facilitate the analysis of the effects of complex soluble gradients and are utilized widely for studies requiring precise spatial and temporal control of soluble environments. (*b*) The optimization of bioreactor platforms is critical for scaling up in vitro culture systems and improving clinical translation capabilities. Figure based on work by Zandstra and colleagues (Csaszar et al. 2012).

Continuous Flow and Gradient Systems

One obstacle to the analysis of cells in vitro is the ability to provide gradients of extracellular signals that more closely mimic the heterogeneous environments present in vivo. As we have highlighted throughout the review, microfluidic approaches can provide additional spatial and temporal control in the design of in vitro systems. In particular, microfluidic platforms can uniquely enable the formation of complex patterns of soluble stimuli owing to laminar flow characteristics in the channels as well as through the large range of channel geometries and device configurations that can be achieved (Kim et al. 2010). For instance, many studies have examined chemotaxis of cells within microfluidic device-generated gradients and have utilized the flexibilities in the designs to explore the effects of chemokine gradients with varied shapes and steepness (Ambravaneswaran et al. 2010, Li Jeon et al. 2002, Lin et al. 2004a, Saadi et al. 2007). The separation of flow streams within microfluidics also has enabled studies examining the localized stimulation of sections of an embryo (Lucchetta et al. 2005) or even a

single cell (Takayama et al. 2001). In vivo, the progressive depletion of nutrients and soluble factors contributes to the formation of signaling gradients. In vitro systems can be designed to simulate these conditions; for example, Allen et al. (2005) used a mesoscale parallel-plate bioreactor to establish steady-state oxygen gradients and to examine the role of this gradient in the zonal expression of drug metabolism enzymes in hepatocytes. In addition, the effect of perfusion on cell function has been explored in microscale flow systems. For instance, such studies have investigated the influence of hydrodynamic shear and nutrient transport on ES cell proliferation and differentiation (Cimetta et al. 2009, Kamei et al. 2009, Kim et al. 2006). It has been suggested also that microfluidic platforms can be applied toward the in vitro analysis of primary tissues and the identification of biomarkers. In recent work, Wood et al. (2012) demonstrated that measurements of blood flow dynamics within a microfluidic device are a biophysical indicator of sickle cell disease patient outcomes. Furthermore, studies employing microfluidic devices containing endothelial cells have built on the extensive work using parallel-plate flow chambers (Chiu et al. 2009) to demonstrate the effects of distinct shear regimes on endothelial cell stimulation and thrombosis (Chin et al. 2011, Tsai et al. 2012, Wang et al. 2011a). Overall, owing to the capabilities for miniaturization and programming of complex flow characteristics, microfluidic platforms have been established as highly tractable tools for both dynamic manipulation of soluble microenvironments and high-throughput screening.

Scale-Up and Translational Applications

Parallel to bioengineering strategies aimed at providing mechanistic insights at the microscale, important studies are focused also on developing in vitro platforms that could contribute to the advancement of translational applications. Specifically, for many organ systems, the development of an effective cell-based therapy will require substantial scale-up over laboratory designs. For example, it is estimated that a clinically effective bioartificial liver (either implanted or extracorporeal) would require approximately 10% of the total liver mass (Chamuleau 2009), corresponding to 1×10^{10} hepatocytes. Accordingly, the fabrication of large-scale engineered tissues will require methods for facilitating nutrient delivery, and numerous bioreactor approaches have been pursued for these purposes. These include strategies incorporating rotating walls (Yu et al. 2004), scalable cartridge configurations (Gerlach et al. 1994), and many methods for integrating perfusion systems with porous scaffolds or microengineered channels (Dvir et al. 2006; Eschbach et al. 2005; McGuigan & Sefton 2006; Radisic et al. 2004, 2006). In addition, the implementation of a cell-based therapy will require an expandable cell source, and methods for obtaining a large number of cells that maintain the proper phenotype and function are required (King & Miller 2007). Various perfusion systems have been explored for the expansion of ES cells (Oh et al. 2005, Thomson 2007) and, in agreement with microfluidics studies, suggest that modulating nutrient delivery and the retention of autocrine factors by tuning flow parameters can significantly influence proliferation. Furthermore, studies performed by Zandstra and colleagues have demonstrated how in vitro hematopoietic stem cell expansion is regulated by feedback signals from differentiated cells (Kirouac et al. 2009, 2010). Building on systems-based modeling of intercellular interactions and bioreactor conditions, modifications in the bioreactor design could be employed and were demonstrated to enhance stem cell proliferation (Csaszar et al. 2012).

CONCLUSIONS AND FUTURE OUTLOOK

In the past two decades, the integration of engineered in vitro culture models and studies in cell and developmental biology has led to tremendous progress in the understanding of the

structure/function relationships regulating cell and tissue processes. Specifically, work at this interface of fields has highlighted the important role of environmental context in determining cell responses and has provided insights into not only chemical signals but also how the physical microenvironment of cells dictates function. In addition, engineering approaches have facilitated the development of a broad range of in vitro applications with clinical relevance. For example, optimized methods for directing and scaling up stem cell differentiation are being applied to cell sourcing challenges in regenerative medicine. Further, improved approaches for stabilizing the phenotype and function of primary cell types in vitro are demonstrating great utility for drug-screening applications.

Despite the substantial progress in improving the fidelity of in vitro culture models and analysis methods, many challenges remain to be addressed. In particular, it remains unclear for many systems how cells process complex, and sometimes even conflicting, microenvironmental signals. Therefore, extensive efforts are aimed at the development of platforms that continue to enhance experimental throughput and improved methods for investigating the bidirectional interactions between multiple cell types within a tissue as well as between cells and tunable material systems. Ideally, the ongoing evolution of these approaches, together with computational models of cell signaling networks, should provide a clearer mechanistic understanding of the in vitro conditions and perturbations that remain primarily empirical to date. Additionally, improved understanding of species-specific requirements will aid in further optimizing in vitro systems for investigating human cell and tissue function as well as the improved translation toward novel therapeutic approaches.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

ACKNOWLEDGMENTS

The authors acknowledge support from the National Institutes of Health (DK56966, EB008396, DK85713 to S.N.B.; EB00262, EB08396, GM74048 to C.S.C.), the RESBIO Technology Resource for Polymeric Biomaterials, and the Center for Engineering Cells and Regeneration of the University of Pennsylvania. S.N.B. is a Howard Hughes Medical Institute Investigator.

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Annual Review of Cell and Developmental Biology

Volume 28, 2012

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Errata

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