Research News

Engineering Microtools in Polymers to Study Cell Biology

By *Xingyu Jiang* and *George M. Whitesides**

Recent advances in surface engineering and soft lithography provide tools to fabricate patterned surfaces and microfluidic devices with dimensions comparable to the sizes of single mammalian cells. These technologies enable the studies of individual cells on spatially well-defined, patterned surfaces, and in contact with patterned liquid media. They provide information about cells impossible to obtain from traditional biochemical techniques.

1 Introduction

In attached cell culture, the cell contacts both the liquid buffer in which it is immersed and the solid surface of the dish to which it is attached. *In vivo*, it is in contact with extracellular liquids, gels and compliant surfaces (the extracellular matrix and the surfaces of other cells). Much of the current understanding of the biology of cells comes from attached cell culture. Historically, the study of cells in culture has used only simple, homogeneous surfaces and solutions, and these studies have provided only limited clues to the ways in which cells sense and respond to their environments. (The physiological environment is, of course, highly heterogeneous in the composition of the surfaces and liquids that contact cells.) Surface engineering and new techniques in microlithography begin to make it possible to pattern the molecular structure and topography of surfaces, and the molecular composition of liquids, in dimensions comparable to individual cells and in physiologically relevant scales [1]. This article outlines some newly developed methods for studying cell biology with spatially well-controlled patterns on surfaces and in fluids. It also describes some other types of microtools useful for the studies of cell biology.

2 Using Molecularly Defined, Patterned Surfaces to Control the Attachment and Spreading of Cells

2.1 Static Patterning of Cells Using Surface Chemistry

In order to control and study single cells in physiologically relevant ways, we started with controlling the attachment of cells onto surfaces with well-defined chemistry. Using soft lithography [2] and self-assembled monolayers (SAMs) of alkanethiolates on gold, we can pattern the adsorption of extracellular matrix (ECM) proteins onto surfaces and thus control the attachment of cells, and the extent and geometry of cell spreading. The key strategy in this work is to differentiate the surface into regions that adsorb proteins from solutions, and regions that do not: adsorbed proteins are required for the attachment and spreading of cells. SAMs provide the capability that is needed. SAMs terminated in methyl groups adsorb proteins irreversibly; SAMs terminated in oligo(ethyleneglycol) groups (-$(EG)_n$, n = 3–6) do not allow proteins to adsorb [3]. Typically, a methyl-terminated alkanethiolate was first printed onto a gold-coated surface (using an elastomeric stamp molded from prefabricated microstructures) to form a patterned hydrophobic SAM. Hydrophobic surfaces adsorb ECM proteins from solutions and promote the attachment of cells. The remaining area of the gold surface not covered by the hydrophobic SAM was then passivated toward adsorption of proteins by adsorbing an $(EG)_n$-terminated SAM. When this patterned surface was exposed to a solution containing a protein that promotes cellular attachment (e.g., fibronectin, laminin, collagen), these proteins adsorbed on the hydrophobic, methyl-terminated regions. These are the only regions that support attachment of cells; cells do not attach to areas where proteins do not adsorb. Fig. 1A gives an example: this figure shows a single bovine capillary endothelial (BCE) cell confined to a square pattern.

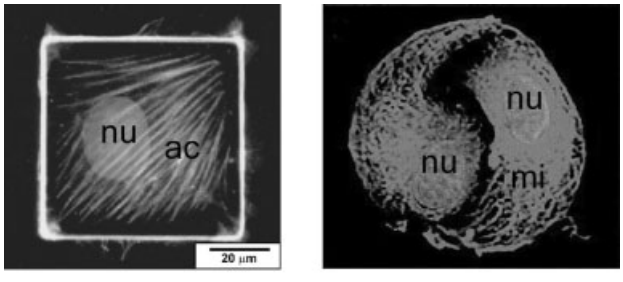

Figure 1. A) A single BCE cell confined to a square pattern. The cell was resting on methyl-terminated SAMs coated with fibronectin; the surrounding is made up of (EG)3-terminated SAM that does not adsorb proteins. The fibres are actin filaments (ac); the ellipse is the nucleus (nu). B) Two BCE cells confined to a circular pattern spontaneously formed a Yin-Yang pattern that rotated (in this case, counterclockwise). The cells were then fixed and stained for mictotubule (mi) and nuclei (nu). The same scale bar in (A) applies.

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Controlling the size and shape of attached cells provides control of cellular attachment, spreading, differentiation and death [4]. Most types of mammalian cells need to attach to surfaces to survive [5]. Their levels of survival and differentiation are directly related to the extent that cells are spread. Whether or not a cell undergoes apoptosis (programmed cell death) is also determined by the extent to which it is spread [6]. By determining the size of the area over which the cells spread, we can control the fate of attached cells: to live or to die. Using combinations of different sizes of cell-substrate attachment areas, we can control the passage of a cell through the cell cycle [7].

Patterning cells also generated novel biological pattern formation on the level of individual cells [8]. Spontaneous symmetry-breaking events occurred when two BCE cells were confined to microislands: two of these cells confined to a circular island rotated counterclockwise continuously (Fig. 1B).

Other groups have also used tools of soft lithography to generate defined patterns of proteins and cells. For example, Bernard *et al.* formed patterns of proteins with micron-scale fidelity by stamping proteins directly onto different kinds of substrates [9]. Chen and coworkers realized high-precision patterning of several proteins and cell types using a single stamp prefabricated to have different levels of topography [10]. Boxer and coworker used micropatterned protein-membrane patterns to control cell adhesion [11].

2.2 Dynamic Patterning of Cells

Patterning cells onto discrete areas of attachment on the surface has one drawback: the pattern cannot be changed once it is formed, and cells are confined to the original pattern. Combining SAM-based technology and organic synthesis, Mrksich and coworkers made substrates that allow the attachment of cells to be controlled electrochemically [12]. The strategy was based on the preparation of a surface that was composed mainly of $(EG)_n$-terminated SAM, but also contained a low density of a SAM terminated in a hydroquinone group. This surface resisted the adsorption of proteins [13]. When the surface was subjected to an oxidative pulse of current, the terminal hydroquinone groups were oxidized to quinone groups. A ligand containing a cyclopentadiene group tethered to the amino acid sequence arginine-glycine-aspartate (RGD – a minimal sequence necessary for the attachment of cells to the ECM [14]) was immobilized onto the quinone-terminated SAM (through a Diels-Alder reaction between the quinone and the dinene). This process converted a surface that was initially inert to cellular attachment into one to which cells could attach, using an electrochemical trigger [12]. Using a related procedure, an electrochemical bond cleavage step made possible the release of RGD moieties from a surface. In this process, attached cells lost their anchors for attachment to the surface, and were released [15].

Another technology based on soft lithography – membrane-based patterning or MEMPAT – used polydimethylsiloxane (PDMS) membranes carrying pores (50 to 500 μm) to position cells precisely on a surface [16]. The membrane was first passivated by adsorption of bovine serum albumin (BSA) onto its surface to resist adsorption of serum proteins and attachment of cells. When this passivated membrane was placed in conformal contact with a plastic surface, and then exposed to a solution containing proteins that allow cellular attachment, the exposed regions on the plastic surface (defined by the pores in the membrane) adsorbed these proteins. Cells would then attach to these regions, and spread and fill the pores as a monolayer. When the membrane that confined the cells was removed (by peeling it away mechanically by hand), the constraints on the cells were eliminated and the cells spread and migrated across the surface. Fig. 2 illustrates this process. Toner and coworkers have used a similar approach to micropattern cells in culture [17].

These new tools – both electrochemical and mechanical – allow cells to be initially confined, and then released; they are promising technologies for studies in mechanistic cell biology and screening of molecules that influence the migratory activities of cells.

2.3 Patterning Cells Using the Topography of Surfaces

In addition to surface chemistry, the behaviors of cell are also influenced by topography on the surface. To study the effects of surface topography on cell growth, we fabricated surfaces simultaneously patterned with tailored topography and controlled surface functionality; Fig. 3 illustrates such an example. Fig. 3A shows "V"-shaped wave structure on the

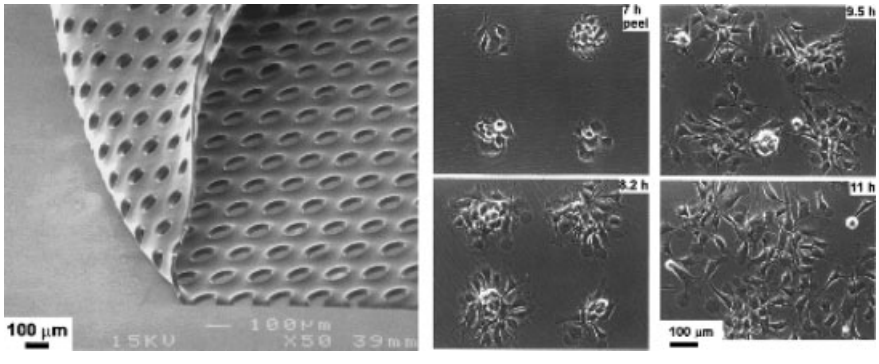

Figure 2. MEMPAT: A) Scanning electron micrograph of a piece of membrane with 100 μm circular holes; this membrane is ca. 50 μm thick. A part of the membrane was lifted out of contact with the substrate to illustrate its flexibility. B) Demonstration that MEMPAT allows the study of cell spreading. BCE cells were patterned on Petri dishes. After 7 h in culture, the membrane was removed ("7 h peel"). Subsequent images show cell spreading as a function of time.

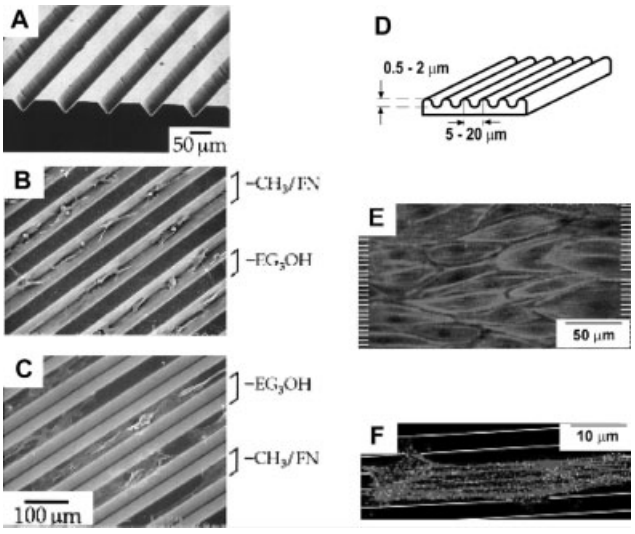

Figure 3. Topographical control of cell spreading and subcellular structures; A) An SEM image of one of the features used. B) Cells only grew in the grooves. C) Cells only grew on the plateaus. "CH_3" indicates stripes patterned with methyl-terminated SAMs. FN, fibronectin adsorbed on methyl-terminated SAM. "EG3OH" indicates areas patterned with SAMs terminated in EG3. D) Schematic of the features used in a diffierent approach in controlling spreading with surface topography alone. E) A monolayer of cells on these features. The grids on both sides of the picture indicated the wavelengths and peaks of the underlying substrates. F) The actin cytoskeleton of a single cell was oriented along the features. The white lines indicated the wavelengh and peak of these features.

surface fabricated by unconventional lithography and etching. Evaporation of a thin layer of gold on the surface of the substrate makes this technology compatible with existing SAM technology: methyl-terminated SAMs promoted adsorption of proteins and attachment of cells, while $(EG)_3$-terminated SAM resisted the adsorption of proteins and attachment of cells. Surface chemistry of the grooves and the plateaus can be controlled separately [18]. Cells can be grown selectively in the grooves or on the plateaus: this technology is a general one capable of preparing optically transparent substrate patterned both in topography and chemistry to study the topographical and molecular interactions between cells and substrates.

In another approach, we developed a technique that forms ordered, sinusoidal waves on the surface of polydimethylsiloxane (PDMS) to control the spreading of cells and the distribution of subcellular structures. This procedure requires only that the PDMS surface be exposed to an oxygen plasma while it is under mechanical stress. The plasma oxidation formed a stiff, thin, oxidized top surface layer; relieving the mechanical stress placed this thin layer under compressive stress and resulted in spontaneous formation of surface waves [19]. Fig. 3D shows a schematic of the features and Fig. 3E shows a monolayer of BCE cells spread on the undulating surface. Not only were the cells aligned, but the subcellular actin filaments were oriented as well (Fig. 3F). The behaviors of cells on these topographies help us understand how cells sense the topographical component of their microenvironment [19].

3 Patterning Fluids in Contact with Cells: Laminar Flows and PARTCELL

Patterning fluids would seem a more difficult task than patterning surfaces. Patterns that consist of stripes of fluid with different compositions are surprisingly easy to prepare, although other types of patterns (e.g., spots or squares) are not. The key to patterning fluids is the use of laminar flows. In multistream flows through small channels that occur at low Reynolds numbers (Re), different layers of fluids flow side by side without turbulent mixing. The Reynolds number (Re, Eq. (1)) is a dimensionless parameter describing the tendency of flowing liquids to develop turbulence. In Eq. (1),

$$\mathrm{Re} = DV\rho/\mu \qquad (1)$$

D is the characteristic dimension of the channel (m), v is velocity of flow (m/s), ρ the density (g/m^{-3}), and μ viscosity (Pa s). Flows with Re > 1000 are usually turbulent (mixing happen quickly by convection); flows with low Re (< 1) are laminar (mixing happens slowly, by diffusion), unless special structures and mechanisms are used to induce mixing. Making D small ensures that the flow of liquids in small capillaries occurs at a low Re, and is thus laminar. Parallel, laminar flows provide a useful, new tool to pattern proteins and cells into stripes. Fig. 4 sketches the protocol used in these types of patterning [20]. The strategy used is to lay down different stripes of proteins side by side using laminar flows, and then employ biospecific ligand-receptor interactions (involving mannose, an *E. coli* surface receptor in this case) to generate patterns of cells.

A *part* of a single cell can also be addressed using laminar flows [21]. In a method we called PARTCELL (short for "partial treatment of cells using laminar flows"), we passed adjacent, laminar flows of different fluids across a cell, with the boundaries between the flows separating the cells into segregated regimes (Fig. 5). This process makes it possible to "paint" different parts of a cell surface with dyes or reagents; when these chemicals diffuse across the membrane, it is possible to use the same technique to localize the concentrations of these materials *inside* the cell.

We have used this technique to obtain information about the motion of intracellular organelles and the cytoskeleton. For example, we passed membrane-permeable red and green dyes across the surface of a BCE cell, and allowed diffusion of the dyes into its interior, to label mitochondria in different parts of the same cell (Fig. 5C); we could then follow the motion of the populations of differently dyed mitochondria. The two populations of mitochondria (here labeled red and green), moved around and intermixed into a homogeneous population over an interval of 2.5 hours (compare Fig. 5C with 5D). We can use this method to monitor the motion of other subcellular organelles or disrupt certain processes within one cell conveniently.

Microinjection and microperfusion experiments attempt to achieve similar goals as PARTCELL. Dotti and coworkers

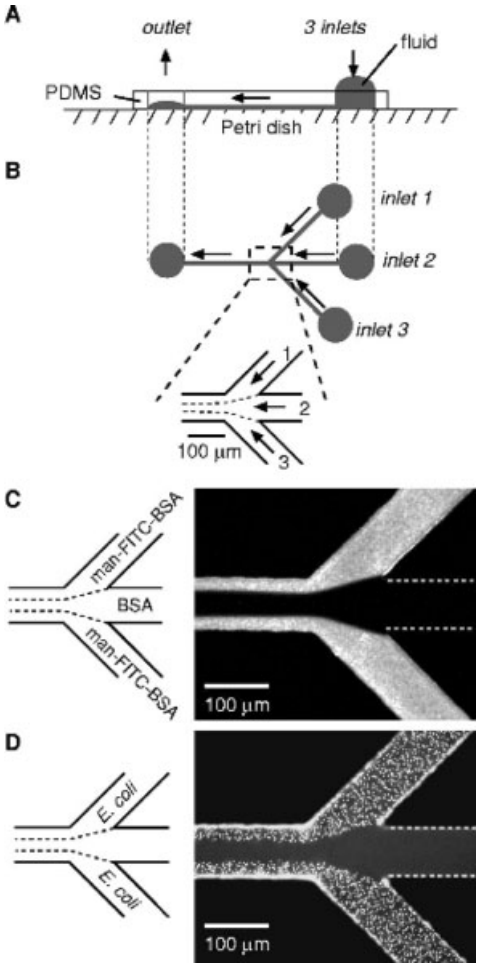

Figure 4. Patterning proteins and cells with laminar flows, A) Side view of the channel system. A PDMS membrane with channels molded in its surface was placed on a Petri dish to form a network of capillaries. B) Top view of the network. Micrographs show the junction where the inlets converge. C) Patterns of adsorbed protein created by laminar flow. Solutions of fluorescently labeled BSA (man-FITC-BSA, BSA derivatized with mannose and fluorescein) that *E. coli* binds to and unlabled BSA, were allowed to flow from the designated inlets into the main channel. D) The channels shown in (C) were filled with *E. coli*, and these bacteria were allowed to adhere and imaged through a nucleic acid dye. White dotted lines identify the boundries of the channels not visible with fluorescence microscopy.

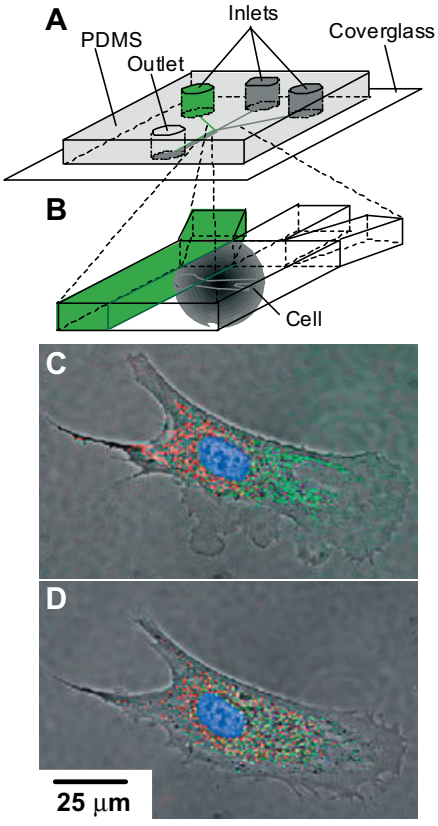

Figure 5. PARTCELL: using laminar flows to manipulate different parts of a BCE cell; A) B) experimental setup; B) is a magnified view at the junction of the channel system. C) Overlay of the phase contrast and fluorescence images of three colors: phase contrast image outlined the cell morphology, the left half of the cell was stained with a red dye that binds to mitochondria, the right half with a green dye that binds to mitrochondria, while the nucleus was stained blue; D) The same cell in (C) 2.5 hours later.

have microinjected cytochalasin D (a drug that disrupts actin filaments) into neuronal cell processes, and found that depolymerization of actin filaments in one of these processes accelerated its growth; this process eventually became the axon of the neuron [22]. This experiment showed that local perturbation of cellular processes can influence cellular development. Fischmeister and coworkers have microperfused frog cardiac myocytes locally with drugs that elevate the levels of cyclic adenyl monophosphate (cAMP, a second messenger that is known to be involved in many cellular processes), and observed localized changes in the activities of Ca^{2+} channels, downstream targets of cAMP [23]. The authors concluded that spatially well-defined cAMP distributions (localized high or low concentrations of cAMP) were the ways in which different parts of a single cell responded to diverse cues on different areas of its surface, using the same messenger system based on cAMP.

PARTCELL has two advantages compared with these techniques. *i)* It is a general method that does not require a specialized cell type (neurons have compartmentalized neurites and myocytes are large cylindrical cells; both geometries prevent rapid diffusion of small molecules to unwanted areas). *ii)* No complicated micromanipulation is required. The interface between microchannels and the macroscopic world is quite straightforward for the experimentalist. Using these tools to pattern the fluids of media in contact with cells, we are starting to understand how local changes and heterogeneities in solution chemistry can affect the activities of cells.

4 Gradients in Solutions and on Surfaces

Gradients of biomolecules are important in a wide variety of biological processes ranging from chemotaxis to embryogenesis [24–26]. For instance, in development, gradients of morphogen are believed to be responsible for the induction

of different levels of gene expression at various locations, thereby achieving cellular differentiation that ultimately leads to formation of tissues and organs [24]. In adult tissues, concentration gradients of chemo-attractant mediate migration of cells to different locations (e.g., at sites where new blood vessels need to form [25]). Unicellular organisms swim along gradients of nutrients for sources of food [26]. The technical limitations in making gradients of desired shapes have made it inconvenient for researchers to study cell biology in various gradients. In order to study the response of cells to different kinds of gradients, we developed technologies to fabricate gradients in arbitrary geometries, using microfluidic networks [27,28]. This method is based on controlled diffusive mixing of species in solutions that flow laminarly, inside a network of microchannels. The microchannel network combined two or three inputs and split them, then recombined and resplit them repetitively. The two initial liquids were made into multiple fluid streams carrying step gradients that were combined into one main channel (Fig. 6A). In the direction perpendicular to the flow, gradient formed in dimensions between 900 and 2500 mm, depending on the size of the main channel. Combining several of these microfluidic networks formed complex gradients (Fig. 6C). Using the same approach under appropriate conditions, we can transfer gradients in solution onto gradients on the surface. We can thus form gradients of many shapes, both in solution and on surfaces. We believe that the gradient generator technology will enable biologists to investigate cell biology in gradients of biomolecules conveniently.

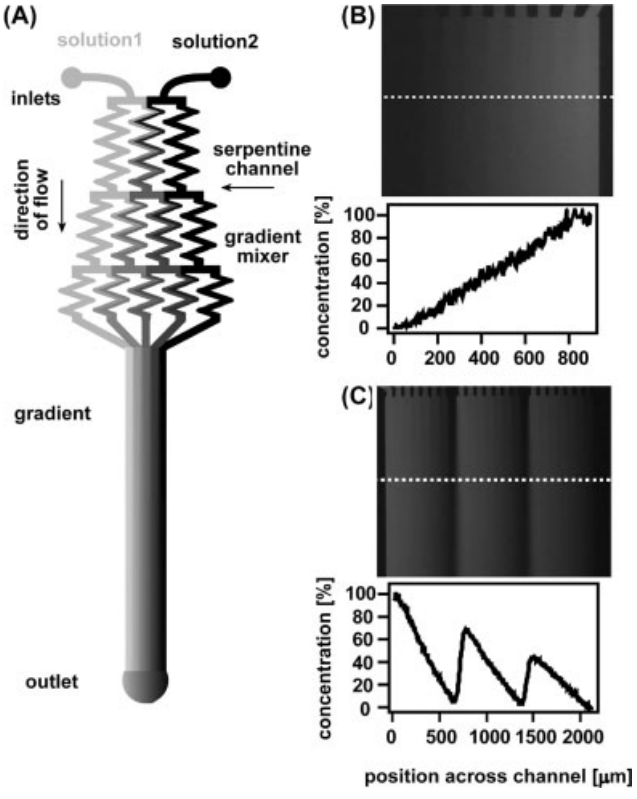

Figure 6. Formation of gradients of arbitrary shapes using microfluidic networks; A) A gradient (perpendicular to the direction of flow) was generated through a networks of small channels that were split and recombined repetitively, and finally recombined. B) shows the fluorescence intensities of a gradient of fluorescein-labelled fibrinogen. C) shows that combination of several gradient generators can produce complex gradients.

5 Other Micro-Scale Methods to Perturb the Cell Locally

Ingber and coworkers have utilized magnetic microbeads (~ 5 μm) to study cellular responses to applied mechanical stress. Using so-called "magnetic twisting cytometry" – a method capable both of applying stress to cell surface and of measuring the cellular response thereafter –, his group directly observed transduction of mechanical signal from the outside into the inside of the cell through integral membrane receptors [29]. It was also possible to quantify the abilities of these receptors to transmit mechanical signals into the cytoskeleton of the cell [30].

Optical methods, such as optical tweezers, have advanced our understanding of biology on the single cell level under different contexts [31–33]. Using lasers focused at small volumes, single cells and organelles can be trapped and moved; these experiments produced information about the influence of forces on these single cells and organelles.

Photoactivation and photobleaching of fluorescent molecules in select local areas of a cell have been used to examine the movement of subcellular components (for example, tubulin in the mitotic spindle [34] and in the axon of a neuron [35]). These techniques generate locally bright or dark regions inside the cells; the movements of these local regions provide information on the motion of subcellular compartments.

Scanning probe microscopy has also begun to find uses in studies of cell biology. Gaub and coworkers have used atomic force microscopy to study the adhesion forces between two cells of *Dictyostelium discoideum* and quantified the unbinding forces and rupture rates [36].

Other groups have also made use of devices and tools microfabricated in polymers to study biology [10,37–46]. Toner and Bhatia used soft lithography to culture hepatocytes and nonparenchymal cells and produced physiologic levels of albumin from the hepatocytes that would otherwise not secrete albumin at such levels [45]. Craighead and coworkers have printed polylysine onto microelectrodes to define the growth of hippocampal neurons [40]. Quake and coworkers used soft lithography to make a fluorescence-activated cell sorter to separate *E. coli* that were fluorescent and from those that were not [37]. Takayama and coworkers have fabricated a disposable microfluidic cytometer based on laminar flows of air and liquid inside small capillaries [46]. Sohn and coworkers have used the so-called "capacitance cytometry" to measure the DNA content of single cells in PDMS microchannels and thus classify cells according to their positions in the cell cycle [42]. Private companies, such as Surface Logix (Cambridge, MA, USA), Fluidigm (South San Francisco, CA, USA) and Micronics (Redmond, WA, USA), have also been instru-

mental in developing novel technologies useful for cell biology and analytical biochemistry.

6 Conclusions

SAMs and soft lithography now make it practical to fabricate microdevices useful in cell biology. These devices offer exquisite control over surface chemistry, e.g., ligands designed for biospecific interactions and inert surfaces that resist adsorption of proteins and ttachment of cells. Soft lithography makes possible the rapid fabrication of patterns on surfaces and in microfluidic channels. Surfaces with defined chemistry and topography generate arrays of cells both in static patterns and arrays of cells that can be released from their original confinement. Microchannel systems allow the use of laminar flows to generate steady-state patterns in flowing liquids used to pattern the surface and interior of cells (PARTCELL, gradients). Using polymers, such as PDMS, to fabricate these microdevices offers other advantages: they make experiments easy to observe (since PDMS is transparent to light); they can be easily fabricated by micromolding or by other soft lithographic tools; they can be coupled to techniques, such as electrochemistry; and they are nontoxic to cells. Polymeric devices whose dimensions are comparable to individual cells are useful in probing how information from the environment is sensed, propagated and processed within a single cell; much of this information is not possible to derive from traditional biochemical studies.

We believe that developing tools capable of even greater spatial and temporal control will open up new avenues in the studies of cell biology: many problems in basic cell biology require physiologically relevant surfaces and liquids with well-defined patterns; tissue engineers would also make use of devices that can accurately define cell-substrate interactions and cell-cell interactions to make artificial tissues or organ replacements.

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