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Patterning proteins and cells using soft lithography

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Abstract

This review describes the pattering of proteins and cells using a non-photolithographic microfabrication technology, which we call 'soft lithography' because it consists of a set of related techniques, each of which uses stamps or channels fabricated in an elastomeric ('soft') material for pattern transfer. The review covers three soft lithographic techniques: microcontact printing, patterning using microfluidic channels, and laminar flow patterning. These soft lithographic techniques are inexpensive, are procedurally simple, and can be used to pattern a variety of planar and non-planar substrates. Their successful application does not require stringent regulation of the laboratory environment, and they can be used to pattern surfaces with delicate ligands. They provide control over both the surface chemistry and the cellular environment. We discuss both the procedures for patterning based on these soft lithographic techniques, and their applications in biosensor technology, in tissue engineering, and for fundamental studies in cell biology.

Keywords: Microfabrication; Microfluidics; Patterning; Cell biology; Tissue engineering; Self-assembled monolayers

1. Introduction

This review describes techniques for patterning the properties and structures of surfaces at the molecular level, and for using these patterns to control both the adsorption of proteins to these surfaces and the attachment of cells to them. The ability to generate patterns of proteins and cells on surfaces is important for biosensor technology [1–4] for tissue engineering [5], and for fundamental studies of cell biology [6–8]. The placement of biological ligands at well-defined locations on substrates is required for certain biological assays, for combinatorial screening, and for the fabrication of biosensors. Biosensors based on living cells [3,9–12] can also be used for environmental and chemical monitoring; accurate positioning of the cells used for sensing on these devices is critical for monitoring the status of the cells. Control over the positioning of cells is also important for cell-based screening, in which individual cells need to be accessed repeatedly to perturb them and to monitor their response. Tissue engineering may require that cells be placed in specific locations to create organized structures. Patterning techniques that control both the size and shape of the cell anchored to a surface, and the chemistry and topology of the substrate to which the cell is attached, are also extremely useful in understanding the influence of the cell–material interface on the behavior of cells [7,8,13,14].

Photolithography is the technique that has been used most extensively for patterning proteins and cells. For example, photolithography can be used to generate patterns by photoablating proteins preadsorbed to a silicon or glass surface [15], by immobilizing proteins on thiol-terminated siloxane films that have been patterned by irradiation with UV light [16], and by covalently linking proteins to photosensitive groups [17]. Although photolithography is a technique that is highly developed for patterning, the high costs associated with photolithographic equipment, and the need for access to clean rooms, make this technique inconvenient for biologists. Photolithography is not well suited for introducing either specific chemical functionalities, or delicate ligands required for bio-specific adsorption, onto surfaces. Photolithography cannot be used to pattern non-planar substrates. While photolithography can be used to

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Table 1
Techniques that have been used to pattern proteins and cells (other than the soft lithographic techniques covered in this review)

Technique	Reference
Photolithography and photochemistry	[15,16,63–79]
Laser lithography	[80]
Laser photoablation	[81,82]
Laser vapor deposition	[83]
Microwriting and micromachining	[42]
3D printing	[84,85]
Ion implantation	[86,87]

produce patterns with features smaller than 1 μm, this resolution may be unnecessary for many applications of patterning in cell biology.

We have developed a set of microfabrication techniques that is an alternative to photolithography for patterning surfaces used in biochemistry and biology. We call this set 'soft lithography' [18–23], because each of the techniques uses stamps or channels fabricated in an elastomeric ('soft') material for pattern transfer or modification. Soft lithographic techniques are not expensive, are procedurally simple, can be used to pattern a variety of different planar and non-planar substrates, and do not require stringent control over the laboratory environment for their successful application.

This review will focus on the patterning of proteins and cells using soft lithographic techniques, in programs carried out in our group and by others. The patterning of proteins and cells by other techniques will not be discussed in this review, other than by reference (Table 1).

2. Key elements of soft lithography

2.1. Elastomeric stamps

Soft lithographic methods use an elastomeric stamp or mold, prepared by casting the liquid prepolymer of an elastomer against a master that has a patterned relief structure (Fig. 1). Photolithography is used only for the fabrication of the masters. Most of the research based on soft lithography has used poly(dimethylsiloxane) (PDMS) as the elastomer. PDMS has several properties [24] that make it well suited for patterning proteins and cells. PDMS is biocompatible, permeable to gases, and can be used for cell culture. It is optically transparent down to about 300 nm. Because it is elastomeric, it can contact non-planar surfaces conformally. Its interfacial properties can be readily modified by treating the surface with plasma and subsequently forming self-assembled siloxane monolayers on the oxidized surface [25]. It is a durable elastomer. We have used the same PDMS stamp approximately 100 times over a period of several

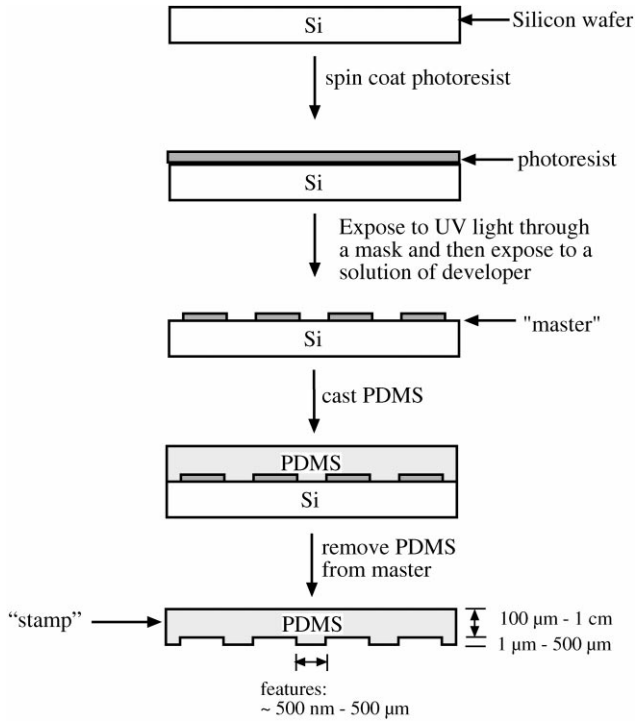


Fig. 1. Schematic illustration of the procedure used to fabricate a PDMS stamp from a master having relief structures in photoresist on its surface.

months without noticeable degradation in its performance.

2.2. Masks and rapid prototyping

An advantage of soft lithography as a method for patterning cells is that at the feature sizes required for this application—2–500 μm—it is often possible to use procedures for making the initial patterns that are substantially more rapid and less expensive than those commonly used to make chrome masks for conventional photolithography. For the fabrication of masters having feature sizes greater than or equal to 20 μm, the patterned mask that is used in the initial photolithographic step can be generated using high-resolution laser printing technology [26] that is inexpensively available commercially. The patterns used are generated using computer programs such as Freehand or AutoCAD, and are printed onto flexible transparencies. The masks can be made in a few hours, at a cost as low as $0.25 per square inch (at the time of writing the paper). For feature sizes greater than 10 μm, the optical reduction of images printed onto transparencies generates patterns in microfiche [27]. Microfiche is then used as the photomask. For smaller feature sizes (between 20 and 2 μm), commercial laser writing can be used to fabricate PDMS stamps relatively inexpensively [28].

The capability to produce features larger than 20 μm rapidly and inexpensively has had a strong impact on the ability of researchers to prototype and produce small numbers of simple microstructures and microsystems. This capability, which we call 'rapid prototyping' [26,29,30], has greatly reduced the barriers to the use of lithographic techniques by researchers in the biological sciences.

3. Soft lithographic techniques

3.1. Microcontact printing

Since many of the studies involving the patterning of proteins and cells using microcontact printing have used self-assembled monolayers (SAMs) of alkanethiols on gold, we begin with a brief discussion of these SAMs. While SAMs can also be formed on silver, and are more ordered on silver than on gold, the cytotoxicity of the Ag^+ released from the silver films when they are exposed to air or other oxidants limits the use of these films in biological experiments involving living cells.

3.1.1. SAMs of alkanethiols on gold

SAMs of alkanethiols on gold are formed by exposing a gold surface to a solution of, or to the vapors of, an alkanethiol (RSH) [31,32]. The nature of the gold–sulfur bond is not yet completely understood [33]. We adopt the view that the species present at the surface is a gold(I) thiolate.

$$R\text{–}SH + Au(0)_n \rightarrow RS\text{–}Au(I)Au(0)_{n-1} + \tfrac{1}{2}H_2\uparrow. \quad (1)$$

Substrates used for experiments are prepared on glass cover slips or silicon wafers by evaporating a thin layer of titanium (1–5 nm) to promote the adhesion of gold, followed by a thin (10–200 nm) film of gold [34]. Gold substrates covered with SAMs are compatible with the conditions used for cell culture and they are not toxic to living cells [35]. While SAMs of alkanethiols on gold can be used in cell culture for periods of days, they desorb on heating above 70°C, when irradiated with UV light in the presence of oxygen, or when exposed to atmospheric ozone. The substrates therefore need to be protected from intense light or excessive temperatures prior to and during experiments.

The structure of the SAMs is shown in Fig. 2. The sulfur atoms of the alkanethiols coordinate to the gold surface, while the alkyl chains are close-packed and tilted by 30° with respect to the surface normal [36]. The terminal functional group (X) of an ω-substituted alkanethiol determines the properties of the interface between the SAM and the contacting liquid, and enables control over the interfacial interactions at a molecular level. The exposure of a gold surface to a solution containing a mixture of alkanethiols forms mixed SAMs (monolayers composed of a mixture of gold(I) thiolates), and allows the density of functional groups on the surface to be varied, although phase segregation might affect the surface properties of certain mixed SAMs.

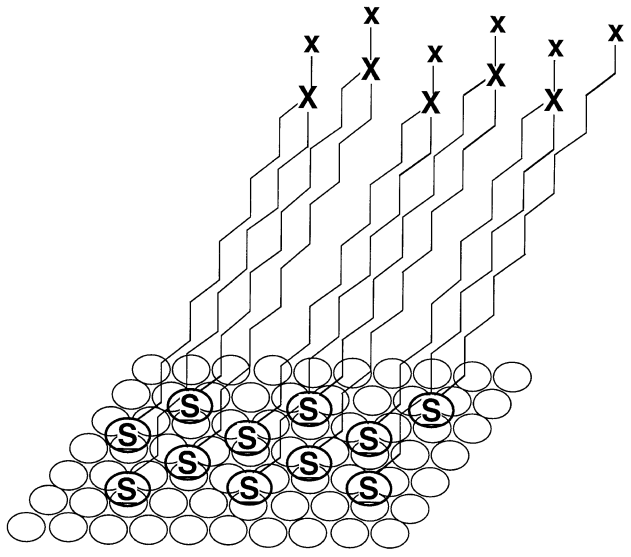


Fig. 2. Schematic diagram of a self-assembled monolayer of an alkanethiolate on gold. The alkyl chains are oriented 30° from the surface normal and packed with nearly crystalline densities; the interfacial properties of the film are largely determined by the chemical properties of the terminal group X.

3.1.2. Adsorption of proteins on SAMs

Understanding and controlling both the specific and non-specific adsorption of proteins to surfaces is important for designing biomaterials and for fundamental studies of biology. The ability to control the nature and density of functional groups presented at the surface of SAMs makes them excellent model surfaces with which to study protein adsorption at surfaces. Sigal et al. [37] studied the non-specific adsorption of several proteins to surfaces presenting different functional groups such as alkyls, amides, esters, alcohols, and nitriles. The adsorption of proteins to uncharged SAMs showed a general correlation with the tendency of water to wet the surface (as determined by the contact angle of water on the SAM under cyclooctane), and on the size of the proteins. While the smaller proteins tested (ribonuclease A and lysozyme) adsorbed only on the least wettable surfaces tested, larger proteins such as pyruvate kinase and fibrinogen adsorbed to some extent on almost all of the surfaces tested. There were exceptions to the general trend of increased adsorption with decreased wettability. For example, fibrinogen adsorbed to a greater extent on SAMs presenting –CN groups than on SAMs presenting $-CH_3$ groups, although the surface presenting –CN groups has a greater wettability. Adsorption of proteins on hydrophobic surfaces was usually kinetically irreversible.

3.1.3. SAMs that resist the adsorption of proteins

Surfaces that resist the adsorption of proteins are essential for certain applications in biomaterials and for research in biosurface chemistry. SAMs presenting oligomers of ethylene glycol, prepared using the alkanethiols $HS(CH_2)_{11}(OCH_2CH_2)_nOH(EG_n)$, resist the adsorption of proteins to surfaces [38,39]. The EG_n-terminated SAMs are not unique in their ability to resist the adsorption of proteins. SAMs presenting tripropylene sulfoxide groups also prevent the non-specific adsorption of proteins [40]. The existence of SAMs that resist the non-specific adsorption of proteins, when coupled with the ability to pattern SAMs using soft lithographic techniques, enables the facile patterning of proteins on surfaces.

3.1.4. Patterning SAMs by microcontact printing

Microcontact printing [18,41] (μCP) is a technique that uses the relief pattern on the surface of an elastomeric PDMS stamp to form patterns of SAMs on the surfaces of substrates (Fig. 3). The stamp is 'inked' with a solution of an alkanethiol in ethanol, dried, and brought into conformal contact with a gold substrate for 10–20 s. The alkanethiol is transferred to the gold substrate only in the regions where the PDMS stamp contacts the substrate. Subsequent exposure of the remaining bare gold substrate to a second alkanethiol generates a surface patterned into regions presenting different terminal groups.

3.1.5. Patterning proteins by microcontact printing

Patterned SAMs generated by microcontact printing can be used to control the adsorption of proteins on surfaces. Lopez et al. [42] used microcontact printing to pattern gold surfaces into regions terminated in oligo(ethylene glycol) groups and methyl groups. Immersion of the patterned SAMs in solutions of the proteins such as fibronectin, fibrinogen, pyruvate kinase, streptavidin, and immunoglobulins resulted in adsorption of the proteins on the methyl-terminated regions. The

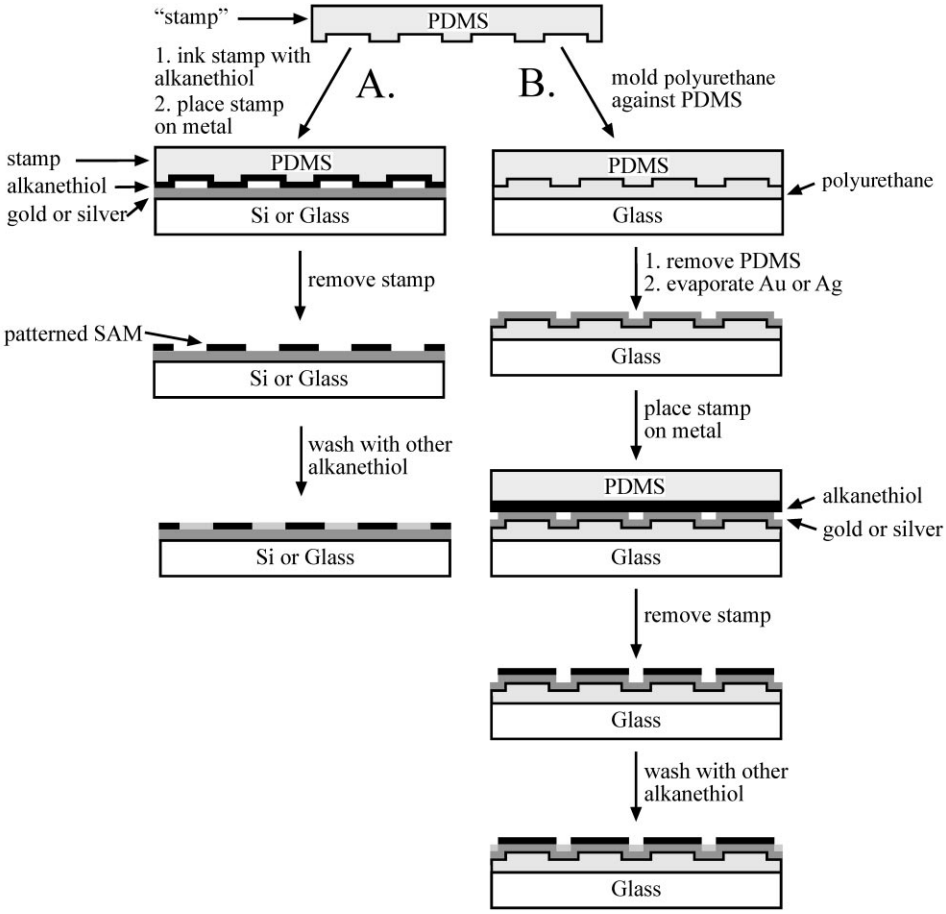


Fig. 3. Microcontact printing (μCP) and fabrication of contoured substrates using soft lithography. (A) A stamp is inked with an alkanethiol and placed on a gold surface; the pattern on the stamp is transferred to the gold by the formation of an SAM on the regions that contacted the substrate. The bare areas of the gold are exposed to a different alkanethiol to generate a surface patterned with an SAM that presents different chemical functionalities in different regions. (B) The PDMS stamp can also be used as a master to mold harder polymers and generate contoured surfaces. After evaporation of a layer of gold, these surfaces can be functionalized by μCP of one alkanethiol with a flat stamp. The grooves of the substrate can then be exposed to an alkanethiol presenting a different functional group to produce a contoured surface with patterned chemical reactivity.

adsorption of proteins could be characterized by scanning electron microscopy, and the layers of adsorbed protein appeared to be homogeneous.

Microcontact printing can also be used to form patterns of oligopeptide ligands by using self-assembling oligopeptides. Zhang et al. [43] 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. The thiol group of cysteine allowed the oligopeptides to form monolayers on gold-coated surfaces. A combination of microcontact printing and these self-assembling oligopeptide monolayers can be used to pattern gold surfaces into regions presenting cell adhesion motifs and oligo(ethylene glycol) groups that resist protein adsorption.

Surfaces can also be patterned by microcontact printing ligands onto reactive SAMs (Fig. 4). Yan et al. [44] generated surfaces presenting interchain carboxylic acid anhydrides by treating SAMs that present terminal carboxylic acid groups with trifluoroacetic acid. Microcontact printing of ligands containing amino groups onto these activated SAMs resulted in the covalent attachment of the ligands to the SAM through amide bonds. Lahiri et al. [45] prepared mixed SAMs from mixtures of thiols presenting terminal tri(ethylene glycol) groups ($HS(CH_2)_{11}(OCH_2CH_2)_3OH$) and terminal hexa(ethylene glycol)-CH_2COOH groups ($HS(CH_2)_{11}(OCH_2CH_2)_6OCH_2COOH$). The carboxylic acid groups were converted to reactive pentafluorophenyl esters. Microcontact printing of amine-terminated ligands onto these activated surfaces also resulted in the covalent attachment of ligands to the SAM through amide bonds. Biotin and benzene sulfonamide printed on the reactive SAMs subsequently bound the proteins streptavidin and carbonic anhydrase, respectively.

Microcontact printing has also been used to generate patterns on different substrates such as glass, silicon, and polystyrene, by using solutions of proteins as the ink [46,47]. A number of proteins printed using elastomeric stamps had activities indistinguishable from those of proteins adsorbed from solutions. Antibody gratings generated by microcontact printing the antibody onto silicon substrates were capable of detecting bacteria by a diffraction-based assay [48].

3.1.6. Patterning cells by microcontact printing

Most mammalian cells are anchorage-dependent—they must adhere to and spread on a substrate in order to live. Extracellular matrix proteins such as fibronectin, vitronectin, and laminin promote the adhesion of anchorage-dependent cells to substrates. The ability to pattern SAMs by microcontact printing, and the resulting control over the adsorption of adhesive proteins, enables the patterning of cells on substrates [13]. Mrksich et al. [35] used microcontact printing to pattern gold or silver substrates into regions presenting oligo(ethylene glycol) groups and methyl groups. After coating 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 pattern defined by the underlying SAMs for 5–7 d. In addition to confining cells to specific regions of a substrate, microcontact printing can also be used to change the size and shape of cells (Figs. 5 and 6).

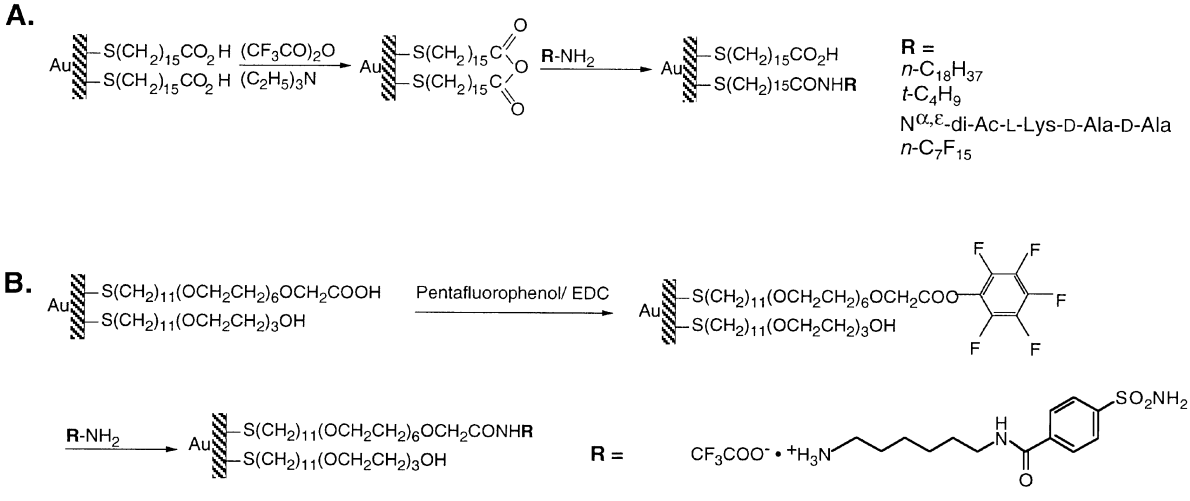
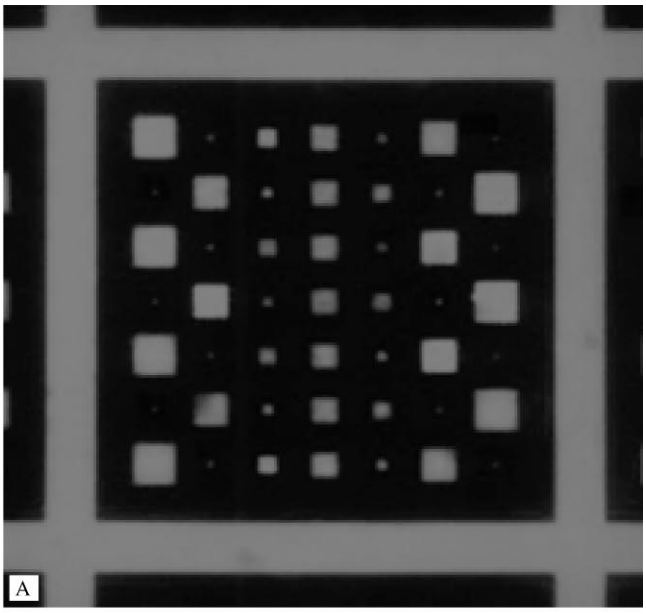


Fig. 4. Formation of mixed SAMs by performing reactions on pre-formed SAMs. Patterns can be generated by stamping the reactive ligand. (A) Alkylamines (in 1-methyl-2-pyrrolidinone) react with interchain carboxylic anhydride groups formed on SAMs of $SH(CH_2)_{15}COOH$ after reaction with trifluoroacetic anhydride and triethylamine in anhydrous *N*, *N*-dimethylformamide. This procedure generates a surface that presents an equimolar mixture of the ligand R and carboxylic acid groups on the alkanethiolate molecules of the SAM. (B) SAMs are formed that comprise a mixture of carboxylic acid-capped hexa(ethylene glycol)- and tri (ethylene glycol)-terminated undecanethiolate. Pentafluorophenol and *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide (EDC) then react with carboxylic acid groups on this surface to form the pentafluorophenyl ester; this active ester subsequently reacts with alkyl amines carrying the ligand of interest, R.



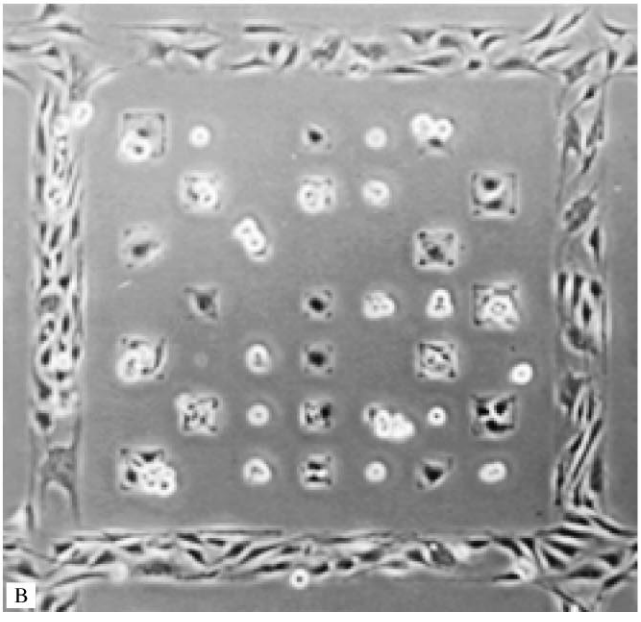


Fig. 5. (A) A gold surface was patterned into regions of hexadecanethiolate and undecanethiolate terminated with tri(ethylene glycol) by μCP. Fibronectin (light) adsorbed on the hydrophobic squares of hexadecanethiolate but not on the tri(ethyleneglycol) terminated alkanethiolate (dark). Patterned substrates were soaked in a solution of fibronectin (50 μg/ml in phosphate-buffered saline (PBS)) for 2 h, fixed using 20% paraformaldehyde (v/v) in PBS buffer and then immersed in a solution of anti-human fibronectin IgG (5 μg/ml) for 1 h followed by extensive rinsing. The substrates were then placed in contact with 100 μl of Texas Red®-labeled goat anti-rabbit IgG (50 μg/ml) for 1 h, followed by mounting in fluoromount-G (Southern Biotechnology Inc.). (B) Bovine capillary endothelial (BCE) cells patterned by culturing on a substrate presenting hydrophobic squares of varying sizes that were coated with fibronectin, prior to incubation with cells using the procedure described in (A).

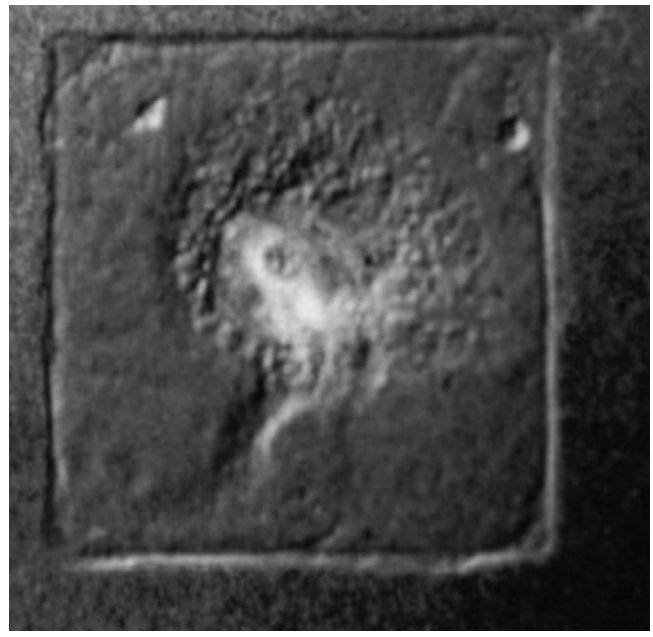


Fig. 6. A single bovine capillary endothelial cell conforming to a 40 μm square adhesive island generated by microcontact printing using the procedures described in Fig. 5.

Microcontact printing has also been used to pattern astroglial cells on silicon substrates [49]. Astroglial cells attached selectively to 50 μm wide adhesive bars generated by microcontact printing *N*-1[3-(trimethoxysilyl) propyl]diethylenetriamine (DETA) on silicon surfaces.

Mrksich et al. developed a simple technique based on microcontact printing and micromolding to control the attachment of cells on contoured surfaces [14] (Fig. 3). Replica molding in PDMS molds having micron-scale relief patterns on their surfaces formed a contoured flim of polyurethane supported on a glass slide. After evaporating a thin film of gold onto these substrates, the raised plateaus of the contoured surface were derivatized with an SAM by stamping with an flat PDMS stamp; a different SAM was formed in the grooves by immersing the substrate in a solution of another alkanethiol. On modifying the raised plateaus with an SAM of hexadecanethiolate, and the grooves with an SAM terminated in oligo(ethylene glycol) groups, and coating the substrates with fibronectin, bovine capillary endothelial cells attached only to the methyl-terminated, fibronectin-coated raised plateaus. A complementary procedure confined fibronectin adsorption and cell attachment to the grooves in the substrate.

3.1.7. Fundamental studies in cell biology using microcontact printed substrates

3.1.7.1. Effect of cell shape on cell function. Singhvi et al. [7] used the ability to control cell shape by microcontact

printing to explore the effect of cell shape on cell function. They plated primary rat hepatocytes on substrates consisting of square and rectangular islands of laminin surrounded by non-adhesive regions. Cells attached preferentially to the laminin-coated islands, and in most cases conformed to the shape of the island. The size and shape of the cells could be controlled by changing the size of the adhesive islands, without changing the density of the adhesive protein, laminin. Singhvi et al. [7] found that the synthesis of DNA was highest on unpatterned surfaces where cells could spread without restriction, and a decrease in the size of the adhesive islands resulted in a progressive reduction in growth. For the smallest islands (less than 1600 μm^2), less than 3% of the adherent cells entered S (DNA synthesis) phase. The differentiated function of hepatocytes cultured on islands of different sizes was also assessed by measuring the secretion of albumin in the culture supernatant. Hepatocytes cultured on unpatterned substrata rapidly lost the ability to secrete high levels of albumin. Albumin secretion rates increased as the size of the adhesive island was decreased. This study demonstrated that the modulation of cell shape provided control over cell growth and protein secretion independent of the density of the adhesive protein laminin.

3.1.7.2. Effect of cell shape on cell life and cell death. Chen et al. [8] used micropatterned substrates to control the shape of human and bovine capillary endothelial cells. Cells were shifted from growth to apoptosis by using substrates that contained extracellular matrix-coated adhesive islands of decreasing size. These results are compatible with two hypotheses: (i) that growth increases with the area of the surface that is in adhesive contact with the cell, and (ii) that growth depends on the extent of cell spreading rather than the area of adhesive contact. To discriminate between these two hypotheses, Chen et al. [8] varied the extent of cell spreading while keeping the total cell–matrix contact area constant by culturing cells on substrates that presented islands with diameters of 20, 5, and 3 μm, separated by 40, 10, and 6 μm respectively. They found that the extent of spreading (the projected surface area of the cell) and not the area of the adhesive contact controlled cell life and death (Fig. 7). Cell shape was found to determine cellular choice between division and apoptosis regardless of the type of matrix protein used to mediate adhesion. Because of the power of the micropatterning technique, it was also possible to define how shape regulates the cell cycle machinery in these cells and thus, finally, to translate the long recognized phenomenon of shape-dependent control of growth into specific molecular terms [50].

3.1.7.3. Effect of cell shape on cell differentiation. Dike et al. [88] found that bovine capillary endothelial cells cultured on fibronectin-coated, micropatterned substrates containing 10 μm wide lines formed extensive cell–cell contacts while cell spreading was restricted to approximately 1000 μm^2. Within 72 h, these cells shut off growth and apoptosis programs and underwent differentiation, as indicated by the formation of capillary tube-like structures containing a central lumen (Fig. 8). Cells cultured on wider (30 μm) lines formed cell–cell contacts, but these cells continued to proliferate and did not form tubes. The use of substrates prepared by microcontact printing revealed that bovine capillary endothelial (BCE) cells could be switched between the three genetic programs of growth, apoptosis, and differentiation, by altering the geometry of spreading.

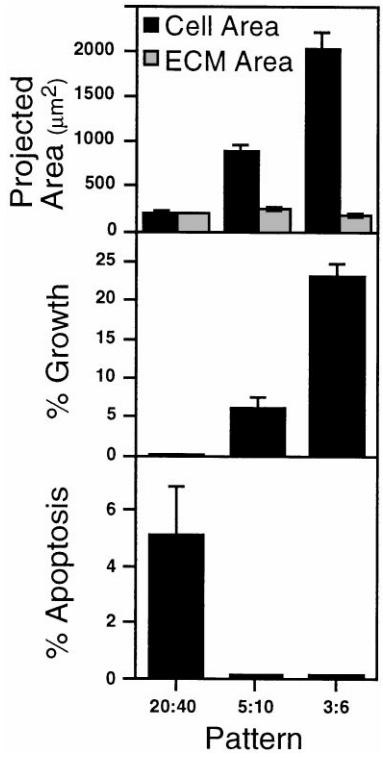


Fig. 7. Graphs of the projected cell area, extracellular matrix (ECM) contact area, growth index, and the apoptotic index (percentage of cells that enter apoptosis) for cells cultured on circular islands having diameters of 20, 5, and 3 μm, separated by 40, 10, and 6 μm, respectively. The results illustrate that the apoptotic index depends on the projected area of the cell and not on the ECM contact area.

3.1.7.4. Effect of surface chemistry on lamellipod extension during chemotaxis. Bailly et al. [51] used micropatterned substrates in studies of the regulation of protrusion shape during chemotactic responses of mammalian carcinoma cells. Since tumor cell motility and protrusive activity are generally correlated with tumor metastatic potentials, such studies increase our fundamental understanding of oncogenic progression. Bailly et al. [51] plated rat mammary carcinoma cells on gold-coated glass coverslips having 10 μm wide adhesive lanes. The

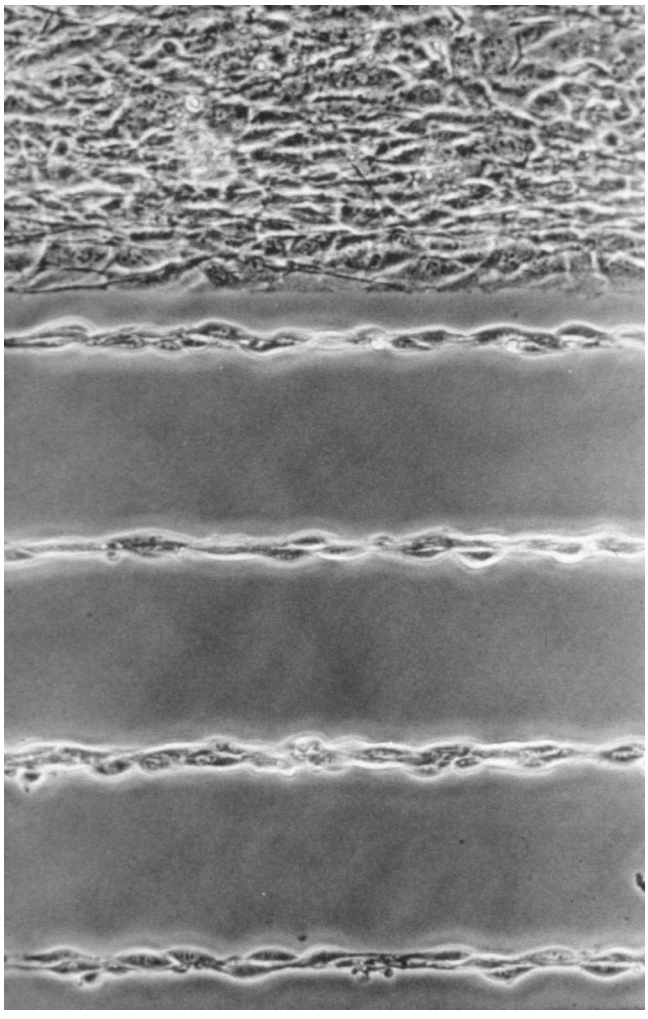


Fig. 8. Capillary tube formation by bovine capillary endothelial cells cultured on 10 μm lines. A larger unpatterned region was included as an internal control. Pattern generation and cell attachment were performed according to procedures described in Fig. 5.

cells attached only to the adhesive lanes. On stimulating the cells with epidermal growth factor (EGF), the cells could extend lamellipods laterally, over the non-adhesive part of the substrate. These results showed that lamellipod extension could occur independent of any contact with the substratum. Contact formation was, however, necessary for stabilizing the protrusion.

3.2. Patterning using microfluidic channels

Microcontact printing relies on the transfer of material (thiols, proteins, etc.) from an 'inked' elastomeric stamp to select regions of a substrate. Patterning can also be carried out by restricting fluid flow to desired regions of a substrate. Kim et al. [21] developed a technique called micromolding in capillaries (MIMIC) for fabricating three-dimensional structures by allowing solutions to flow into microfluidic channels formed by bringing a PDMS mold into conformal contact with a substrate. MIMIC is not restricted to patterning curable prepolymers, and has also been used to pattern a wide variety of materials such as precursor polymers to glassy carbon or ceramics [52,53], sol–gel materials [54], inorganic salts [55], polymer beads [56], and colloidal particles [55].

Delamarche et al. [57,58] extended MIMIC to the patterning of biological molecules such as immunoglobulins. They patterned biomolecules with submicron resolution on a variety of substrates including gold, glass, and polystyrene, by allowing solutions of the biomolecules to flow through microfluidic channels. Only microliters of reagent were required to cover square millimeter-sized areas. The technique enables simultaneous and highly localized immunoassays for the detection of different IgGs.

Patel et al. [59] developed a method of generating micron-scale patterns of any biotinylated ligand on the surface of a biodegradable polymer using microfluidic channels. A biotin molecule was introduced into the end group of the poly(ethylene glycol) (PEG) block of a polylactide–poly(ethylene glycol) copolymer to produce the biodegradable polymer, PLA–PEG–biotin. Films of PLA–PEG–biotin were patterned by allowing solutions of avidin to flow over them through elastomeric microfluidic channels. The avidin moieties bound to the biotin groups on the surface, and served as a bridge between the biotinylated polymer and biotinylated ligands. Patel et al. [59] used their method to achieve spatial control over the adhesion and spreading of bovine aortic endothelial cells and PC12 nerve cells on films of PLA–PEG–biotin. Neurite extension on the polymer surface was found to be directed by patterned features composed of peptides containing the IKVAV sequence.

Folch et al. [60,61] also used microfluidic channels to produce patterns of cells on biocompatible substrates. They created protein templates on surfaces by the adsorption of proteins from solutions that were passed through elastomeric channels. Micropatterns of collagen or fibronectin were used to cause cells to adhere selectively on various biomedical polymers and on heterogeneous or microtextured substrates. On removing the elastomeric stamp, the bare substrate areas could be seeded with more adhesive cell types such as fibroblasts, thereby producing micropatterned co-cultures. By allowing different cell suspensions to flow through different microchannels, patterns of cells could be generated on surfaces (Fig. 9).

3.3. Laminar flow patterning

Laminar flow patterning, a method recently developed by our group [62], adds a new capability to the patterning of microfluidic channels. Microfluidic systems have distinctive properties as a result of their small

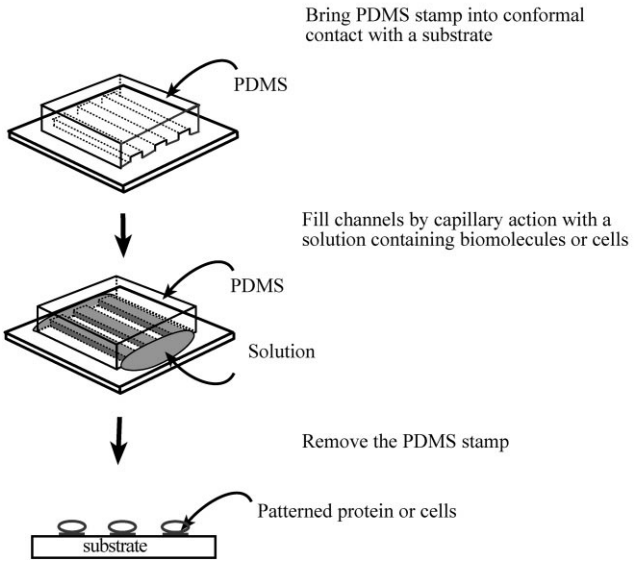


Fig. 9. Illustration of the procedure used to pattern proteins and cells using microfluidic channels.

dimensions. One notable feature is that the flow of liquids in capillaries often has a low Reynolds number (Re) and is laminar (Re is a dimensionless parameter relating the ratio of inertial to viscous forces in a specific fluid flow configuration, and is a measure of the tendency of the liquid to develop turbulence). Operating in laminar flow conditions allows two or more layers of fluid to flow next to each other without any mixing other than that taking place by diffusion of their constituent molecular and particulate components across the interface. This ability to generate and sustain parallel streams of different solutions in capillaries provides a unique opportunity to pattern cells and their environment—that is, the molecular structure of the surface to which the cells are attached, the nature and position of other cells in their vicinity, and the composition of the fluid medium surrounding them. Although we have discussed methods that allow the patterning of surfaces and cells, laminar flow patterning is the only method that allows patterning of the culture medium itself in a highly controlled fashion.

A typical setup for laminar flow patterning experiments, together with images from representative patterning experiments, is outlined in Fig. 10. A network of capillaries with multiple inlets that converge into a single main channel is fabricated by bringing a PDMS membrane with the pattern of channels molded into its surface into contact with the flat surface of a Petri dish. By allowing different patterning components to flow from the inlets, patterns of parallel stripes are created in the main channel (Fig. 10A). Micrographs show the patterns

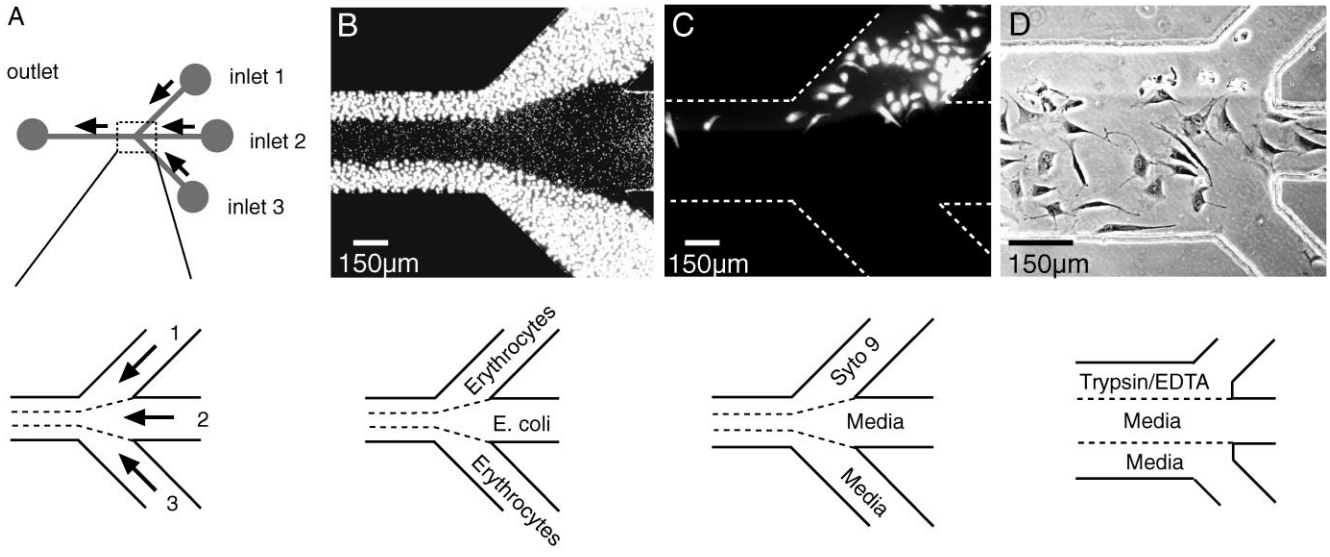


Fig. 10. Schematic representation of a laminar flow patterning experiment. (A) Top view of the capillary network. A poly(dimethylsiloxane) (PDMS) membrane containing micron-sized channels molded in its surface was placed on the flat surface of a Petri dish to form a network of capillaries. Micrographs were obtained for the area of the capillary system at which the inlet channels converge into a single main channel. (B) Two different cell types patterned next to each other. Chick erythrocytes and *Escherichia coli* were deposited selectively in their designated lanes by patterned flow of cell suspensions. Adherent cells were visualized with a fluorescent nucleic acid stain (Syto 9). (C) Pattern of selectively stained BCE cells. A suspension of BCE cells was introduced into the capillary network (pretreated with fibronectin) and allowed to attach and spread. After removing non-adherent cells by washing with media, Syto 9 and media were allowed to flow from the designated inlets. (D) Patterned detachment of BCE cells by treatment with trypsin/EDTA. Cells were allowed to adhere and spread in a fibronectin-treated capillary network, and non-adherent cells removed by washing. Trypsin/EDTA and media were allowed to flow from the designated inlets. Pictures B and C are fluorescence micrographs taken from the top through PDMS. Picture D is a phase contrast image observed by an inverted microscope looking through the polystyrene Petri dish. White dotted lines identify channels not visible with fluorescence microscopy.

Table 2
Examples of patterning proteins and cells using soft lithographic techniques

Technique	Substrate	Feature	Initial component patterned	Proteins patterned	Effect observed (cells patterned)	Ref.
Microcontact printing	Gold	5 μm lines	Alkane thiols terminated in various functional groups including oligo-ethylene glycol	Patterned adsorption of ribonuclease A, pyruvate kinase, fibrinogen, fibronectin, streptavidin, and immunoglobulins	Adsorbed proteins were visualized using scanning electron microscopy	[42]
	SAM of interchain carboxylic acid anhydride on gold	10 μm squares	*n*-Hexadecylamine, cystamine, 3-amino-1-propanesulfonic acid and other amine containing molecules		Gold nanoparticles (~20 nm) adhered selectively to regions patterned with cystamine	[44]
	Glass, polystyrene, silicon, various SAMs on gold	Arbitrary patterns of 1 μm and larger	Proteins	Antibodies, phosphatase, cytochrome c, bovine serum albumin, streptavidin, protein A, proteinase K, peroxidase, chymotrypsin, NgCAM (cell adhesion molecule)	The amount of protein adsorbed onto surfaces is similar to adsorption from solutions, however, the non-solution environment inevitable with stamping sometimes compromises protein function	[47]
	Silicon	10 μm stripes separated by 30 μm	Antibodies	Anti-*E. coli* O157 : H7 antibodies	Diffraction-based detection of *E. coli* O157 : H7 cells using an antibody grating created by microcontact printing	[48]
	Mixed SAMs of terminal oligo(ethylene glycol) and oligo-ethylene carboxylate thiols on gold	5–50 μm squares	Amine compounds (derivatives of biotin or benzene sulfonamide)	Anti-biotin antibodies or streptavidin bound to regions patterned with biotin	Patterned binding of streptavidin. Bound streptavidin can be used to capture any biotinylated ligand or protein in so-called 'sandwich' experiments	[45]
	Gold	2–80 μm rectangles	Hexadecane thiol, hexa(ethylene glycol) terminated alkane thiol	Laminin adsorbed to patterned hexadecane thiol SAMs	Primary rat hepatocytes adhere and spread only on laminin-coated islands. Cell shape controls cell growth and function	[7]
	Gold	60 μm lines separated by 120 μm	Hexadecane thiol, tri(propylene sulfoxide) terminated alkane thiol	Fibronectin adsorbed to patterned hexadecane thiol SAM	BCE cells were confined to the hexadecane thiol SAM regions for 1–2 d then started to spread into other areas	[40]
	Thin gold film (<12 nm) on poly urethane	25–50 μm ridges and grooves	Hexadecane thiol, tri(ethylene glycol) terminated alkane thiol	Fibronectin adsorbed to patterned hexadecane thiol SAMs	Patterned adsorption of fibronectin. Cells are confined to tri(ethylene glycol) terminated alkane thiol SAM regions for at least 5 d	[14]
	Gold	5–40 μm squares and circles	Hexadecane thiol, tri(ethylene glycol) terminated alkane thiol	Fibronectin, vitronectin, collagen, anti-integrin β_1 antibody, or anti-integrin $\alpha_v\beta_3$ antibody adsorbed to patterned hexadecane thiol SAMs	Extent of cell spreading and not the area of adhesive contact controls life and death of bovine capillary endothelial (BCE) cells	[8]
	Gold and silicon	Circles and squares of 5–80 μm	Hexadecane thiol, tri(ethylene glycol) terminated alkane thiol	Fibronectin adsorbed to patterned hexadecane thiol SAMs	Cell cycle progression of BCE cells were controlled by cell shape and cytoskeletal tension	[50]

	Gold	10 μm lines	Hexadecane thiol, hexa(ethylene glycol) terminated alkane thiol	Vitronectin adsorbed to patterned hexadecane thiol SAM	Chemotactic response of MTLn3 metastatic rat mammary adenocarcinoma cells. Lamellipod extension is independent of contact with the substratum	[51]
	Silicon	Features of 1 μm and larger	Octadecyltrichlorosilane (OTS), *N*(3-(trimethoxy-silyl)propyl)diethylene-triamine (DETA), polylysine		LRM 55 cells (astroglial cell line) selectively attached to DETA patterned surfaces in the presence of serum containing media. Rat hippocampal neurons attach to polylysine patterned regions	[49]
	Gold	Squares and lines of 20 μm and larger	Peptides containing cell adhesion motifs and cysteine, hexa(ethylene glycol) terminated alkane thiol		Human epidermoid carcinoma A431 cells, primary human embryonic kidney 293 cells, bovine aorta fibroblasts	[43]
	Gold	10 or 30 μm width lines and 5 or 10 μm squares	Hexadecane thiol, tri(ethylene glycol) terminated alkane thiol	Fibronectin adsorbed to patterned hexadecane thiol SAMs	BCE cells can be switched between growth, apoptosis, and differen-tiation by altering the geometry of spreading	[88]
Microfluidic networks	Gold, glass, silicon, polystyrene	Lines with widths of 3 μm and bigger	Immunoglobulins	Immunoglobulins and bovine serum albumin (BSA)	Simultaneous delivery of functionally distinct proteins onto targeted regions of a surface	[57,58]
	Polylactide–biotinylated poly(ethylene glycol) block copolymer	12–70 μm lines	Avidin	Biotinylated ligands bind to regions with avidin attached to it	Biotinylated RGD peptide pro-motes bovine aortic endothelial cells attachment and IKVAV peptides promote PC12 nerve cells attachment	[59]
	Various polymers	20 μm lines	Protein	Fibronectin or collagen	Selective attachment of hepato-cytes and 3T3-J2 fibroblasts onto protein patterned areas	[61]
	Tissue culture-grade plastic	200 μm channels separated by 200 μm	Cells		Fluorescently labeled 3T3-J2 fibroblast cells attached selectively in the areas over which they flowed. Spatially separated co-cultures of two different cell types were created	[60]
Laminar flow patterning	Polystyrene, glass, gold, silicon	Lines with widths of 10–100 μm	Proteins, cells, and media	Wheat germ agglutinin, BSA, and fibronectin were patterned onto the substrate Trypsin was a component of patterned media flow.	E. coli RB 128 cells, chick erythrocytes, and BCE cells. Patterning of substrate, cell position, and media.Media can be patterned to flow selectively over half of a cell	[62]

created at the junction where the inlet channels converge into a single main channel. Fig. 10B shows patterning where cells (*E. coli* and erythrocytes) are used directly as the patterning component. The cells adhere only to those areas over which they were allowed to flow. Fig. 10C shows an example of using patterned culture media to selectively deliver chemicals to cells. In this experiment, BCE cells covered the entire bottom face of the capillary; this fluorescence micrograph shows selective staining only of those cells over which a medium containing a fluorescent dye was allowed to flow. Fig. 10D demonstrates patterned release of attached BCE cells using trypsin/ethylenediaminetetraacetic acid (EDTA). Digestion of fibronectin on the channel surface, and sequestering of calcium by EDTA, causes cells to detach and contract. When a solution of trypsin/EDTA flowed over only a portion of a cell, the treated part of the cell detached and contracted; the untreated part remained spread (for example, see inside dotted square in Fig. 10D). Since no physical barriers are required to separate the different liquid streams, different liquids can flow over different portions of a single cell.

Laminar flow patterning has some features that makes it complementary to other patterning techniques used for biological applications. It takes advantage of the easily generated, multiphase laminar flows to pattern fluids and to deliver components for patterning. This mild delivery method allows the use of cells themselves as the patterning component. Laminar flow patterning is experimentally simple. Multiple-component patterns can be made in a short sequence of steps, without the need for multiple stages of pattern transfer with registration required by other methods. Neither patterning of the growth medium itself, nor patterning over delicate structures such as mammalian cells, is possible by other techniques.

4. Conclusions

The soft lithographic techniques described in this review are a powerful set of tools for controlling the cell–material interface. These techniques offer several advantages over conventional photolithographic techniques. They are inexpensive, and are accessible to chemists and biologists. They allow the patterning of delicate ligands on a variety of substrates, including biocompatible substrates. They can be used to pattern non-planar substrates and to make three-dimensional microstructures. Soft lithographic techniques can be used to control not only the surface chemistry, but also the cellular environment.

The soft lithographic techniques complement each other well. Microcontact printing provides the highest resolution; it has been used to make features smaller than 1 μm. It also provides the greatest flexibility in the shapes of the pattern generated. It is the best technique for controlling the chemistry of the surface at a molecular level. It is most useful when one only needs to pattern two types of ligands. Patterning multiple ligands requires sequential registered stamping steps with different inks, and is more complicated.

Microfluidic channels are well suited for patterning delicate objects like proteins and cells on a variety of substrates. They are useful when multiple ligands need to be patterned, although the range of possible patterns is limited. They can also be used to pattern multiple cell types.

Laminar flow patterning has greatly enhanced the capabilities of patterning using microfluidic channels. The technique enables the patterning of multiple ligands or cells without the problem of registration. It is best suited for patterning parallel lines, although more complicated patterns can be created with additional steps. The ability to pattern over the surface of delicate materials like mammalian cells is unique to this technique (Table 2).

The use of substrates patterned using microcontact printing has already led to fundamental insights into the effects of cell shape on cell function. The other soft lithographic techniques, used either by themselves, or in conjunction with microcontact printing, enhance our ability to control the cellular environment, and should help increase our understanding of fundamental cell biology. The capabilities described in this review should pave the way for engineering cells and tissues for use in biosensors and other 'hybrid' systems that combine living and non-living components.

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