# Microfabricated three-dimensional environments for single cell studies

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Most of what is known about cells and their functional regulation has been derived from cell cultures performed on flat, mostly rigid culture surfaces, such as the ubiquitous dish introduced by Julius Petri in 1877. However, results obtained in two-dimensional cell cultures (2D) often lacked the power to predict, for example, the toxicity of drugs in a whole organism[1] or the biocompatibility of synthetic materials.[2] Increasing evidence suggests that placing a cell on a flat 2D substrate versus into a three-dimensional (3D) matrix can have major effect on cell behavior, from adhesion and differentiation to apoptosis.[3,4] For example, the phenotype of breast cancer cells can be reversed, when cultured in a 3D collagen matrix, to a normal phenotype by blocking a specific integrin receptor, an effect which has never been observed in standard 2D cultures.[5] Cells deposited onto detergent-insoluble 3D fibronectin (FN)-rich matrices developed a type of adhesive contact, termed 3D matrix adhesions, with a molecular composition different from their 2D counterparts.[4] These discrepancies are in part due to the nonphysiologically high stiffness of traditional culture substrates relative to their *in vivo* extracellular matrix (ECM) counterparts, the induction of apical/basal polarity in normally nonpolar cells, the development of abnormal shapes on planar surfaces, or the absence or presence of cell-cell contacts.[21]

Materials derived from animals or cell cultures clearly mimic the *in vivo* situation more closely through presentation of copious amounts of molecularly distinct binding sites in a spatially organized fibrillar structure, and our understanding of cell behavior in 3D substrates has greatly benefited from these matrices. However, the microstructural, biochemical, and mechanical properties of cell- and tissue-derived matrices are highly complex and correspondingly difficult to control in a systematic and quantitative manner. If we look more closely at the microenvironment that a single cell experiences in a confluent cellular monolayer [Fig. 1(a)] or a native environment *in vivo* [Fig. 1(b)], the heterogeneous and spatially organized adhesive structures and forces present *in vivo* must be reconsidered if we are to accurately emulate the cell's *in vivo* microenvironment in an engineered 3D culture system *in vitro*.

Alternative, synthetic approaches to biomaterials with better controlled 3D properties have therefore been successfully put forward, for instance self-assembled peptide nanofibers,[6] synthetic hydrogels,[7] and fibrous collagen-based matrices.[8] By direct photopatterning of poly(ethylene glycol) (PEG) gels containing cells[9] or soft lithography techniques to structure collagen gels[10] [Fig. 2(a) (V)], complex 3D organizations of multiple cell types were achieved with structures on the order of 200 $\mu$m. Myocytes cultured on 3D microtextured poly(dimethylsiloxane) (PDMS) substrates, exhibiting a combination of grooves and pillars, showed a difference in cell shape, gene expression, and protein distribution[11] [Fig. 2(a) (IV)]. The distribution of sarcomeric striation was highly influenced by the adhesion of the cells to the vertical pillars. These widely varying approaches offer vast potential for instance in tissue engineering applications where multiple cell types and molecularly and mechanically distinct matrix components must be organized in 3D patterns and forms.[12] However, it becomes difficult to interpret and compare results obtained with dissimilar 3D culture systems in the absence of a widely accepted standard for 3D culture [Fig. 2(a)]. In addition, while gel- or engineered polymer-based 3D systems allow some control over bulk mechanical properties of the matrix, cells respond to the local rigidity which is often not well defined and may become heterogeneous due to cellular remodeling of their extracellular network structures.

Despite our rich appreciation for the importance of 3D cellular environments for normal cell function, no established experimental 3D culture systems currently exist which allow for control of the shape of individual cells or cell cultures. However, a quantitative analysis of the external factors which regulate cell function in a 3D context requires cell shape control since it is well established from 2D studies that constraining cell shape or degree of spreading via micropatterned adhesive islands in a noninteractive background determines whether cells proliferate or apoptose,[13] whether hu-

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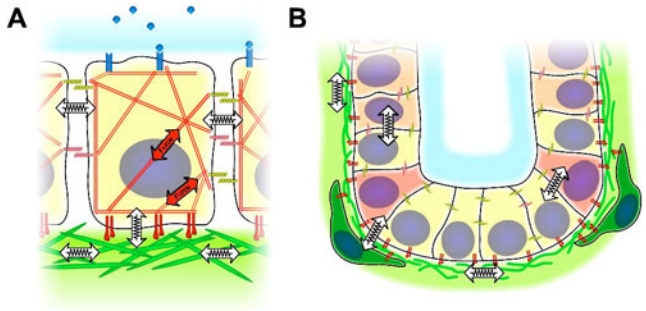


FIG. 1. (a) Schematic representation of an adherent cell in a confluent monolayer showing different cues of the microenvironment which govern cell function [adapted and modified from Pirone *et al.* (see Ref. 39)]. Cells are influenced by soluble cues such as growth factors and other media conditions and by insoluble cues that are adhesive and mechanical in nature. The latter include attachment of the cell to the extracellular matrix (ECM, green) and binding to other cells. Forces are generated by the actin/myosin machinery and transmitted to and through the ECM and to neighboring cells; they control the overall 3D shape of the cells and play an important role, together with all other cues, in governing cell behavior. (b) Schematic representation of the microenvironment (or so called "niche") in an intestinal stem cell (ISC) niche of the mammalian gut crypt *in vivo*. Stem cells (red) are found in specific locations above the paneth cells (yellow) present at the crypt base. Stem cell progeny (orange), known as transit amplifying cells, move upwards and differentiate. Underlying mesenchymal cells (green) send signals that help regulating stem cell activity. This represents only a simplified model of the 3D organization of the ISC.

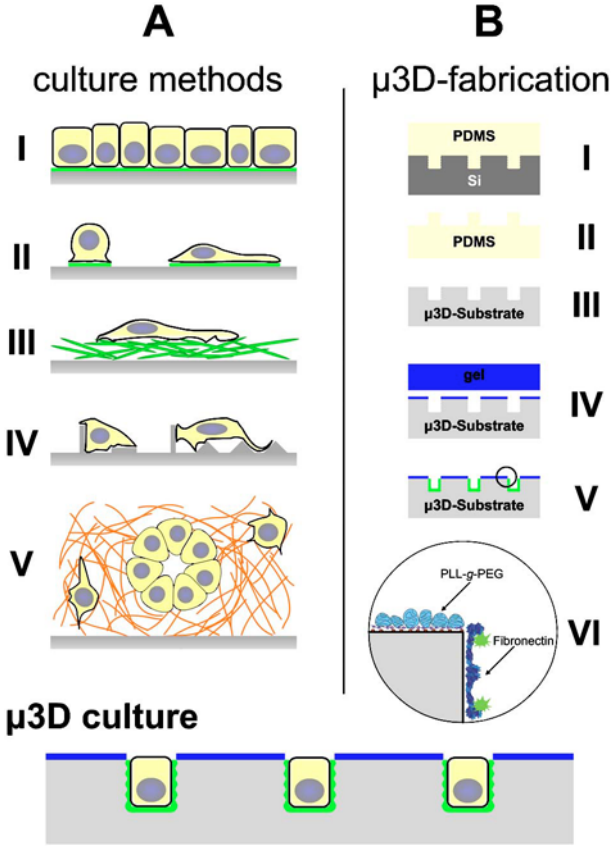


FIG. 2. (a) Different methods to culture cells *in vitro*. (I) Dense monolayer culture of epithelial cells with 3D aspects of adhesipn due to cell-cell contacts. (II) Cells with different shapes on 2D adhesive islands of different size (see Ref. 13). (III) Cells on top of so-called 3D cell-derived FN matrices (see Ref. 4). (IV) Cells interacting with topographically structured substrates (see Ref. 11). (V) Single cells or aggregates inside a 3D collagen gel (see Ref. 8 and 10). (b) Fabrication of $\mu$3D culture substrates (I–III) Overview of process steps for the replication of a primary master structure into various materials such as PS, PDMS, or PEG hydrogels using an intermediate PDMS master replicated from microfabricated Si. (IV–VI) Scheme of the inverted microcontact printing method. The plateau surface is contacted with a flat stamp to transfer a PEG-*graft*-copolymer rendering those areas noninteractive. The surface of the microwells is backfilled with a cell-adhesiye protein, such as FN. Comment: Although (a) (III), (IV), and (V) are considered 3D culture concepts, these approaches generally result in heterogeneity with respect to cell morphology, cell polarity and local mechanical properties of the matrix. The $\mu$3D culture approach may reduce these limitations.

man mesenchymal stem cells differentiate into adipocytes or osteoblasts,[14] and might drive cell polarity during mitosis through orientation of the cytoskeleton.[15] We envision model culture systems that allow for quantitative control of 3D cell shape independent of the other properties of the microenvironment and therefore advance our understanding of how form and function are related in single cells, cellular ensembles, and finally in organs and organisms, a highly philosophical subject that has interested intellectuals since the dawn of modern man.[16–20]

To allow for a quantitative control of the shape of either individual cells or cell clusters in 3D, microfabricated wells are needed that allow tight regulation of relevant physical and biochemical parameters. Substrate rigidity must also be tightly tunable in an attempt to more closely match the microenvironment of cells *in vivo* (as reviewed by Discher *et al.*[21]). Typical soft tissues *in vivo* present a range of elastic properties, with a Young's modulus in the range of hundreds of pascals (Pa), while modified extracellular matrix production or components contribute to stiffnesses of up to a few thousands of Pa in contractile healing wounds[22] or tumor stroma.[23] Polyacrylamide gels and PDMS with variable mechanical properties were used to demonstrate that substrate stiffness regulates cell spreading, cell migration speed,[24] focal adhesion formation,[25] and differentiation of cells,[26] and these findings ultimately led to the current paradigm that numerous mechanoresponsive cell signaling pathways exist (as reviewed by Vogel and Sheetz[3] and Chen *et al.*[27]).

We thus explored the fabrication of micro-3D ("$\mu$3D") culture systems exhibiting arrays of microwells with different shapes and dimensions made from different materials such as polystyrene (PS), (3 MPa), PDMS with tunable mechanical properties (1–1000 kPa), and PEG hydrogels (100–1000 Pa). By combining replication techniques with inverted microcontact printing of a protein-resistant PEG-*graft*-copolymer on PDMS or PS substrates, we have successfully limited protein adsorption and cell adhesion to the inside of the microwells.[28] The surfaces, walls and floor, of this first generation of microwells were homogenously coated by fibronectin.

First studies of single endothelial cells captured in individual microwells indicate unique distributions of cytoskeletal and other subcellular components, which were highly influenced by the 3D shape of the microwells (Fig. 3). Interestingly, we found less prominent actin structures at the bottom or apical surface of cells in microwells in comparison to

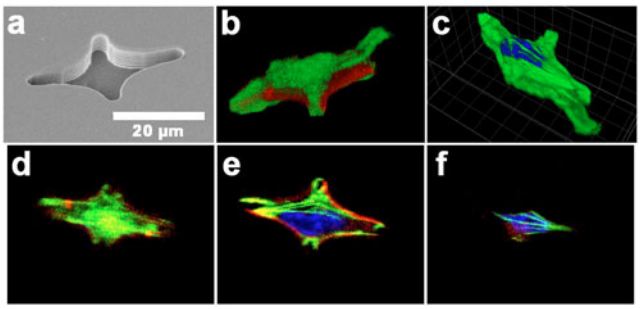


FIG. 3. Primary human endothelial cell inside a spindle-like microstructure after 16 h of culture. (a) Scanning electron microscopy image of the empty microstructure fabricated in PDMS according to Fig. 2(b). (b)–(c) CLSM 3D reconstruction of the cell viewed from the top and from below, respectively. (d)–(f) Confocal $z$ stacks of the cell (taken at different distances from the surface): top of cell (d), center (e), bottom (f). Actin is shown in green, FN in red, nucleus in blue. The actin cytoskeleton organization is strongly guided by the shape of the microwell, with the fibers aligned in direction of the long axes and influenced by the two corners of the short axis. The fibers are distributed in 3D throughout the cell volume. The nucleus shows a unique distorted shape as a result of constraints induced by the geometry of the microwell. The nucleus is embedded in the oriented actin fiber network.

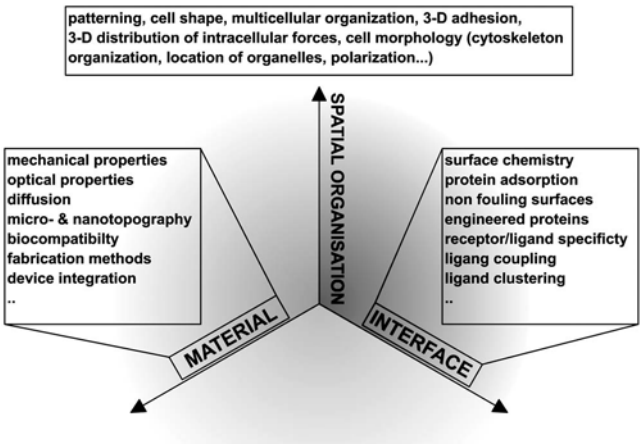


FIG. 4. Three different aspects of how the microenvironment of cells can be engineered: Choice of material type and fabrication techniques, the biointerface, and the spatial organization of cues taking into account the 3D aspect of a biological system in nature.

2D glass controls.[29] Exact control of the full 3D shape of cells turned out to be more challenging than patterning adhesive contact area, which merely limits degree and geometry of spreading in 2D. The third dimension adds another degree of freedom for cell adhesion, and proper control of cell shape was only achieved if the volume of the cell exactly fit the volume of the microwells, which is only the case for a fraction of cells due to their broad volume distribution in a given cell culture. Distinct advantages of our $\mu$3D substrates is the cost effectiveness of production, ease-of-use, the compatibility with high-resolution confocal microscopy, and the possibility to produce them in large numbers and store them until use in a biology laboratory without the need of having access to expensive microfabrication facilities.

In the long term, the ability to control the 3D cell shape of single cells will allow new biological questions to be addressed. The surface chemistry and spatial distribution around a single cell may be important deterministic parameters of cell behavior. The spatial organization of ligands in 2D, for example, can regulate T-cell activation[30] and control cell adhesion and spreading.[31–33] It may also be possible to decouple the contributions from surface anchored molecules to the biochemical communication provided by adjacent cells. To pursue such goals, one challenge that must be overcome is to selectively coat the walls and floor with molecularly distinct ligands, which would more accurately mimic both cell-ECM and cell-cell interactions. The relevance of such studies is highlighted by recent findings that cells in a confluent monolayer seem to loose their rigidity response to the underlying 2D substrate,[34] and that stress concentrations within populations of cells on a patterned 2D surface lead to a locally differentiated proliferative response among the cells that experience an increased contractile stress.[35]

Another possible direction is to incorporate a controlled number of different cell types organized in designed microenvironments, for instance adult stem cells in combination with other niche cells to create *in vitro* models of stem cell niches,[36] as shown in Fig. 1(b). Those niches are thought to present a complex microenvironment of multiple cell types and ECM to the stem cells, and their ability for self-renewal or differentiation probably depends highly on proper spatial organization.[37,38]

Finally, arrays of engineered 3D cell substrates have significant potential to probe in high-throughput screens the relationship between drug efficacy and the physical and biochemical parameters of given cell environments, thereby improving their predictive power. This approach allows for detection of anomalous points of outliers, within a single population, information which would be missed when only comparing population averages between groups. These future directions highlight our current approach to engineer environments for single cells or aggregates, where aspects including materials properties, interface functionalization, and spatial organization should be considered (Fig. 4). Progress will heavily depend on collaborative efforts and open communication between material scientists, to develop smart functional materials serving as sensing and actuating elements, biomedical engineers and molecular biologists, to provide engineered proteins and cells, computer scientists, to expedite analysis of rapidly growing data sets, and engineers, to finally integrate these systems into high-throughput lab-on-a-chip devices.

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