Chapter Nineteen

Micro-Scale Patterning of Cells and Their Environment

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I. INTRODUCTION

Control of the cellular environment is crucial for understanding the behavior of cells and for engineering cellular function (Jiang and Whitesides, 2003; Whitesides *et al.*, 2001; Xia and Whitesides, 1998). This chapter describes the use of a set of tools in microfabrication, called *soft lithography*, for patterning the substrate to which cells attach, the location and shape of the areas to which cells are confined, and the fluid environment surrounding the cells, all with micrometer precision. We summarize examples where these tools have helped to control the microenvironment of cells and have been useful in solving problems in fundamental cell biology. The methods described here are experimentally simple, inexpensive, and well suited for patterning biological materials.

How do tissues assemble *in vivo*? How do cells interact with each other in tissues? How do cells respond to stimuli? How do abnormal stimuli give rise to pathological conditions? Answering these fundamental biological questions and using the information thus obtained for medical applications requires understanding the behavior of cells in well-controlled microenvironments. Many of the challenges in trying to control the environment experienced by individual cells lie in the relevant scales of size as well as the character of the stimuli. These scales of size range from angstroms (for molecular detail), through micrometers (for an individual cell), to millimeters and centimeters (for groups of cells); the types of stimuli that must be addressed include the molecular composition of the liquid in which the cell is immersed, the topographical and chemical composition of the surface to which the cells attach, the nature of neighboring cells, and the temperature.

Microfabrication and micropatterning using stamps or molds fabricated from elastomeric polymers (*soft lithography*) provide versatile methods for generating patterns of proteins and ligands on surfaces, microscale chambers for culturing cells, and laminar flows of media in capillaries, all in the size range of 0.1–100 micrometers (Gates *et al.*, 2005; Jiang and Whitesides, 2003; Whitesides *et al.*, 2001; Xia and Whitesides, 1998). Soft lithographic methods are relatively simple and inexpensive. The elastomeric polymer most often used in these procedures — polydimethylsiloxane (PDMS) — has several characteristics (optical transparency, ease of manipulation, and low cost) that make it attractive

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for biological applications (McDonald and Whitesides, 2002). Although a new technology when compared to molecular biology, soft lithography is being increasingly used in cell biology, due to its biocompatibility, simplicity, and adaptability to biological and biochemical problems. This chapter gives an overview of the application of soft lithography to the patterning of cells and their fluidic environment, using micro-scale features and laminar flows.

Researchers have used a number of techniques to pattern cells and their environment (Letourneau, 1975). Before the 1990s, the most common was photolithography. This technique has been highly developed for the microelectronics industry; it has also been adapted, with varying degrees of success, for biological studies (Kleinfeld *et al.*, 1988; Letourneau, 1975; Ravenscroft *et al.*, 1998). Examples have included topographical features that confine the growth of snail neurons to silicon chips, as first demonstrations of interfacing natural computation with artificial ones (Mérz and Fromherz, 2005; Zeck and Fromherz, 2001). As useful and powerful as photolithography is (it is capable of mass production at 70-nm resolution of multilevel, registered structures), it is not always the technique best suited for biological studies. It is an expensive and time-consuming technology; it is poorly suited for patterning nonplanar surfaces; it provides too little control over surface chemistry to pattern sufficiently diverse types of biomolecules on surfaces; it is poorly suited for patterning materials such as hydrogels; the equipment required to use it is rarely routinely accessible to biologists; and it is directly applicable to patterning only a limited set of photosensitive materials (e.g., photoresists).

II. SOFT LITHOGRAPHY

Soft lithography solves many of the problems that required the application of microfabrication to biological problems (Chen *et al.*, 2005; Jiang and Whitesides, 2003; Whitesides *et al.*, 2001). Soft lithographic techniques are inexpensive, are relatively procedurally simple, are applicable to the complex and delicate molecules often dealt with in biochemistry and biology, can be used to pattern a variety of different materials, are applicable to both planar and curved substrates (Jackman *et al.*, 1995), and do not require stringent control (such as a clean-room environment) over the environment in which they are fabricated beyond that required for routine experiments with cultured cells (Whitesides *et al.*, 2001; Xia and Whitesides, 1998). Access to photolithographic technology is required only to create a master for casting the elastomeric stamps or membranes; even then, the requirement for chrome masks — the preparation of which is one of the slowest and most expensive steps in conventional photolithography — can often be bypassed in favor of high-resolution printing (Deng *et al.*, 2000; Linder *et al.*, 2003). Soft lithography offers special advantages for biological applications, in that the elastomer most often used (PDMS) is compatible with most types of optical microscopy commonly used in cell biology, is permeable to gases such as O_2 and CO_2, is mechanically flexible, seals conformally to a variety of surfaces (including most types of petri dishes), is generally biocompatible (Lee *et al.*, 2004), and can be implantable *in vivo*. The soft lithographic techniques that we discuss include microcontact printing, micromolding, patterning with microfluidic channels, and laminar flow patterning.

III. SELF-ASSEMBLED MONOLAYERS (SAMs)

Introduction to SAMs

Since many of the studies involving the patterning of proteins and cells using soft lithography have been carried out on self-assembled monolayers (SAMs) of alkanethiolates on gold, we give a brief introduction to SAMs (Allara and Nuzzo, 1985; Bain and Whitesides, 1988; Jiang *et al.*, 2004b; Prime and Whitesides, 1991; Ulman, 1996). SAMs are organized organic monolayer films normally formed by exposing a surface of a gold film to a solution containing an alkanethiol (RSH). SAMs allow control at the molecular level by chemical synthesis of derivatized alkanethiol(s); this molecular control, in turn, gives control over the properties of the interface. The properties of surfaces covered with SAMs are often largely or entirely determined by the nature of the terminal groups of these alkanethiols. The ease of formation of SAMs and their ability to present a range of chemical functionality at their interface with aqueous solution make them particularly useful as model surfaces in studies involving biological components. Furthermore, SAMs can be easily patterned by simple methods such as microcontact printing (μCP) with features down to 100 nanometers in size (Love *et al.*, 2005; Xia and Whitesides, 1998). These features of SAMs make them structurally the best-defined substrates for use in patterning proteins and cells. SAMs on gold are used for many experiments requiring the patterning of proteins and cells, because they are biocompatible, easily handled, and chemically stable. SAMs on silver, although better defined structurally than those on gold, cannot be used in most experiments with cultured cells, due to the toxicity of silver (Ostuni *et al.*, 1999). SAMs on palladium and platinum are just starting to be explored (Jiang *et al.*, 2004a; Petrovykh *et al.*, 2006).

The substrates for SAMs are easy to prepare; once formed, SAMs are stable for weeks under conditions typical for culturing cells. Gold substrates are prepared on glass coverslips or silicon wafers by evaporating a thin layer of titanium or chromium (1–5 nm) to promote the adhesion of gold to the support, followed by a thin layer of gold (10–200 nm) (Lopez *et al.*, 1993). SAMs formed on these gold substrates are stable to the conditions used for cell culture, but care should be taken to avoid strong light and temperatures above ~70°C since both can result in degradation of the SAM (J. Huang and Hemminger, 1993; Love *et al.*, 2005).

Preventing Protein Adsorption: "Inert Surfaces"

Proteins play an integral part in the adhesion of cells with surfaces: Cells require adsorbed proteins (or peptides that mimic parts of a protein) to adhere to the surface (Ruoslahti and Pierschbacher, 1987). Control of the interaction of proteins with a surface, therefore, enables the control of the interactions of cells with that surface. Most solid surfaces — especially hydrophobic surfaces — adsorb proteins. Thus, the main challenge in controlling the interactions of proteins and cells with surfaces lies in finding surfaces that resist nonspecific adsorption of proteins (surfaces that we call *inert*, for brevity). Inert surfaces provide the background necessary for spatially restricting protein adsorption or for preparing surfaces that bind only specific proteins and are used in patterning proteins and cells, as biomaterials (Andrade *et al.*, 1996; Han *et al.*, 1998), and in the construction of biosensors (Mrksich and Whitesides, 1995).

SAMs terminated in oligo(ethylene glycol) (EG_n, $n > 2$) resist the adsorption from solution of all known proteins and their mixtures (Prime and Whitesides, 1991, 1993; Whitesides *et al.*, 2001). We know that EG_n groups are not unique in their ability to make inert SAMs. For example, several polar functional groups that do not contain H-bond donors often make good components of inert surfaces (Chapman *et al.*, 2000; Kane *et al.*, 2003). The combination of inert and adsorptive surfaces with soft lithographic techniques enables the facile patterning of proteins and cells. Patterns of hydrophobic regions (for example, SAMs terminated in methyl groups) and regions that are "inert" provide the basis for most work using patterned cells.

A number of other substances also make the surface more or less inert. Many of them are used in connection with soft lithography, for example, bovine serum albumin (BSA) and related proteins (Sweryda-Krawiec *et al.*, 2004), man-made polymeric materials (e.g., polyethyleneglycol, or PEG) (Jeon *et al.*, 1991), and dextran (Frazier *et al.*, 2000).

IV. MICROCONTACT PRINTING AND MICROFEATURES USED IN CELL BIOLOGY

Patterning Ligands, Proteins, and Cells Using Microcontact Printing on SAMs

Microcontact printing (μCP) is a technique that uses topographic patterns on the surface of an elastomeric PDMS stamp to form patterns on the surfaces of various substrates (Fig. 19.1) (Xia and Whitesides, 1998). The stamp is first "inked" with a solution containing the patterning component, the solvent is allowed to evaporate under a stream of air, and the stamp is brought into conformal contact with the surface of the gold film for intervals ranging from a few seconds to minutes. The thiol transfers to the gold film in the regions of contacts. Other components used as ink for μCP include activated silanes that react with the SiOH groups ($RSiCl_3$ or $RSi(OCH_3)_3$) present on the surface of silicon (with a native film of SiO_2) and various ligands (such

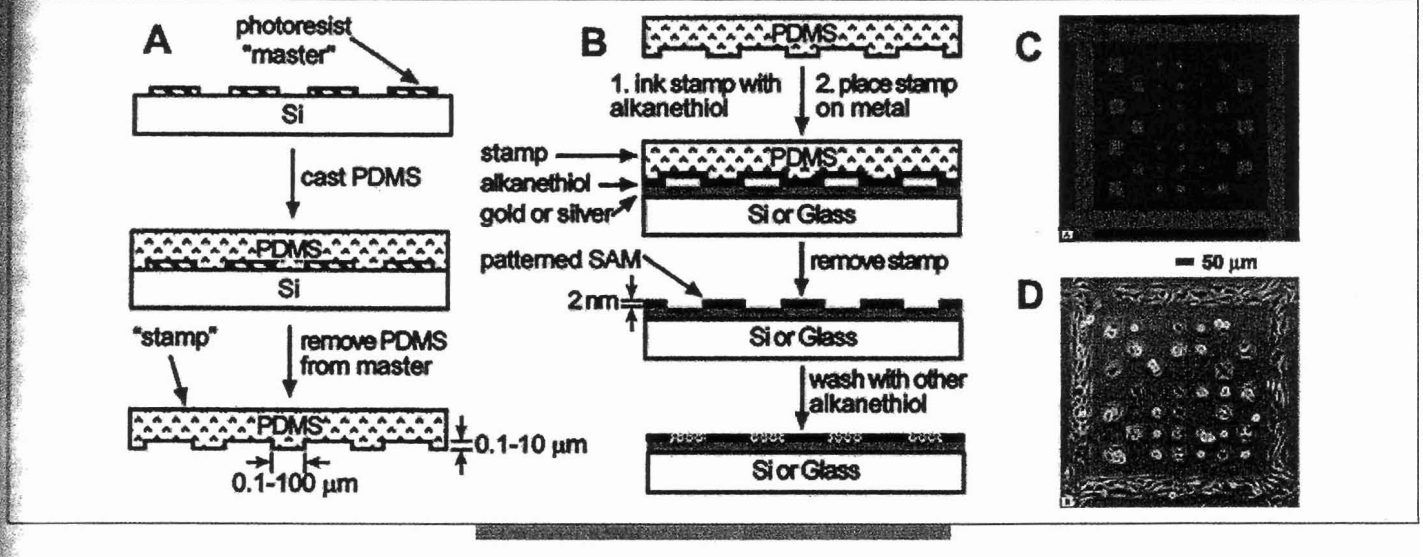

FIG. 19.1. Molding from a master, microcontact printing (μCP), and patterning of proteins and cells. **(A)** A method for generating stamps (also applicable for channels and other molds) of PDMS for μCP. A PDMS stamp is prepared by pouring PDMS liquid prepolymer on a "master" (generally generated by photolithography), followed by curing the PDMS and removing it (as an elastomeric solid) from the master. **(B)** A typical procedure used for μCP. A solution containing the patterning component of interest (ink) is applied to this stamp and the solution allowed to dry. This inked stamp is placed on a substrate to allow the ink to transfer to the substrate. A substrate patterned with SAMs remains after removal of the stamp. **(C)** Selective adsorption of fibronectin onto a surface patterned by SAMs into areas that either promote or resist the adsorption of proteins, by μCP, visualized by immunostaining. **(D)** Patterned attachment and spreading of cells on the protein-patterned substrate in C.

as amine-containing compounds) that react with activated SAMs (usually resulting in the formation of a peptide bond that tethers ligands with surfaces) (Lahiri *et al.*, 1999; Yan *et al.*, 1998).

The most general and reliable method for patterning proteins is accomplished by preparing areas of SAMs that promote the adsorption of proteins, surrounded by regions that resist adsorption of proteins (regions that we call *inert background*), and allowing proteins to adsorb onto the adsorbing regions from solutions. For example, we used microcontact printing to pattern gold surfaces into regions terminated in methyl groups and then filled the rest of the surface of gold with an oligo(ethylene glycol)-terminated thiol to form inert regions (Lopez *et al.*, 1993). Immersion of the patterned SAMs in solutions of proteins such as fibronectin, fibrinogen, pyruvate kinase, streptavidin, and immunoglobulins resulted in adsorption of the proteins exclusively on the methyl-terminated regions (Lopez *et al.*, 1993). Characterization of patterns of adsorbed proteins with electron and optical microscopy shows that the layers of adsorbed protein appeared to be homogeneous. Alternatively, proteins can be anchored to ligands patterned onto surfaces by μCP; for example, μCP of biotin onto activated SAMs allows the biospecific immobilization of avidin on the surface (Lahiri *et al.*, 1999).

The ability of μCP to create patterns of ligands and proteins allows the patterning of many anchorage-dependent cells (most normal cells in multicellular organisms are anchorage dependent) (Alberts *et al.*, 2002); this patterning confines them to specific regions of a substrate and allows the precise control of the size and shape of the cells (Fig. 19.1). For example, μCP allows the partition of the surface of gold into regions presenting EG_n groups and methyl groups (Mrksich and Whitesides, 1996). After we coated the substrates with fibronectin, bovine capillary endothelial cells attached only to the methyl-terminated, fibronectin-coated regions of the patterned SAMs. The cells remained attached in the patterns defined by the underlying SAMs for five to seven days. We have also used SAMs on palladium for confinement of mammalian cells (Jiang *et al.*, 2004a). EG-terminated SAMs on palladium allow the patterning of individual cells, groups of cells, as well as focal adhesions (subcellular complexes that enable cell-substrate attachment, FA) for over four weeks; similar SAMs on gold confined cells to patterns for one to two weeks.

Other Types of Microcontact Printing

It is possible to pattern certain proteins (ones that can withstand drying onto the surface of the stamp and stamping) directly onto surfaces (Bernard *et al.*, 1998, 2000, 2001; Mayer *et al.*, 2004; St. John *et al.*, 1998). Direct patterning of proteins, however, is typically applicable only to structurally stable proteins and is usually more demanding experimentally than patterning via SAMs (Kam and Boxer, 2001). The surface of the PDMS stamp used in this type of procedure must be rendered hydrophilic by exposure to a plasma before use (Bernard *et al.*, 2000).

Patterning cells directly with μCP was thought to be unfeasible, because most cells are too delicate to be dried or stamped. Recently, however, we have demonstrated the stamping of proteins and cells directly with a soft hydrogel stamp (agarose) that contains large amounts of water (Mayer *et al.*, 2004; Stevens *et al.*, 2005). The resolution of this technique (tens of micrometers) is not comparable to μCP with PDMS and SAMs, but it makes patterning cells at this large size range easier than patterning with SAMs.

Other workers have used μCP for different types of cells on other types of surfaces. Craighead and coworkers patterned polylysine on surfaces of electrodes to confine the growth of neurons (James *et al.*, 2000). Zhang *et al.* have synthesized oligopeptides containing a cell-adhesion motif at the N-terminus connected by an oligo (alanine) linker to a cysteine residue at the C-terminus (Zhang *et al.*, 1999). The thiol group of cysteine allowed the oligopeptides to form monolayers on gold-coated surfaces. They used a combination of microcontact printing and these self-assembling oligopeptide monolayers to pattern gold surfaces into regions presenting cell-adhesion motifs and oligo(ethylene glycol) groups that resist protein adsorption. Wheeler *et al.* created patterns of covalently bound ligands and proteins on glass coverslips and used these patterns to control nerve cell growth (Branch *et al.*, 1998). In addition, polymers of EG and supported phospholipids have been used on a series of different substrates for pattering cells (Amirpour *et al.*, 2001; Kam and Boxer, 2001; Michel *et al.*, 2002; Tourovskaia *et al.*, 2003).

Dynamic Control of Surfaces

It is possible to modulate the ability of surfaces to promote the adhesion of cells by controlling the composition of the surfaces. A relatively simple method to achieve this control is to desorb EG-terminated SAMs electrochemically from a substrate patterned with cells in a buffer containing proteins that promote attachment of cells (Jiang *et al.*, 2003). Electrochemical desorption converts inert areas into regions that can promote the adsorption of proteins and adhesion of cells, and thus it allows initially confined cells to move out of their patterns (Fig. 19.2). Mrksich and coworkers have used electrochemical conversion of a hydroquinone-terminated SAM into a quinone-terminated SAM to allow the attachment of a cyclopentadiene-tether peptide (which allows the immobilization of cells via the binding of integrin receptors on cell surface) to "turn on" an otherwise-inert SAM for adhesion of cells (Yousaf *et al.*, 2001a). Mrksich has used this technique for the patterning of multiple types of cells on surfaces (Yousaf *et al.*, 2001b). A newer technique from the same group involves first desorption of an immobilized ligand (patterned in certain areas on the surface) and detachment of cells adhered to the surface via this ligand, on application of an electrical reduction, and a subsequent electrical oxidation of the substrate for immobilizing another

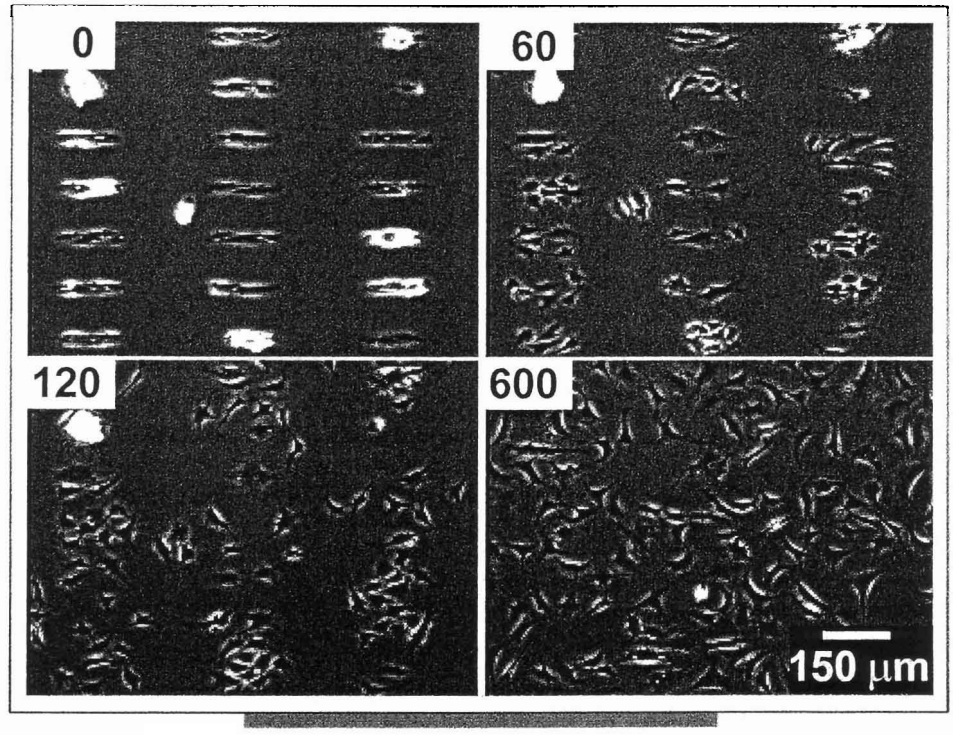

FIG. 19.2. Using electrochemical desorption of EG-terminated SAMs to release confined BCE cells and allow their free movement. Initially, bovine capillary endothelial cells are confined to a patterned array of hydrophobic regions (SAMs terminated in methyl groups) and surrounded by regions that are "inert" (SAMs terminated in EG-terminated groups). Application of a cathodic voltage desorbs the EG-terminated SAMs to allow cells initially confined to move freely on the surface. The numbers indicate time after application of electrochemical treatment (in minutes).

ligand that allows the migration of adherent cells (that were initially confined to certain areas on the surface) (Yeo *et al.*, 2003). Some of the methods for preparing the appropriate tools employed by Mrksich *et al.* may be too complicated for routine use in a regular cell biology laboratory, but the demonstration of such sophisticated control of the interactions between the cell and solid substrates is unprecedented. Another set of electrochemical methods can be used to activate BSA-rendered inert surfaces for cell adhesion (Kaji *et al.*, 2004, 2005).

There is a set of photochemical methods that allow the tuning of the inertness of SAMs. Mrksich and coworkers have devised a SAM that is initially inert but has in it a nitroveratryloxycarbonyl-protected hydroquinone, which can be oxidized photochemically to generate a benzoquinone group, which, in turn, allows the attachment of a ligand for immobilization of cells on surfaces (Dillmore *et al.*, 2004). Another method for photoactivation of the surface utilized a photochemical process that desorbed a ligand from SAMs on surfaces. BSA, initially physically adsorbed on the surface via a photocleavable 2-nitrobenzyl group–terminated SAM, made this surface inert; photochemistry-mediated desorption of the 2-nitrobenzyl group, and therefore BSA, allowed cells to adhere to the surface (Nakanishi *et al.*, 2004). Both techniques allow the use of the mercury lamp on a fluorescent microscope of the kind typically used for experiments with cultured cells to carry out the required electrochemistry.

Takayama and his coworkers fabricated substrates that allow the dynamic control of focal adhesions (FAs). They first generated a slab of PDMS with a brittle surface by means of oxygen plasma treatment and then made the surface inert by means of physical adsorption of a polymer containing moieties of PEG (Zhu *et al.*, 2005). By stretching the slab, they created cracks on the surface, which are not covered with the polymer-containing PEG; these cracks could thus promote the formation of FAs and adhesion of cells. Releasing the stress on the slab PDMS closed these cracks and again prevented the adhesion of cells. This stretch-and-release process could be recycled multiple times.

A number of other techniques also allow the patterning and dynamic patterning of proteins and cells in time and space. These techniques are related to soft lithography in one way or another (Co *et al.*, 2005; Kumar *et al.*, 2005; Ryan *et al.*, 2004).

Patterning with Microtopographies

Microtopographies include membranes with microholes, microwells, microneedles, and grooves or steps on surfaces. It is possible to confine cells to micropatterns using either elastic membranes that carry holes or microwells (Folch *et al.*, 2000; Ostuni *et al.*, 2000, 2001). Some of these techniques also allowed the initial confinement, then release, of groups of cells (Folch *et al.*, 2000; Ostuni *et al.*, 2000).

Chen and his coworkers fabricated stamps with multiple levels that allowed the patterning of several different types of proteins and cells at once (Tien *et al.*, 2002). Chen *et al.* also used bowtie-shaped microwells of agarose gel both to confine individual cells to particular shapes and to allow cells to be close to each other without mutual contact (Nelson and Chen, 2002). Positioning cells next to each other while preventing their direct contact is difficult to achieve with μCP alone. They also fabricated arrays of micropillars (in sizes much smaller than a single cell) to

probe forces that cells apply to the substrate as they adhere to and migrate on solid surfaces (Tan *et al.*, 2003). Tien and coworkers succeeded in molding microstructures in hydrogels of resolution larger than 5 micrometers and used these structures to generate arrays of cells in three dimensions (Tang *et al.*, 2003).

We also studied the issue of topographical contact guidance — how cells interact with chemically homogeneous surfaces that have topographical features. These studies provide simple methods for further studies of this interesting and complex type of interactions (Jiang *et al.*, 2002; Lam *et al.*, 2006; Takayama *et al.*, 2001b).

Fundamental Studies in Cell Biology Using Patterned Substrates

The ability to pattern proteins, groups of cells, single cells, and their FAs has led to new studies on the effect of patterned surface environments and cell shape on cell behavior.

Our first attempt in these studies was to prepare (via μCP) substrates consisting of square and rectangular islands of laminin surrounded by inert regions and to study the behavior of rat hepatocytes on them (Singhvi *et al.*, 1994). The cells conformed to the shape of the laminin patterns; the patterning allowed the control of cell shape independent of the density of ligands in the extracellular matrix (ECM). We observed that cell size, regardless of ECM ligand density, was the major determinant of cell growth and differentiation. We then used μCP to prepare substrates that presented circular cell-adhesive islands of various diameters and interisland spacings (Chen *et al.*, 1997; Dike *et al.*, 1999). Such patterns allowed the control of the extent of cell spreading without varying the total cell-matrix contact area. We found that the extent of spreading (the projected surface area of the cell), rather than the area of the adhesive contact, controlled whether the cell divided, remained in stationary phase, or entered apoptosis.

Dike *et al.* (1999) used μCP to prepare substrates with cell-adhesive lines of varying widths. They found that bovine capillary endothelial cells (BCE) cultured on 10-μm-wide lines underwent differentiation to capillary tube–like structures containing a central lumen. Cells cultured on wider (30 μm) lines formed cell–cell contacts, but these cells continued to proliferate and did not form tubes.

Recent progress in understanding how cell adhesion regulates cell physiology has used methods related to micropatterning. In several model types of cells, the strength of cell–substrate adhesion increased as the allowed area of cell adhesion increased, for small areas (typically less than 300 μm^2); but the strength of adhesion remained constant for larger areas (Gallant *et al.*, 2005; Tan *et al.*, 2003). These results related cell spreading and the strength of cell attachment empirically. Within the focal adhesion (FA), integrin receptors need to aggregate in order to activate the appropriate biochemical pathways for adhesion of cells (Assoian and Zhu, 1997; Hotchin and Hall, 1995; Schwartz *et al.*, 1991). It has not been clear, however, at what maximum separation integrin receptors can still perform their normal functions. Bastmeyer has used different combinations of micropatterns to determine that cells could adhere to surfaces with arrays of circles with area of 0.1 μm^2, when the spacing between these circles is less than 5 μm; but when the separation between these circles is larger than 30 μm (and when the circles are larger than 1 μm^2), cells fail to adhere and spread on these surfaces (Lehnert *et al.*, 2004). This result gives a semiquantitative description of the geometrical requirements for clustered integrins.

Using a combination of self-assembly of nanoparticles and micropatterns, Spatz and colleagues definitively determined the maximum distances (73 nm) between individual integrin receptors in order for normal cell adhesion to occur (Arnold *et al.*, 2004). Another recent report shows that when FAs mature into larger-than-normal sizes, they appear to exert four times as much stress on the surface than normal FAs; this result might be important for myogenesis, the process of the formation of muscle fibers (Goffin *et al.*, 2006).

Micro-scale features enable the studies of the movement of mammalian cells. The extension of lamellipodia is an important process in the movement of cells. Bailly *et al.* (1998) used micropatterned substrates to study the regulation of lamellipodia during chemotactic responses of mammalian carcinoma cells to growth factors. On stimulation with epidermal growth factor, the cells extended their lamellipodia laterally out of their patterns of confinement, over the inert part of the substrate. This result showed that the extension of lamellipodia could occur independent of any contact with the substrate. Contact formation was, however, necessary for stabilizing the protrusion. We further observed that when endothelial cells were confined to patterns with corners (such as triangles and squares), their lamellipodia tended to spread most actively from the corners of these shapes (Brock *et al.*, 2003; Parker *et al.*, 2002).

Most moving mammalian cells adopt asymmetric shapes. We have examined whether the asymmetry in the shape of a cell is connected to the direction of its movement (Jiang *et al.*, 2005a) (Fig. 19.3A). Since moving cells often appear to have a teardrop shape, we confined cells to teardrop-shaped patterns and then used electrochemistry to allow free cell movement. It is tempting to assume that the released cells would move toward the sharp end of the teardrop pattern, considering that lamellipodia tend to extend from sharp corners; but a teardrop-shaped cell resembles a naturally moving cell (with the *blunt* end being the front) (Fig. 19.3). The conflicting observations left unclear the direction in which a teardrop-shaped cell would move once released. We released teardrop-shaped cells electrochemically and determined that these preshaped cells appear to prefer moving toward their blunt ends.

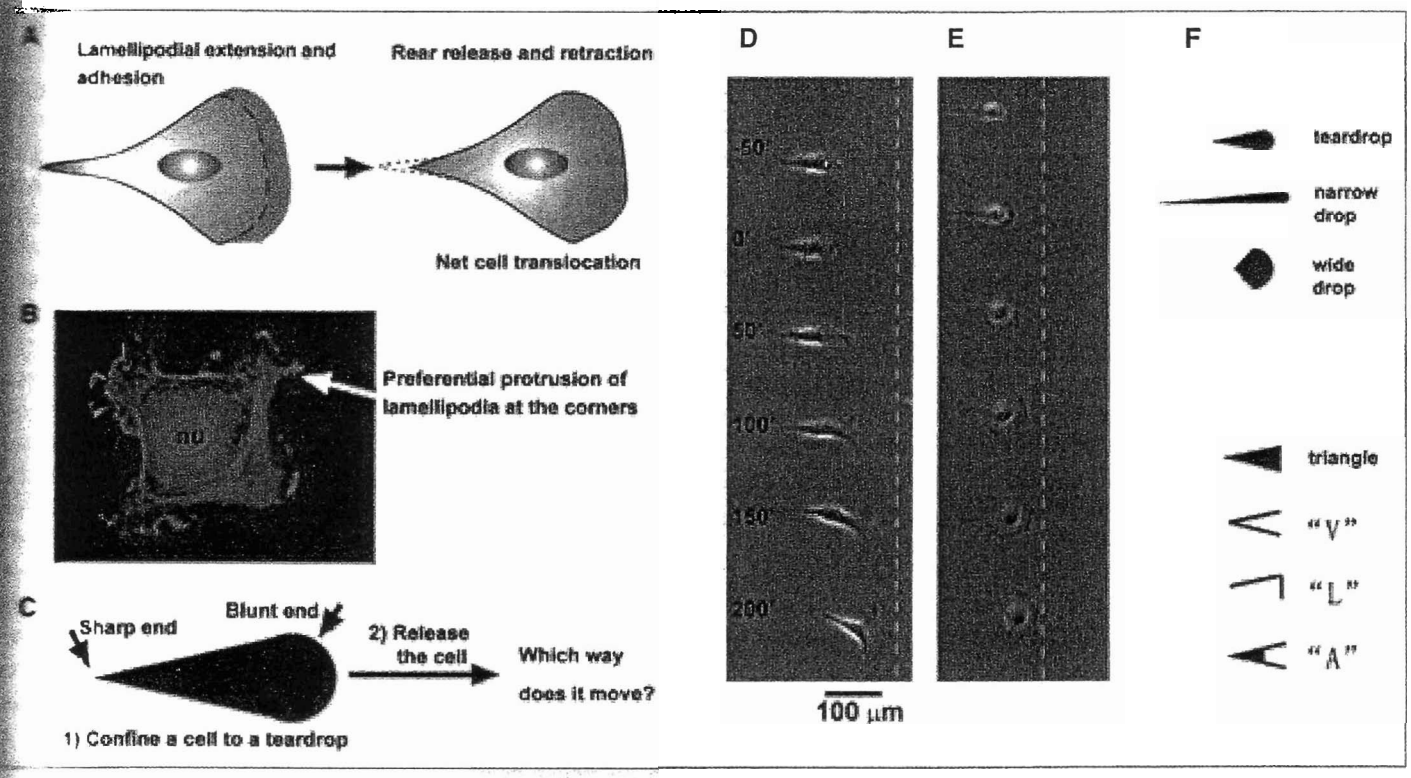

FIG. 19.3. Understanding the relationship between cell shape and the direction of cell migration. **(A)** A typical migrating cell has an asymmetric, teardrop-like shape and moves toward the blunt end. **(B)** When confined to shapes that have sharp corners, lamellipodia tend to extend out of the sharp corners. **(C)** A cell initially confined to a teardrop shape and then subsequently released may choose which way it moves: toward its blunt or sharp end. **(D) (E)** Individual 3T3 cells and COS cells, respectively, were initially confined to teardrop shapes and then released to move freely across the surface. The movement was predominantly toward the blunt end. Numbers indicate time after the application of electrochemical potential (minutes). **(F)** Different initial patterns used to confine cells.

To understand the issue in detail, we varied the initial shapes. It appeared that "narrow drops" (regardless of details of the shape) had a similar capability to direct cell migration, while "wide drops" failed to do so. Triangular patterns (having a similar aspect ratio to the teardrop) also direct cell migration, to the same extent as the teardrop, further confirming that the asymmetry of the initial shape alone could direct cell movement. In contrast, symmetric patterns such as rectangles, squares, and circles failed to direct cell migration. To determine whether it was the shape of the spread cell or the uneven distribution of FAs underneath the cell that was responsible for directed cell motion, we started cells on "L"-, "V"-, and "A"-shaped patterns. Because individual cells could span these patterns, the immobilized cells resembled each other in overall geometry, and their geometry was similar to that of cells confined to triangular patterns. But these patterns allowed different distributions of FAs. Triangles and "L"-shaped cells allowed more FAs in the blunt end, "V"-shaped patterns allowed the same amount of the FAs in the blunt end as in the sharp end, while "A"-shaped patterns allowed more FAs in the sharp end. All these patterns appeared to direct cell migration to the same extent, thus confirming that the overall shape of cells was the determining factor in directing cell motion.

Micron-scale tools based on soft lithography have also been used in studies of cell division. Even though most mammalian cells round up and almost completely detach from the substrate when they divide, Bornens' group has used μCP to show that the shape of the ECM to which cells initially attach determines the direction of cell division (Thery *et al.*, 2005).

Micropatterns could also bias the differentiation of human mesenchymal stem cells: When allowed to adhere and spread, the stem cells became osteoblasts; when spreading was prohibited by small patterns of confinement, stem cells became adipocytes (McBeath *et al.*, 2004).

These tools offer opportunities to study not just single cells, but groups of cells. For example, Chen and coworkers devised experiments to control the size and the contact between a pair of two cells, thus definitively proving that cell–cell contact, not soluble factors alone, enable cell contact–mediated proliferation of cells in culture (Nelson and Chen, 2002). Toner and colleagues showed, by patterning of hepatocytes and nonparenchymal cells with precise geometrical parameters, that the interface between the two types of cells is critical for the function of hepatocytes (Bhatia *et al.*, 1999). Ingber and his coworkers discovered, using patterned groups of two or more endothelial cells, that

spontaneous ordering arises and patterns that resemble the Chinese Yin-Yang ideograph would emerge while endothelial cells migrate on the patterns (Brangwynne *et al.*, 2000; S. Huang *et al.*, 2005). Studying groups of tens to hundreds of cells patterned into defined geometries, Chen and coworkers realized that the shapes of sheets of cells influence the mechanical forces each cell within experiences, and these forces affect the physiology of individual cells differently, as a function of where in the sheets these cells are located (Nelson *et al.*, 2005).

V. MICROFLUIDIC PATTERNING

The use of microfluidic channels allows patterning surfaces by restricting the flow of fluids to desired regions of a substrate. The patterning components — such as ligands, proteins, and cells — are deposited from the solution to create a pattern on the substrate.

Delamarche *et al.* used microfluidic patterning (μFP) to pattern immunogloblins with submicron resolution on a variety of substrates including gold, glass, and polystyrene (Delamarche *et al.*, 1997). Only microliters of reagent were required to cover square millimeter-sized areas. Patel *et al.* (1998) developed a method to generate micron-scale patterns of any biotinylated ligand on the surface of a biodegradable polymer. These investigators prepared biotin-presenting polymer films and patterned the films by allowing solutions of avidin to flow over them through 50-μm channels fabricated in PDMS. The avidin moieties bound to the biotin groups on the surface and served as a bridge between the biotinylated polymer and biotinylated ligands. Patterns created with biotinylated ligands containing the RGD or IKVAV oligopeptide sequences determined the adhesion and spreading of bovine aortic endothelial cells and PC12 nerve cells.

Both our group and Toner's group used μFP to produce patterns of adsorbed proteins and adherent cells on biocompatible substrates (Chiu *et al.*, 2000; Folch *et al.*, 1999; Folch and Toner, 1998). We formed micropatterns of proteins deposited from fluids in separately addressable capillaries. By allowing different cell suspensions to flow through different channels, we could pattern two types of cell on surfaces with high spatial precision. After the adhesion of two types of cells in different areas on the surface, we could remove the elastomeric stamps to allow the studies of the movement of two types of cells. By filling individual channels with different fluids, multiple components could be patterned at the same time without the need for multiple steps or the accompanying technical concerns of registration (although registration was required in the fabrication of the stamp itself).

VI. LAMINAR FLOW PATTERNING

Laminar flow patterning (LFP) is a technique that can pattern surfaces and the positions of cells on them in useful ways (Takayama *et al.*, 2001b). It can also pattern fluids themselves (Takayama *et al.*, 1999). This technique utilizes a phenomenon that occurs in microfluidic systems as a result of their small dimensions — that is, low-Reynolds-number flow (Squires and Quake, 2005; Stroock and Whitesides, 2003). The Reynolds number (Re) is a parameter describing the ratio of inertial to viscous forces in a particular flow configuration; it is a measure of the tendency of a flowing fluid to develop turbulence. The flow of aqueous fluids in capillaries usually has a low Re and is laminar. Laminar flow allows two or more streams of fluid to flow next to each other without any mixing other than by diffusion of their constituent molecules across the boundary between them (which is usually fairly slow). Diffusional motion of particulate components (e.g., cells) is even slower.

In a typical setup for LFP experiments, a network of capillaries is made by sealing a patterned PDMS slab with a glass slide or the surface of a petri dish (Fig. 19.4) (Takayama *et al.*, 1999). By passing streams of fluid with different compositions from the inlets, patterns of parallel stripes of flowing fluid are created in the main channel. It is possible, therefore, to treat different parts of a single cell with different reagents if a cell happens to span these stripes. Fig. 19.4

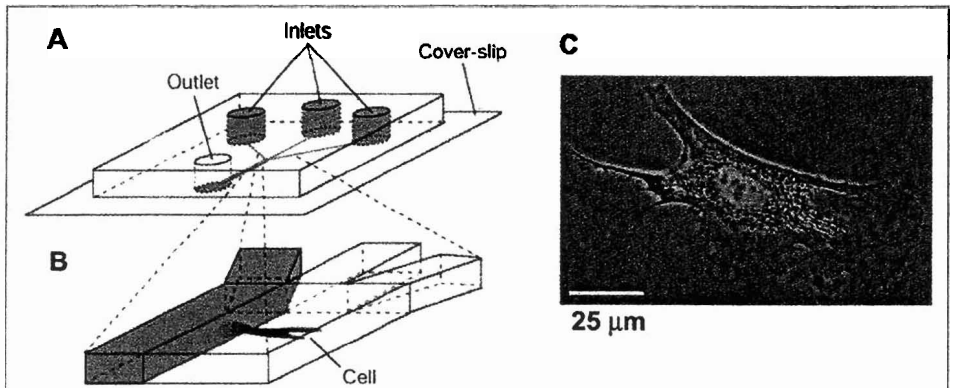

FIG. 19.4. Manipulation of two regions of a single bovine capillary endothelial cell using multiple laminar flows. **(A)** Experimental setup; **(B)** shows a close-up of the point at which the inlet channels combine into one main channel. **(C)** Fluorescence images of a single cell after treatment of its right pole with Mitotracker Green FM and its left pole with Mitotracker Red CM-H2XRos. The entire cell is treated with the DNA-binding dye Hoechst 33342.

illustrates the painting of a single cell with dyes that stained mitochondria located in different parts of the same cell (Takayama *et al.*, 2001a, 2003).

Using this technique, the positions and microenvironments of cells can be controlled simultaneously in several stripes in the same channel (Gu *et al.*, 2004; Sawano *et al.*, 2002). Using a similar approach, we could pattern the substrate with different proteins and cells (Takayama *et al.*, 1999). We can pattern the culture media over an individual cell by delivering chemicals selectively to cells. Since no physical barriers are required to separate the different liquid streams, different liquids can flow over different portions of a single cell.

Ismagilov and colleagues have used LFP to generate a step gradient in temperature to study the development of the embryos of the fruit fly *Drosophila melanogaster* (Lucchetta *et al.*, 2005). They treated the anterior (front) and posterior (back) of the embryo with media of different temperatures and observed that the fly embryos developed normally under such a condition. They concluded that in complex biochemical systems, there exist mechanisms for compensation. They further showed that if they reverse the temperature gradient within a certain time, embryos failed to develop normally. This observation shows there is a limitation of time for the mechanisms for compensation.

Another type of LFP generates gradients with parallel streams of flow of increasing or decreasing concentrations. We have generated gradients of biomolecules both in solution and on surfaces (Fig. 19.5) (Dertinger *et al.*, 2001; Jeon *et al.*, 2000). Because we can control the input concentration and the width of the microfluidic channel, it is possible to generate gradients of virtually any characteristics (e.g., the length and the slope), both in solution and on surfaces. We studied the generation of neuronal polarity — the process of the selective formation of one axon and several dendrites from a number of initially equivalent neurites projecting from a single neuron — and found that a surface gradient of laminin was sufficient to guide the orientation of this process (Fig. 19.5) (Dertinger *et al.*, 2002). We further quantified the slope of the gradient and determined the minimum slope of the gradient required for this process to take place. We have also studied the chemotaxis of neutrophils in a solution of gradient of interleukin-8 (IL8) (Jeon *et al.*, 2002). The neutrophils migrate directionally toward increasing concentrations of IL8 in linear gradients. Neutrophils halt abruptly when encountering a sudden drop in the chemoattractant concentration (from maximum directly to zero). When neutrophils encounter a gradual increase and decrease in chemoattractant (from maximum gradually to zero), however, the cells initially cross the crest of maximum concentration but then head back toward the maximum. It would be very difficult to carry out experiments to answer questions of these types — questions covering the response of cells to the details of gradients over scales of microns — without the ability to form precisely controlled gradients.

LFP has some features that make it complementary to other patterning techniques used for biological applications. It takes advantage of easily generated multiphase laminar flows to pattern fluids and to deliver components for patterning. The ability to pattern the growth medium itself is a special feature that cannot be achieved by other processes. This method can pattern even delicate structures, such as portions of a mammalian cell. This type of patterning is difficult by other techniques. LFP can also give simultaneous control over the surface patterns, cell positioning, and the fluid environment in the same channel.

One may ask if the fluid flow required in the generation of laminar flows would cause problems for certain

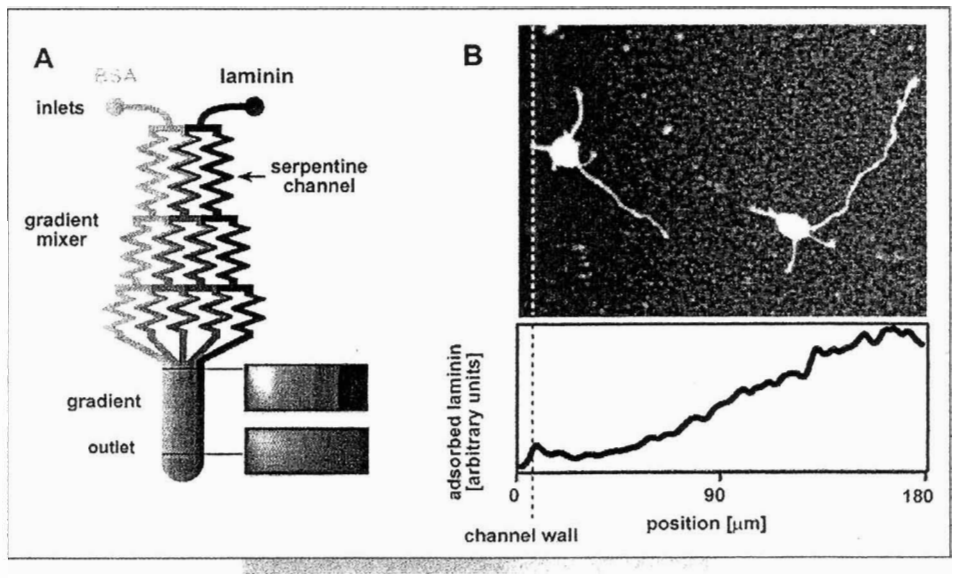

FIG. 19.5. Generation of gradients using microfluidic networks, and use of these gradients to study neuronal differentiation. **(A)** In appropriately designed microfluidic channels, flows generate gradients of BSA and laminin in solution. **(B)** The gradient in solution became a gradient on the surface when proteins adsorb; and when rat hippocampal neurons grow on the gradient, the neurons extend their longest process (the presumed axon) toward the higher concentrations of laminin in the gradient of proteins on the surface.

experiments, such as the measurement of chemotaxis. Wikswo and coworkers addressed this issue by measuring the motility of HL60 leukemia cells (which express CXCR2 receptors) in a gradient of CXCL8 (Walker *et al.*, 2005). They found that high rates of flow can affect the motility of cells. Reasonably low rates of flow, however, do not affect measurements on motile cells.

A few recent examples have combined patterned substrates with patterned flows. We have fabricated gradients of proteins on surfaces in microchannels whose floors carry patterns generated by μCP (Jiang *et al.*, 2005b). Jeon and his coworkers have combined substrates patterned in topography and patterned flows to form a model system that conveniently isolates axons of rat hippocampal neurons from the rest of the cell for studies of their molecular biology (Taylor *et al.*, 2005). Langer and coworkers have fabricated microchannels that have micropatterns within them to immobilize proteins and cells (Khademhosseini *et al.*, 2004). Folch and coworkers have used micropatterns to form myotubes from myoblasts and then used laminar flows to deliver agrin, a proteoglycan found in the neuromuscular junction, precisely to these myotubes. In these experiments, he monitored the clustering of acetylcholine receptor (AChR), and his results corroborated the hypothesis that focalized release of agrin causes the clustering the AChR (Tourovskaia *et al.*, 2006).

VII. CONCLUSION AND FUTURE PROSPECTS

Soft lithography brings to microfabrication low cost, simple procedures, rapid prototyping of custom-designed devices, three-dimensional capability, easy integration with existing instruments such as optical microscopy, molecular level control of surfaces, and biocompatibility (Jiang and Whitesides, 2003; Whitesides *et al.*, 2001). These techniques allow patterning of cells and their environments with convenience and flexibility at dimensions smaller than micrometers. We have described several complementary soft lithographic techniques — microcontact printing, patterning with microtopography, patterning using fluids in microfluidic channels, and laminar flow patterning — that are useful in their ability to pattern the microenvironment of cultured cells.

Microcontact printing is perhaps the simplest method for patterning surfaces. It also provides the highest resolution in patterns with the greatest flexibility in the shape and size of the patterns generated. It provides the best control when one needs to pattern only two types of ligands or proteins. Microtopographies can be useful for certain experiments where micropatterning alone is not sufficient. Microfluidic channels are well suited for patterning surfaces using delicate objects such as proteins and cells. They are also useful when multiple ligands, proteins, or cells need to be patterned. Laminar flow patterning is similar to patterning with individual microfluidic channels, except the individual flows are kept from mixing with each other by laminar flow, not by physical walls. The ability to pattern the fluid environment is the distinguishing feature of this method, and it enables laminar flow to be used to pattern the distribution of different fluids over the surface of a single mammalian cell. This capability allows patterning of portions of a single cell and remodeling of the cell culture environment, both in the presence of living cells. The combination of two or more of these techniques is starting to become useful for more sophisticated experiments.

Soft lithography is still practiced by a relatively small number of biologists, but its use is growing rapidly. There are many cell culture environments and related technologies that we have not discussed in this chapter; many of them relate to soft lithographic methods. For example, there are a number of methods of manipulation of chemistry on SAMs and tools of micropatterns to allow for molecular level control at the cell–substrate interface (Chapman *et al.*, 2000; Kandere-Grzybowska *et al.*, 2005; Kato and Mrksich, 2004).

Although we are starting to have more techniques for fabrication in three dimensions, patterning of cells in three dimensions is still difficult (Gates *et al.*, 2004, 2005; Shin *et al.*, 2004). We are making rapid progress, however, in the fourth dimension, i.e., time. Since we can turn the surface on and off for adhesion of cells and change the media at will in laminar flows, real-time monitoring of temporal changes in individual cells is possible (Jiang *et al.*, 2003; Takayama *et al.*, 1999; Yousaf *et al.*, 2001a). The optical transparency of PDMS makes it straightforward to pattern the intensity of light in cell cultures (Whitesides *et al.*, 2001). The gas permeability of PDMS may be useful in patterning the gas surrounding cells. PDMS is electrically insulating, and molding or fabricating electrically conducting wires in it should allow patterning of electric fields around cells (Kenis *et al.*, 1999; Takayama *et al.*, 1999). Gravitational fields can also be affected: Microfluidic culture chambers with adherent cells can be turned upside down without loss of the culture media. Temperature, fluid shear, and other factors may also be accurately patterned.

The functional potential of a cell is determined by its genetics. Realization of that potential depends, *inter alia*, on whether the cell is exposed to the appropriate environment for expression of particular sets of genes. Soft lithography provides tools for patterning cells and their environment with precise spatial control. This capability aids efforts to understand fundamental cell biology and advances our ability to engineer cells and tissues. The ease with which electronic components or other "nonbiological" components can be fabricated with soft lithography also paves the way for the engineering of cells and tissues for use in biosensors and hybrid systems (e.g., interfaces between semiconductor-based computation and biological computation) that combine living and nonliving components.

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