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MICROENGINEERING OF CELLULAR INTERACTIONS

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■ **Abstract** Tissue function is modulated by an intricate architecture of cells and biomolecules on a micrometer scale. Until now, in vitro cellular interactions were mainly studied by random seeding over homogeneous substrates. Although this strategy has led to important discoveries, it is clearly a nonoptimal analog of the in vivo scenario. With the incorporation—and adaptation—of microfabrication technology into biology, it is now possible to design surfaces that reproduce some of the aspects of that architecture. This article reviews past research on the engineering of cell-substrate, cell-cell, and cell-medium interactions on the micrometer scale.

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INTRODUCTION AND HISTORICAL BACKGROUND

Cellular processes such as adhesion, migration, growth, secretion, and gene expression are triggered, controlled, or influenced by the biomolecular three-dimensional organization of neighboring surfaces. This organization cannot be straightforwardly reproduced in the laboratory. Furthermore, cells respond to local concentrations of a variety of molecules that may be dissolved in the extracellular medium (e.g. enzymes, nutrients, and small ions) or present on the underlying surface [e.g. extracellular matrix (ECM) proteins] or on the surfaces of adjacent cells (e.g. membrane receptors). In traditional cell culture, these factors are distributed homogeneously on the substrate. Microfabrication techniques enable the researcher to design, with micrometer-level control, the biochemical composition and topology of the substrate (otherwise homogeneously adherent to cells), the medium composition, and the types of cells in the vicinity of each cell.

In the past, cell biologists have resorted to clever approaches to recreate different degrees of cellular organization in the laboratory. Harrison, for example, studied cell migration on spiderwebs as early as 1912 (1). Others studied cell behavior on surface features such as milled grooves on mica (2), polystyrene replicas of diffraction gratings (3), polyvinyl chloride music records (4), dried protein spots (5, 6), crystals (7), and scratches in agar (8), in phospholipid films (9), or in ECM protein (10). Albeit ingenious, the technology utilized in these studies could not address the structural dimensions, chemical heterogeneity, and/or precise repeatability over large areas found in live tissue. Microfabrication technology offers the potential to control cell-surface, cell-cell, and cell-medium interactions on a micrometer scale.

For the vast body of literature on protein patterning, which is technologically pertinent to cellular micropatterning but is generally aimed at applications such as antibody-based sensors, the reader is referred to the review by Blawas & Reichert (11).

MICROFABRICATION TECHNOLOGIES

A cellular patterning method may adopt one of two strategies: (*a*) promote selective cell attachment indirectly by first creating a thin adhesive template (of metals, polymers, proteins, etc) to which the cells adhere preferentially through some (often undetermined) molecular recognition process, or (*b*) provide a physical barrier that can be removed after cell attachment without inflicting damage to the cells. In this section, we present an overview of the basic principles of microfabrication technology and its advantages and limitations for use in micropatterning biological material ("biopatterning").

Photolithography

Modern microfabrication technology is based on photolithography, the patterning of a layer of photosensitive polymer ("photoresist") by means of light (12). In

general, as schematized in Figure 1*a* (see color insert), ultraviolet (UV) light is shone through a "mask" containing the desired pattern in the form of opaque features (made of chrome or a black emulsion) on a transparent background (glass or plastic). The photoresist is usually spun on a flat substrate from solution to a thin film ($\sim$1 μm thick; a prerequisite for high resolution) and dried before exposure through the mask. The exposure chemically alters the photoresist by modifying its solubility in a certain "developer" solution.

Many biopatterning methods utilize photoresist-based photolithography at some point in the process. Unfortunately, since the smallest dust particle distorts the spreading of the photoresist dramatically during spinning, photolithography must be carried out in clean-room facilities, which require a level of expertise uncommon in biological laboratories and are costly to build and maintain. Overall, the application of photolithography to biopatterning requires the circumvention of two major obstacles: (*a*) most chemicals used in standard photolithography are toxic to cells and denature biomolecules; and (*b*) biological solutions, owing to their rich ionic and molecular content, are a menace to the finely tuned conductivity of a semiconductor circuit and are, therefore, banned from most clean-room facilities originally designed for microelectronics applications.

Soft Lithography

More recently, Whitesides and colleagues have pioneered a family of sister techniques, collectively dubbed "soft lithography," which have in common the utilization of a microstructured surface made with poly(dimethylsiloxane) (PDMS)—essentially transparent rubber—to generate a pattern [see review by Xia & Whitesides (13)]. This method is inexpensive because (*a*) the PDMS surface can be replicated nondestructively from a microfabricated master wafer, thus substantially reducing clean-room processing expenses; and (*b*) biopatterning does not damage the PDMS surface. Soft lithography is an enabling technology for biologists because many polymeric materials universally used in tissue culture (e.g. polystyrene) are attacked by organic solvents and must therefore be ruled out as photolithography substrates.

Microstamping This method, originally named "microcontact printing," was devised to print organic molecules (14). It is based on the contact-transfer of the material of interest from a PDMS stamp onto a surface only on the areas contacted by the stamp (see Figure 1*b*, color insert). Because of its additive nature, microstamping can be applied to nonplanar surfaces (15–17). It is not clear whether the areas contacted by PMDS are entirely free of dimethylsiloxane monomers. Multiprotein patterns can be microstamped as well (18–21). We wish to emphasize that the generalization from alkanethiol to proteins is not straightforward. When the solvent of an alkanethiol solution (generally ethanol) evaporates on a stamp, the alkanethiol molecules plausibly form a nonvolatile "oily" thin film on the stamp, with a high molecular mobility, which ensures efficient transfer. Proteins, however, tend to form crystalline aggregates when dried. Why do proteins prefer

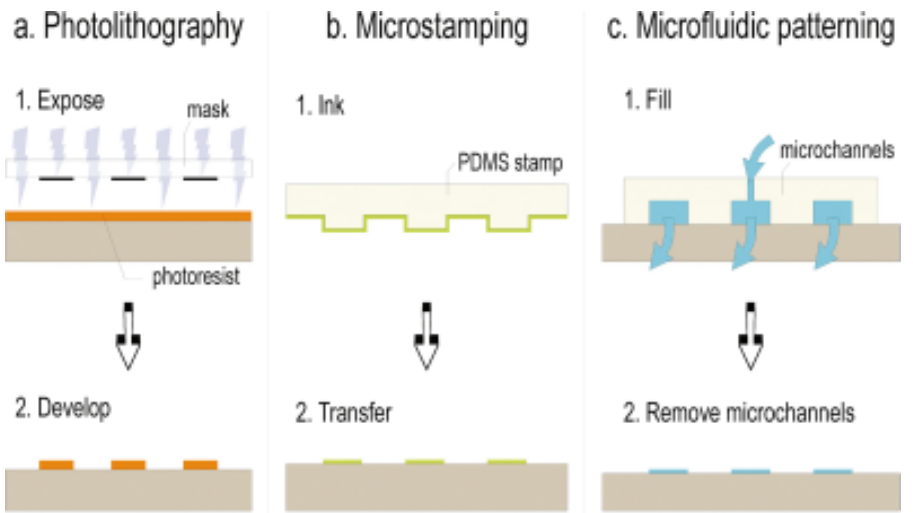

Figure 1 Cross-sectional schematics of the principles of pholithography (*a*), microstamping (*b*), and microfluidic patterning (*c*). (*a*) The photoresist layer is usually spin-coated from solution and dried before exposure. (*b*) The "ink" is transferred only to the areas where the stamp contacts the surface. (*c*) The material of interest is left only on the areas where the elastomeric stamp does not contact the surface.

the substrate to the PDMS stamp? Because virtually all surfaces in air are covered by a molecularly thin aqueous layer, water could act as the intermediary solvent for efficient transfer. If that is the case, one would expect that the transfer efficiency depends greatly on the ambient humidity. To the best of our knowledge, no work presented to date has addressed whether the PDMS surface, after being exposed to a protein solution, is covered by a thin layer of physisorbed protein or by a thick layer of dried protein, and how much of these layers are solubilized in adsorbed water. PDMS is naturally hydrophobic. Increasing the hydrophilicity of the stamp—for example, by exposing it to an oxygen plasma (22–24)—may help to create an evenly dried, thick protein layer but could result in uneven coverages depending on the exact drying protocol. It should be noted that, technically, such dried layers would not be physisorbed because they become instantly solubilized when exposed to an aqueous solution.

Microfluidic Patterning The microstructured PDMS surface can be designed to form a network of microchannels on the areas where the stamp does not contact the surface. The microchannels can thus be used to deliver fluids to selected areas of a substrate, a strategy we refer to as microfluidic patterning (Figure 1*c*, see color insert). Note that in microfluidic patterning, as opposed to microstamping, the material is added at sites where the PDMS does not come into contact with the surface. The fluids are blocked from wetting the substrate in the areas where the PDMS contacts the surface thanks to a unique property of PDMS: It self-seals reversibly against another smooth, dry surface. This is probably due to a combination of its elastomeric nature, allowing for a highly conformal contact, and the hydrophobicity of its surface, which impedes wetting of liquids at the PDMS-substrate interface. The fluid can, depending on its composition, be either cured into a solid itself (25–27), used as a vehicle to deposit a material that remains when the microchannels are peeled off (28–30, 32), or used to remove underlying material (32). Originally, Kim et al (25) micromolded polymer patterns by allowing a liquid precursor of the polymer to fill the microchannels by capillary action, a technique they dubbed micromolding in capillaries. Microchannels featuring deeper and wider profiles can be manufactured (33, 34) and filled by pressure-driven flow over large areas (30, 35). With this method, proteins can be chemically immobilized (28, 29) or physisorbed (30) only on the areas exposed to the protein solution, and cells can be selectively delivered to desired areas of a substrate (34, 36).

Stencil Patterning Micropatterning by means of a thin piece of material containing holes (a "stencil") constitutes a straightforward strategy: The substrate is physically prevented from coming into contact with the material to be patterned. Indeed, stencils have been used for decades to shadow the deposition of metal vapors. When the stencil is applied to the substrate, metal deposits only on the areas exposed through the holes. Although stencils made of thin metal foil are inconvenient for patterning biological material from the liquid phase because they do not form a seal with the substrate, they have been used for patterning cell suspensions (37). Schwarz et al (38) made laser-ablated holes on a thin membrane of

poly(ethylene terephthalate) (PET) and polyethylene to pattern proteins from solution. PDMS has been micromolded into a self-sealing stencil that can be applied to a substrate to protect the areas that are contacted by PDMS and leave the substrate exposed through the holes for adding or removing inorganic materials (39, 40) or living cells (40a). When cells are seeded on the stencil-covered substrate, cellular "islands" with the same shape as the hole remain after cell attachment (both to the substrate and to the stencil). The stencil is straightforward to use: It can be applied by hand or tweezers to the everyday cell culture surface, peeled off after cell attachment without harming the cells, combined with virtually all cell types and substrates (even curved or moist), and reused after a simple ethanol wash.

MICROENGINEERING OF CELL-SUBSTRATE INTERACTIONS

The molecular mechanisms by which cells recognize certain substrates as suitable for attachment have been largely elucidated. Cell adhesion is mediated by cell membrane–bound receptors. In particular, integrins, a family of heterodimeric transmembrane proteins that are linked to the cytoskeleton on the cytoplasmic side of the membrane, recognize specific peptide sequences present in the fibrillar protein meshwork found in vivo and known as the ECM. Thus, integrins establish a mechanical link not only between the membrane and the ECM substrate but also between the ECM and the cytoskeleton. Moreover, integrins aggregate in organized structures termed focal contacts (41–43). More importantly, in most cell types, certain biochemical signals essential for cell growth, function, and survival are triggered by integrins upon attachment; without attachment, the cell undergoes apoptosis (programmed cell death) (44). Since many cell types secrete ECM, an artificial substrate may support cell adhesion even if it is not initially coated with an ECM protein.

Success in creating cellular micropatterns thus rests on the ability to control the size, geometry, and chemical nature of the adherent layer. We stress that cellular interactions between the cell and a microtopographically patterned substrate (i.e. containing three-dimensional structures) are not covered here because they do not generally result in cellular micropatterns; they have been reviewed in detail elsewhere (45, 46). However, work that exploits the three-dimensional nature of the substrate to create cellular micropatterns is covered. In addition, cells are sensitive to other physical parameters of the substrate, such as local temperature, which may be used for selective attachment of cells to surfaces (47). Materials other than physiological biomolecules were the first to be explored owing to the constraints imposed by early micropatterning techniques.

Interaction of Cells with Metallic Micropatterns

Carter (48–50) was the first to produce, as early as 1967, surfaces with micropatterned cellular adhesiveness (see Figure 2, color insert). He observed that L-line

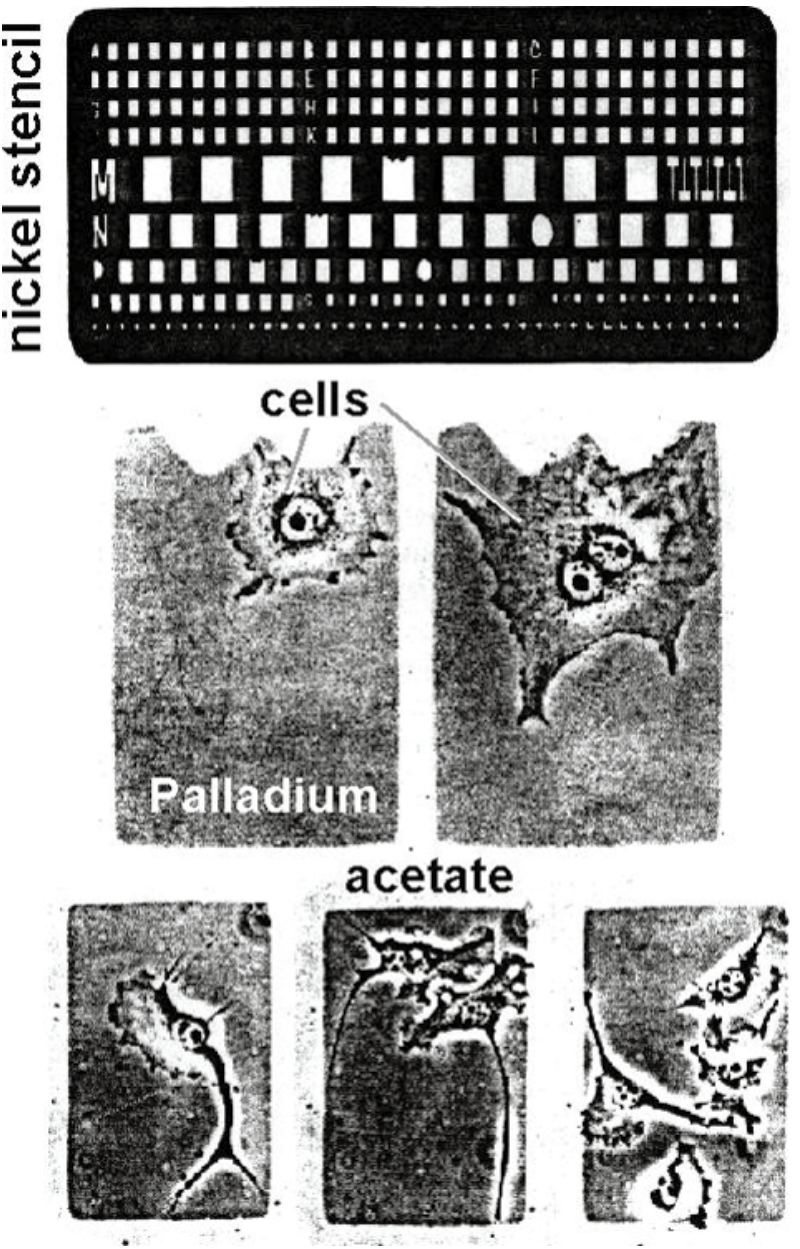

Figure 2 SB Carter created the first micropatterned cell culture [with permission from (40)]. By shadow evaporating palladium through a stencil mask (*top*), cell-adhesive metal islands on acetate were created (*middle* and *bottom*).

fibroblasts adhered to and spread on ~100-by 150-μm palladium (Pd) islands created on a piece of (nonadherent) acetate film by evaporating the metal through a 15-μm-thick nickel stencil mask (48). The micropatterns enabled Carter to observe and control the directionality of cell motion, or haptotaxis, in response to a surface chemical gradient (49, 50); he also foresaw the feasibility of massively parallel single-cell clonogenic assays (48). The idea of patterning by means of a removable (and reusable) stencil mask is attractive in its conceptual simplicity, compatibility with a variety of surfaces, low toxicity, and small cost. Since the work of Carter, this technology has been used by a number of researchers, in combination with metallic stencil masks (51–54), transmission electron microscopy grids (55–59), or photolithography (60), to pattern a variety of cell-adhesive materials, such as gold (58), glass (51), and metal oxides (60).

The observation of cells on metallic micropatterns has provided valuable insights into cell-substrate adhesion mechanisms. Letourneau (56) studied axon guidance in chicken embryo neuron cultures and found a hierarchical preference of growth cones for polyornithin or collagen over Pd micropatterns on tissue culture plastic. Albrecht-Bueler (57) found evidence that the directional preference of 3T3 cells during migration is a response to the substrate adhesiveness as sensed by the filopodia: he analyzed the exploratory function of filopodia during spreading of 3T3 cells onto micropatterns of gold on glass to find that cells did not extend lamellipodia unless some filopodia, in a rapid scanning motion, had contacted a gold area. Experiments performed by Westermark (59) and Pontén & Stolt (52) demonstrated that cell contacts are not necessary to produce a quiescent growth state by showing, for a variety of cell types, that cells seeded on Pd islands stopped growing once confluence was reached even though they had a free edge bordering the air-dried agarose background. Ireland and colleagues found that cytoskeletal organization in 3T3 cells depends on the shape of the Pd island, e.g. the focal points tend to accumulate at the periphery of the islands, whereas the total focal contact area is independent of the shape of the cell (61).

Interaction of Cells with Polymer Micropatterns

Many researchers have micropatterned polymers to control cell-substrate interactions. Poly(*N*-isopropylacrylamide) is a thermoresponsive polymer that, below a certain temperature (32°C in pure water and 25°C in pH 7.4 saline), becomes soluble in aqueous solutions and thus ceases to support cell attachment (62–64). This principle has been extended to photosensitized copolymers of poly(*N*-isopropylacrylamide) to produce micropatterns of fibroblasts on polystyrene (65); the detachment selectivity is dependent on the physisorbed protein layer (66), cell type (64), and polymer layer thickness (67). Yamazaki et al (68, 69) were able to optimize attachment/detachment selectivity to the point that cellular spheroids of specified size could be generated upon detachment. Photoreactive copolymers have been developed by Matsuda and coworkers to control cell attachment (70–74). Of all photosensitive polymers, the most widely available are

the photoresists used in microelectronics processing, and their photochemistry is extremely well characterized. Rohr et al (75–78) made photoresist patterns on glass to study the patterned growth of neonatal rat heart cells in culture; the photoresist resisted the attachment of cells and did not show signs of cytotoxicity, as assessed by morphology and electrophysiology measurements, for up to 17 days of culture. Nicolau et al (79) found that the attachment selectivity, tested with neurons and glial cells on a range of photoresists, depended strongly on the photoresist type.

However, metals and polymers, by virtue of being chemically foreign to in vivo cellular environments, might arguably trigger unexpected, nonphysiological cell responses. Hence, there arises a need to control not only the sizes of the cell-adhesive features but also their biochemical compositions.

Interaction of Cells with Micropatterns of Self-Assembled Monolayers

Surface chemistry methods have been used to tailor the adhesiveness of biomolecules and cells on artificial surfaces for a long time (80) [see the review by Drumheller & Hubbell (81)]. Usually, these methods seek the formation of an intermediate monolayer of organic molecules bearing specific functional groups by reaction of their reactive end groups with a clean surface of the appropriate reactivity. In general, the heterobifunctional nature of these molecules ensures that the thin film is monomolecular. Upon formation of a complete monolayer, the chemical nature of the surface is no longer defined by the composition of the underlying substrate but rather by the exposed functional groups of the monolayer.

Some organic molecules form closely packed self-assembled monolayers (SAMs) [see the review by Ulman (82)]. SAMs of different end-group functionalities have been used to tailor surface properties such as adhesion (83), lubrication (84), wettability (85), and protein physisorption (86). Moreover, SAMs may be functionalized with reactive groups to which biological material is subsequently attached. Biomolecules may be immobilized either directly or by attaching an intermediate tether or cross-linker to the monolayer (81). The creation of SAMs whose end-group functionalities are recognized by cell membrane receptors (87, 88) or by tagged proteins (89, 90) is also possible. Although organic polymers have poorly characterized surface compositions, they are also amenable to SAM derivatization after certain chemical treatments (22–24, 81, 91–94).

Cell function data obtained using monofunctional SAM substrates should be interpreted with caution. Recent results indicate that some cell types may be sensitive to substrate surface functionalities even if the SAM substrate is coated with protein prior to cell seeding (95). Kapur & Rudolph (96) observed that cellular morphology and strength of adhesion were strongly influenced by the composition of the underlying silane monolayer. Tidwell et al (97) showed that in serum-containing medium, the degree of endothelial cell growth (but not protein adsorption or elutability) varied significantly with the functional group and, most importantly, that it was at least three times higher on polystyrene tissue culture

dishes than on any of the SAM surfaces studied. This suggests that the rich (yet poorly defined) chemical complexity of tissue culture polystyrene surfaces is advantageous for cell culture. Despite the issues of monolayer order and biointeractions, the use of SAMs in biopatterning is appealing because the adhesiveness of the surface is engineered at a molecular level (98) and, at the same time, biopatterning is reduced to patterning the SAM (99). SAMs have been micropatterned by using photolithography (100), a previously patterned heterogeneous surface (101), microstamping (14), laser ablation (102), UV irradiation (103), electron beams (104), ion beams (105), and scanning probe microscopes (106).

Kleinfeld et al (100), in an attempt to create functional in vitro neuronal networks, cultured embryonic mouse spinal cells and perinatal rat cerebellar cells on micropatterns consisting of alternating stripes of aminosilane and methyl-terminated alkylsilane SAM. Patterns with cerebellar cells were preserved for at least 12 days, which allowed for the development and observation of electrical excitability. Their choice of aminosilanes was justified to mimic poly(D-lysine), a polyamino acid containing amino side groups, which is an adhesive surface coating used widely in neuronal culture. Indeed, only the areas derivatized with diamines and triamines—but not monoamines—promoted the adhesion of embryonic mouse spinal cells and perinatal rat cerebellar cells, and cell morphology was assessed to be very similar to that of cultures on poly(D-lysine). The goal of using methyl-terminated alkylsilanes was to recreate the hydrophobicity of certain surfaces that inhibit cell adhesion. This is arguable; as we will see, other researchers have created cellular micropatterns on which cells attach precisely to methyl-terminated areas. For a given surface, cellular adhesiveness appears to be highly dependent on a number of parameters, including cell type and medium composition.

Early on, Clark and colleagues emphasized the importance of medium composition for cell-substrate interactions (107–109). Fibroblast-like baby hamster kidney cells attached to and aligned with aminosilane patterns on a methylsilane background, but cell adhesiveness contrast was reduced (by ~60%) as the time of exposure to serum-containing medium was increased (24 h). Healy et al (110) found that bone-derived cells did not comply with the pattern unless they were seeded in the presence of serum or unless serum proteins were prephysisorbed onto the aminosilane/alkylsilane pattern. It was determined later that for regioselective attachment and spreading, the presence of vitronectin (an ECM protein) in the serum was required and that the difference in cell adhesion strength between aminosilane and alkylsilane vanished after 2 h of culture, presumably due to physisorption of endogenously secreted ECM (111, 112). Interestingly, when culture was extended for 15 to 25 days, the matrix synthesized by the cells was preferentially mineralized on the aminosilane regions (113).

Given the propensity of proteins to physisorb to most surfaces (114–117), surface micropatterns exposed to serum may not even be "seen" by the cell, and therefore confounding results may be obtained. Aebischer and colleagues cultured neuroblastoma cells on aminosilane SAM patterns created on a fluorinated ethylenepropylene surface (118, 119), and they found that the highest differentiation

rates and lowest attachment selectivities were obtained for prephysisorbed fibronectin or fetal calf serum (120, 121), whereas cells attached best when they were seeded on aminated fluorinated ethylenepropylene that had been coated with albumin (118); this finding is intriguing because albumin, lacking integrin-specific peptide sequences, is a nonadhesive protein. The confusion is exemplified by a contradicting study that showed that laminin physisorbed preferentially onto alkylsilane SAM areas versus aminosilane SAM areas, allowing for the preferential attachment of neurons to the alkylsilane SAM (122). A recent report stated that if laminin was allowed to physisorb onto an aminosilane SAM pattern on glass, it could be removed from the glass background simply by thorough rinsing of the substrate with deionized water (123); no data on the absolute laminin coverage on glass before and after the water and ethanol rinses, or after the micropatterning step, were provided, but more than 80% of the neurons attached preferentially to the lines and ~66% showed a preferential neurite outgrowth along the lines. In another study, the ability of a micropatterned perfluoroalkylsilane SAM to contain the attachment of endothelial and neuronal cells to, and their growth on, the aminosilane SAM areas was attributed solely to electrostatic attraction between the positive charges present on the amino-terminal SAM and the negative charges present at the cell surface (124–126). However, proteins adsorbed from the serum-containing medium could have played an important role as well. The same applies to other serum-based cellular studies of SAMs with different foreground and/or background chemistries, such as aminosilane/octadecyltrichlorosilane on glass or silicon (127–129) or 3-mercaptopropanoic acid/octanethiol on gold (130). Another study showed that prephysisorption of human fibronectin onto aminosilane SAMs on glass did not affect the adhesion, spreading, or proliferation of human umbilical vein endothelial cells when these cells were seeded in medium containing 2% fetal bovine serum (131). Importantly, human umbilical vein endothelial cells were shown to differentiate into oriented neovascular cords after addition of basic fibroblast growth factor in long-term (7- to 10-day) culture (131). In light of the undefined surface composition resulting from the use of serum-containing medium, Corey et al used serum-free medium for seeding of rat hippocampal neurons onto micropatterns of (cell-adhesive) aminosilane SAM and phenyltrichlorosilane SAM (132).

Despite the difficulties in controlling physisorption of proteins from the medium onto SAMs, Stenger et al (133) have recently been able to bias the polarity of embryonic hippocampal neurons in a specified direction by means of (adhesive) aminosilane SAM patterns on a (nonadhesive) fluoroalkylsilane SAM background. Cell bodies adhered to a 25-μm-diameter island and were allowed to extend processes along four 5-μm-wide paths in orthogonal directions, with the particularity that three of the paths were interrupted in 10-μm-long segments, whereas the fourth was continuous. The axons of most cells grew along the continuous path and their dendritic processes grew along the other three paths, as identified by immunostaining of microtubule-associated proteins and neurofilament polypeptides that are process specific. This work represents an outstanding example of micrometer-scale engineering and control of cell function.

Interaction of Cells with Micropatterns of Extracellular Matrix Proteins

Letourneau and colleagues are credited with creating the first biomolecular micropatterns (134). Their method was based on the observation that cell adhesiveness of laminin could be inactivated by selective UV irradiation through a microfabricated metal stencil. Thus, they were able to create nonadhesive islands on an adhesive background. Only nonirradiated laminin was recognized by antilaminin antibodies and supported attachment of embryonic chick dorsal root ganglia neurons and growth of neurites. Interestingly, this differential response was not observed for irradiated fibronectin and was quantitatively correlated with the concentration of laminin initially applied to the substrate (134, 135). To investigate further the hypothesis that axons during development follow pathways of high-level adhesiveness, they developed a complementary method to create adhesive islands on a nonadhesive background of an air-dried mixture of albumin and agarose (136). They found that although the filopodial length of dorsal root ganglia growth cones was <30 μm, occasionally a >50-μm-wide nonadhesive gap between islands was spanned. Similarly, Corey et al (137) showed that hippocampal neurons were deterred from attaching to laser-ablated polylysine and that they migrated within 1 to 3 days to the areas where the local area of unablated polylysine was higher, such as the nodes of intersecting lines. However, this method produced toxic by-products when used with polyimide-coated electrode arrays (132).

Physisorbed protein layers are not stable in aqueous solutions. Also, proteins present in the medium or secreted by the cells after attachment may displace an underlying protein layer (138). For this reason, many researchers have focused on the chemical immobilization of proteins via cross-linkers or photoreaction schemes (11). Even though chemical (or physical) immobilization of proteins is likely to induce partial denaturation of the protein structure, which can affect cell function (139, 140), the peptide sequences necessary for attachment seem to remain largely exposed. Denaturation is to be expected when the micropattern is exposed to solvents during the micropatterning step. Notwithstanding, it is found that cells attach, spread, grow, and function on denatured ECM proteins, either physisorbed (141, 142) or chemisorbed (143, 144), presumably because the degree of denaturation does not mar integrin binding to the ECM peptide fragments. This is consistent with the fact that such denatured micropatterns, which are routinely visualized by secondary immunofluorescence, exhibit affinity binding with their natural antibodies. However, other long-term functions, more sensitive to the state of denaturation of the underlying ECM protein or to the presence of residuals from the solvent-stripped material, might be impaired by this culture technique. Tai & Buettner (144) studied nerve growth cone dynamics on micropatterned stripes of (solvent-exposed) chemisorbed laminin on a physisorbed albumin background. The mean outgrowth length along 20- or 30-μm-wide laminin stripes was observed to be smaller than that on uniform laminin surfaces, while outgrowth direction was strongly biased in the direction parallel to the stripes. To circumvent the problems

associated with photoresist removal, Wheeler's group microstamped proteins onto a glutaraldehyde-aminosilane-derivatized surface; protein patterns were aligned on titanium micropatterns, and the compliance of B104 neuroblastoma cells to the pattern was studied (20). By using elastomeric stencils, Folch et al (40a) have been able to create a micropattern of liver cells embedded in a collagen gel; since in this "sandwich" configuration hepatocytes exhibit increased levels of differentiation compared to cultures on physisorbed collagen cultures (145), this method may represent a further step in avoiding denaturation-related effects.

Proteins readily physisorb from solution onto most materials (114–117), a phenomenon that has not been thoroughly elucidated [reviewed by Chinn (146)]. The common belief, often encountered in the cellular micropatterning literature, that protein adhesiveness correlates with the hydrophobicity of the surface is not solidly founded, since neither very hydrophilic surfaces such as agarose gels nor very hydrophobic surfaces such as Teflon support protein adhesion. Even though physisorbed protein layers may elute from the surface as a function of time, physisorption procedures promoting cell attachment are simple and have been used in cell culture for decades on a variety of materials. Protein physisorption onto polymers may be enhanced by plasma polymerization (147) or plasma glow discharge (e.g. the tissue culture-grade polystyrene petri dishes) of the polymer surface. These treatments are believed to introduce a rich variety of chemical functionalities that attract proteins through electrostatic or dipolar interactions.

Combining the facts that it is surprisingly difficult to completely remove a physisorbed protein layer and that cell anchorage is exquisitively sensitive to trace amounts of ECM protein, several researchers have been able to produce cellular patterns on microfabricated templates of physisorbed protein, avoiding chemical immobilization methods altogether. Rudolph and coworkers (148) fabricated microtextured surfaces with deep trenches in a variety of biomedical polymers and selectively physisorbed proteins onto the mesas by carefully dipping the microstructures in a protein solution. As a result, only the mesas, and not the trenches, were coated with protein solution. With this method, they demonstrated the selective attachment of NCTC-929 cells to fibronectin-coated PDMS mesas (149). Folch & Toner (30) have created cellular micropatterns on a variety of biocompatible polymers and heterogeneous surfaces by microfluidic patterning of physisorbed ECM templates (Figure 3, see color insert). Craighead et al (129) selectively attached hippocampal neurons to microstamped poly(L-lysine) patterns after 2 to 4 h in medium containing 10% serum. Elastomeric stencils may be used to mask the physisorption of protein or the direct attachment of cells on a surface (40a); stencils are convenient for patterning cell types, such as fibroblasts, which feature poor adhesiveness selectivity and for patterning cells on homogeneous surfaces.

The design of the nonadhesive region is often crucial. Great research efforts have focused on the development of materials that do not support platelet or microbial adhesion for implantation and surgery (150). Examples of surfaces that exhibit low levels of protein physisorption and cell attachment are cellulose

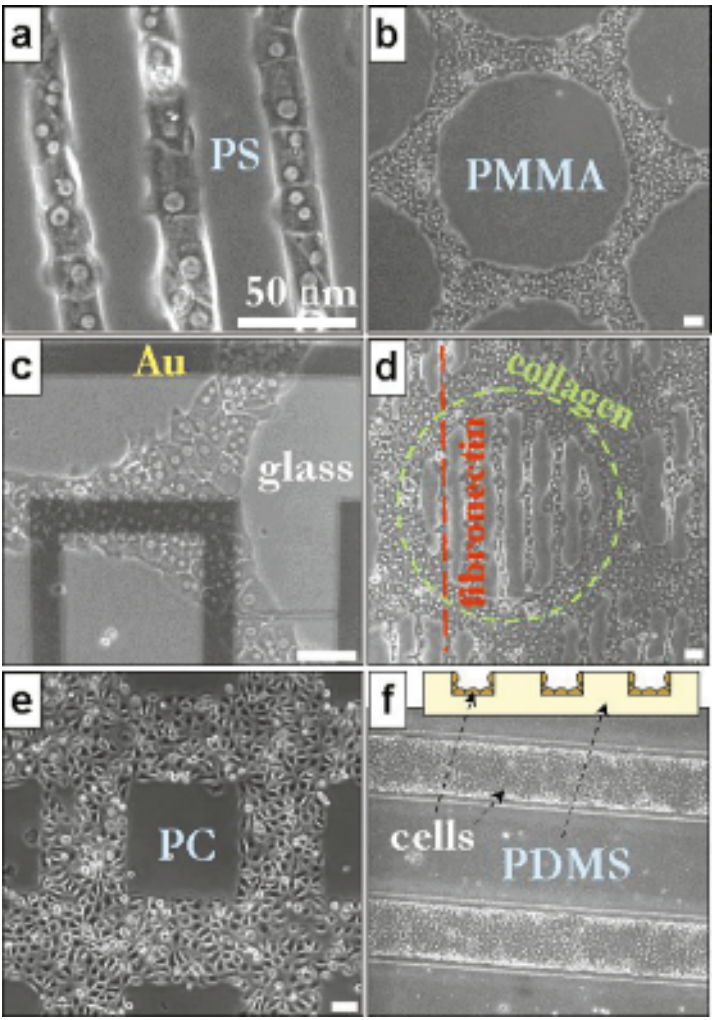

Figure 3 Cellular micropattens on templates of physisorbed ECM proteins on biocompatible materials. The templates were created by microfluidic patterning on polystyrene (*a*), poly(methyl methacrylate) (*b*), a gold microelectrode pattern on glass (*c*), an underlying protein micropattern on polystyrene (*d*), and microtextured PDMS (*e*). The template, made of collagen (*a*, *c–f*) or fibronectin (*b*,*d*), induced the selective attachment and spreading of hepatocytes (*a–d*, *f*) or keratinocytes (*e*).

acetate (151), paraffin (152), agarose (52), sulfonate-terminated alkylsilane SAMs (102, 103, 153), poly(ethylene glycol) (PEG) (92) [reviewed by Harris & Zalipsky (154)], PEG-derivatized alkanethiol SAMs (86, 155), interpenetrated copolymers of PEG (156–159), alkanethiol SAMs functionalized with tri(propylene sulfoxide) (160), fluorocarbon polymers (161), poly(vinyl alcohol) (162), multilayers of PEG functionalized with $SiCl_3$ (163, 164), or surfaces exposed to the surfactant Tween 20 (165). In addition, immobilization of albumin, a protein that does not contain any known integrin-binding sequences, is a common method used in cell culture to deter cell attachment.

Singhvi et al were able to engineer the shape of attached cells by microstamping PEG-derivatized alkanethiol SAMs (166). As shown in Figure 4 (see color insert), the spreading and shape of individual cells can be constrained within adhesive "islands"—physisorbed with fibronectin—surrounded by HEG (hexacthylene glycol)-terminated areas. Singhvi et al (166) observed that a reduction in DNA synthesis and an increase in apoptosis rates in micropatterned hepatocyte cultures correlated with a reduction of island size [graph in Figure 4 (see color insert)]. In addition, albumin secretion rates, which are known to decrease with increasing culture time as part of a hepatocyte dedifferentiation process, decreased at a slower pace for smaller islands. This indicates that, to some extent, cell function can be tailored by modifying cell shape. The elegance of the experiment resides in the fact that unlike previous work in which cell shape and function were modulated by varying the density of the ECM on the substrate (167) or by limiting the area available for attachment on microspheres (168), cell spreading was varied independently of ECM density.

Chen et al (169, 170) subsequently addressed the question of whether the critical parameter in the switching of cells between apoptosis and survival is the projected cell spread area or the total area of focal contacts. By breaking up islands into smaller (adhesive) separated spots, they were able to control cell spreading independently of the total integrin-ECM contact area. When cells were cultured on arrays of closely spaced 3- or 5-μm-diameter spots, they spread across several spots over the nonadhesive areas. Vinculin staining showed that cells formed focal contacts only on the fibronectin-coated spots. DNA synthesis increased with increasing total projected area at a nearly constant integrin-ECM focal contact area. This indicates that cell shape, and not cell-ECM contact area, determines cell fate. Island geometries, such as continuous 10-μm-wide lines of fibronectin that constrained spreading to intermediate degrees, neither supporting cell growth ($>1500\ \mu m^2$) nor inducing apoptosis ($<500\ \mu m^2$), caused endothelial cells to differentiate into capillary tube-like structures (171). It was also found that in contrast with fully spread cells (either on homogeneous plastic or on closely spaced arrays of 5-μm-diameter spots), human capillary endothelial cells that were prevented from spreading outside of small ($\sim$30-μm/side) square islands failed to progress through G_1 and enter S phase of the cell division cycle and to regulate essential cell cycle proteins (172).

Bailly et al (173) investigated the chemotaxis of cells at the edge of nonadhesive areas. Rat mammary carcinoma cells were free to move along lines of

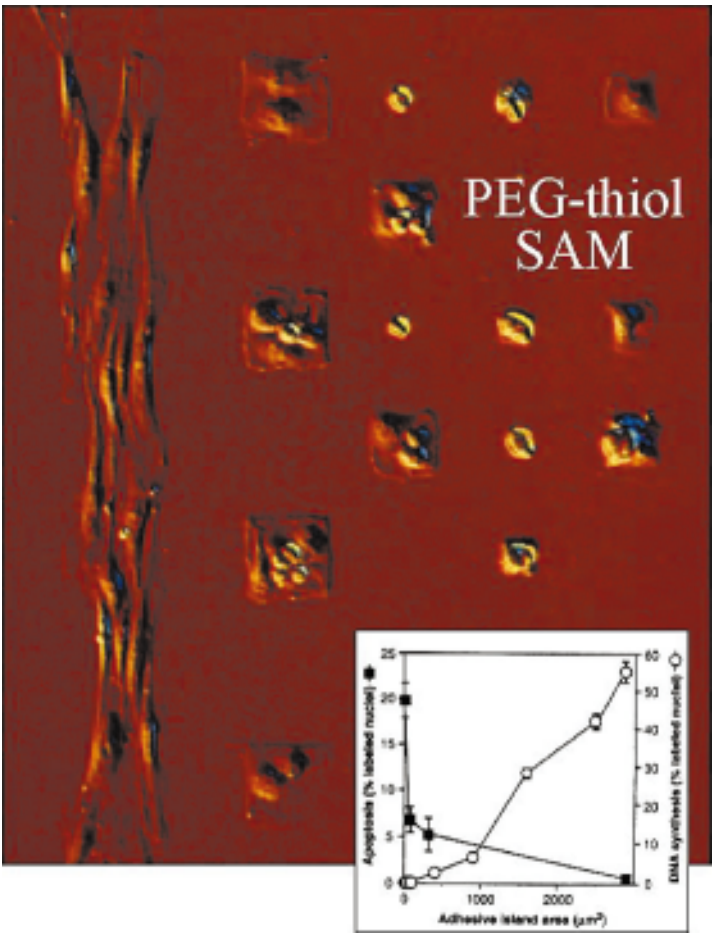

Figure 4 Control of cell shape by means of self-assembled monolayers of PEG-thiolate. A methyl-terminated alkanethiol pattern was microstamped on gold. The remaining gold surface was chemisorbed with PEG-terminated alkanethiol, which deters protein physisorption and endothelial cell attachment/spreading. The graph shows that, for similarly patterned hepatocyte cultures, apoptosis and proliferation rates decrease and increase, respectively, as island size increase. Adapted from Frankel & Whitesides, *On the Surface of Things*, Chronical Books, 1999 and (166).

hexadecanethiol SAM microstamped on gold that were bordered by a nonadhesive PEG-terminated alkanethiol SAM. Lamellipod extension was stimulated in specified directions by delivering chemoattractant with a pipette. When a cell was attracted into a nonadhesive region, it still extended lamellipods over the PEG-derivatized areas but rapidly retracted them. The absence of cell-substrate contacts on the nonadhesive areas (as determined by immunostaining of talin, one of the proteins that links the cytoskeleton to integrins) indicates that lamellipod extension can occur without establishment of focal contacts but that focal contacts are required for stabilization.

Healy and coworkers (174) have also demonstrated precise control of the shape of individual cells by micropatterning thin layers of PEG copolymers (159, 175). This provides additional evidence that regardless of the chemistry employed, micropatterning of PEG is a successful strategy for confining cell spreading. Vitronectin preferentially physisorbed onto aminosilane-derivatized glass areas surrounded by a PEG copolymer background and, as shown in Figure 5 (see color insert), directed the selective attachment and spreading of osteoblasts. Cells maintained on small ($<$900-μm^2) islands were not able to organize their cytoskeletons, whereas for larger islands that still constrain cell spreading, long-term (21-day) cytoskeletal organization was dictated by the shape of the adhesive area, confirming results by O'Neill et al (61) obtained with shadow-evaporated metal islands on polyhydroxyethylmethacrylate.

Other nonadhesive surfaces are being investigated for controlling cell attachment and spreading. Lhoest et al (176) exposed a micropattern of alternating stripes of hydrophilic and hydrophobic polystyrene to a solution containing the surfactant Pluronic F68 and ECM protein (either collagen or fibronectin; $\sim$33 μg/ml) and found that adsorption of Pluronic onto the hydrophobic areas predominated, effectively blocking protein physisorption. Rat adrenal pheochromocytoma cells were shown to adhere preferentially to the ECM-coated hydrophilic areas. Hydrogels such as agarose, which is known to deter protein physisorption and cell attachment (59), can be micromolded into the recessed regions of a PDMS stamp to create PDMS "islands" surrounded by the hydrogel (30). Hepatocytes were observed to attach only to the fibronectin-coated islands and to limit their spreading precisely to the border of the island for several days. Nakayama et al (177) have been able to create micropatterns of a (nonadhesive) thiolated poly(vinyl alcohol) hydrogel on gold; the gold was deposited by shadow evaporation onto a poly(ethylene terephtalate) (PET) sheet, and a hydrogel micropattern formed only on the gold areas. After physisorption of ECM protein onto the PET background, bovine endothelial cells were observed to attach and spread only on the PET areas for at least 2 days.

Interaction of Cells with Micropatterns of Cell-Adhesion Peptides

In 1984, Pierschbacher & Ruoslahti identified the short peptide sequences that are present in ECM and are recognized by cell membrane receptors which trigger cell attachment and spreading (178). Massia & Hubbell (87, 88) first showed that

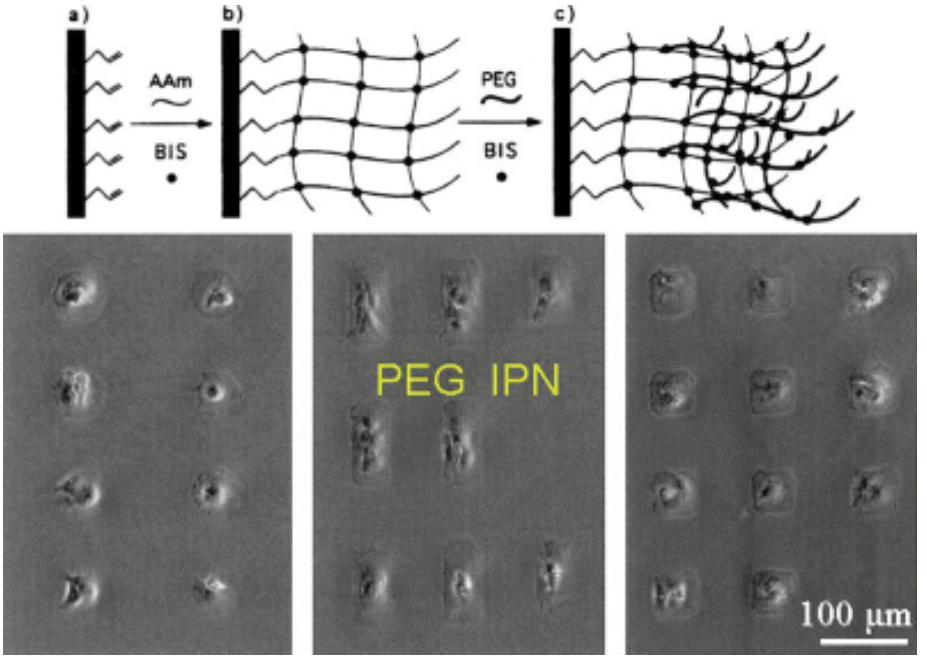

Figure 5 An interpenetrated network (IPN) of PEG and acrylamide immobilized on an allyltrichlorosilane SAM is photolithographically defined to produce cell-adhesive islands surrounded by the nonadhesive IPN background. The cell-adhesive areas are derivatized with aminosilane and physisorbed with vitronectin. (*Top*) Schematics of the chemical grafting scheme for the IPN-PEG background: (*a*) allyltrichlorosilane SAM; (*b*) acrylamide grafted onto the SAM; (*c*) PEG interpenetrates into the acrylamide network. (*Bottom*) Osteoblasts confined to single-cell islands of various shapes. Adapted with permission from (174).

cell attachment and spreading can be promoted on surfaces derivatized with the tripeptide sequence Arg-Gly-Asp (RGD) and similar peptide sequences identified by those cell adhesion receptors. These peptide layers are more stable under heat treatment and pH variation than ECM protein coatings (179). The advantage of promoting cell attachment via covalently linked biospecific peptide sequences is that surface coverage and composition are easily controlled and temporally stable [reviewed by Drumheller & Hubbell (81)]. In light of these findings, several groups have made efforts to micropattern cell attachment peptides on a range of materials.

Ranieri et al showed that neuroblastoma cells attach preferentially to the laminin fragments YIGSR and IKVAV (180) when a micropattern of such peptides is immobilized onto an otherwise nonadherent fluoropolymeric surface. A similar preference of neuroblastoma cells for micropatterns of the laminin fragment CDPGYIGSR immobilized onto glycophase glass, poly(vinyl alcohol), and hydroxylated fluorinated ethylenepropylene, was observed (181). Likewise, Matsuzawa et al (182) created micropatterns of a synthetic peptide derived from the neurite outgrowth-promoting domain of the B2 chain of laminin. The peptide-modified areas supported the growth of rat hippocampal neurons with a morphology resembling that of neurons cultured on laminin-coated surfaces. The guided neuronal outgrowth could be observed at high resolution by atomic-force microscopy (183). The same chemistry has been used for adherence of neurons to a field-effect transistor array (184). Sugawara & Matsuda (162) selectively attached bovine endothelial cells to micropatterns of RGD on poly(vinyl alcohol). Saneinejad & Shoichet (185) showed that hippocampal neurons seeded under serum-free conditions attached selectively to the peptide-modified areas only and displayed neurite extension statistics similar to or greater than those of neurons seeded on laminin. They could use commercially available peptide sequences labeled with cysteine (and thus containing a gold-reactive thiol group) that were immobilized on gold islands created by shadow evaporation onto a PEG-aminosilane SAM surface. This approach uses essentially the same micropatterning technology as that employed by Carter 33 years earlier, but the chemical compositions of both the adhesive and nonadhesive areas are now a rational design based on present-day knowledge of cell and protein interactions with surfaces at the molecular level. Zhang et al (186) used a similar chemistry in combination with microstamping to demonstrate the selective attachment of cells (human epidermoid carcinoma A431 cells, 3T3 mouse embryo fibroblasts, or primary human embryonic kidney 294 cells) to the peptide areas in medium containing 10% serum.

However, micropatterning peptides on surfaces that support protein physisorption immediately raises the question of whether cells will, sooner or later, attach to ECM proteins present in the medium or secreted by the cells. Increased control over cell-substrate interactions may be achieved by immobilizing cell adhesion peptides on PEG copolymers (157) or PEG-functionalized SAMs (187); since the surface does not support ECM protein physisorption, the precise surface concentration of adhesive ligands is known. Patel et al (188) micropatterned RGD-like peptide sequences to demonstrate the selective attachment of bovine

aortic endothelial cells and of PC12 nerve cells, respectively, in serum-free medium. Interestingly, they were able to synthesize a nonadhesive PEG copolymer that is biodegradable and contains biotin groups; the peptides were microfluidically patterned and immobilized via biotin-avidin binding. Park et al (189) have used an inkjet printer to polymerize, layer by layer, three-dimensional scaffolds of biodegradable poly(L-lactide) with immobilized peptide sequences to promote hepatocyte attachment. The surface density of RGD-like peptides (and hence the adhesiveness of the surface) can also be tailored by using a combination of synthetic photosensitive oligopeptides and laser illumination. By scanning a laser beam at various speeds, Herbert et al (190) were recently able to create oligopeptide (adhesive) gradients on a PEG-thiol background. As expected, fibroblasts attached to the gradients at a density that increased with increasing oligopeptide density. This technology allows for unprecedented control over cell attachment ligand densities and enables molecular-level studies of haptotaxis.

Laser writing offers great potential for creating three-dimensional biomolecular patterns. Aebischer and coworkers promoted neurite outgrowth in three dimensions by seeding neurons onto agarose gels that had been derivatized with laminin oligopeptides (191). The oligopeptides were selectively immobilized within a transparent agarose gel by means of a laser beam (192). This system, provided the gel does not perturb diffusion of neurotransmitters at the chemical synapse, may have a clinical application in nerve regeneration.

Interaction of Cells with Micropatterns of Other Bioactive Molecules

Biorecognition between cells and surfaces goes both ways. As much as cells recognize passive ligands on surfaces for attachment suitability or migration guidance, they also encounter surfaces (such as other cells) that display enzymatic or immunological activity ("bioactivity") when in contact with them. The interest in immobilization of bioactive molecules on solid surfaces is not new (80). However, only recently has it been possible to create micropatterns of enzymes (193) or antibodies (153) that retain their bioactivity.

Imanishi's group showed that insulin photoimmobilized on tissue culture polystyrene enhanced the growth of anchorage-dependent cells such as Chinese hamster ovary cells and mouse fibroblasts (194), and with greater mitogenic activity than free insulin (195). Micropatterns of insulin could be straightforwardly created on PET by exposing the substrate to UV light through a mask in the presence of a photoreactive insulin solution (196). The immobilized insulin did not enhance cell attachment but transduced a growth signal to the cells. When the medium was depleted of serum, cell growth was only observed for the cells on immobilized insulin. A similar experiment was done with micropatterns of mouse epidermal growth factor (197). Although cells attached everywhere across the substrate, seeding at low densities resulted in segregated cellular stripes when the stripes were far apart ($\sim$100 μm) because cell growth was observed only in the epidermal growth

factor-immobilized areas. When pattern line widths were smaller than the cells ($\sim$2 μm), patterned cell growth did not occur because all cells proliferated.

The immunorecognition affinity of antibodies for certain cell-surface ligands is the basis of panning, a method commonly used to sort cells. When a glass or plastic plate is coated with antibodies against proteins present on the membrane of a certain cell type, the plate will immunocapture that cell type preferentially. Micropatterns of antibodies, then, can be used to create cellular micropatterns. Rather than relying on the cell's ability to recognize—and, thus, preferentially attach to—a biomolecular template, St. John et al (198) demonstrated that a physisorbed micropattern of an antibody against *Escherichia coli*, made by microstamping onto an unmodified silicon surface, selectively "captures" the bacteria on the antibody-covered areas. Similarly, Le Pioufle et al (199) have demonstrated the selective immunocapture of human T helper lymphocytes by means of a clever antibody immobilization method. A fluorescein isothiocyanate-labeled conjugate of anti-CD3 antibody was immobilized on a gold micropattern. When a lymphocyte suspension was exposed to this pattern, cells were captured on the gold-antibody pattern in less than 1 min. The concept could potentially be used for patterning several cell types simultaneously, if different antibodies to selected membrane proteins of each cell type featuring small cross-reactivities can be produced in a practical manner.

MICROENGINEERING OF CELL-CELL INTERACTIONS

Kleinfeld et al (100) were the first to attempt to control in vitro synapse formation between pairs of neurons on a microfabricated network. Patterns of the cerebellar cells were preserved for at least 12 days, which allowed for the development and observation of electrical excitability. Unfortunately, the interpretation of the electrophysiology data is not straightforward owing to the variety of cell types that attached to the pattern simultaneously. Ravenscroft et al (200) were able to create circuit-like micropatterns of hippocampal neurons and recorded spontaneous as well as evoked electrical activity by means of dual patch clamp recording. Fully functional synapses were identified on aminosilane surfaces but were absent from fluoroalkylsilane surfaces (201).

It should be emphasized that in vitro neuronal networks, like most in vitro systems, are inherently simple. The human brain contains around 10^{11} neurons of 10,000 different types, and each neuron may form synapses with thousands of other neurons (202). Moreover, in contrast with other organs featuring well-known physiologies and multicellular three-dimensional architectures (such as the liver), the connectivity of the brain is still under intense investigation. Therefore, it is not yet clear whether relevant information can be extracted from such simple systems. Assuming that artificially formed neuronal networks can be functionally meaningful, one still faces the challenge of recording from ensembles of neurons. Micropatterned electrodes have been used to record from neuron cultures

(203), semi-intact systems (such as slices), and retinas (204) for a long time. The challenge then lies in causing the neurons to attach to the electrodes in a useful manner. Pine's group (205, 206) micromachined grids of "neurowells" in silicon to mechanically trap neurons onto electrodes and yet allow them to freely grow processes and synapse with each other. A similar approach has implemented a flow-through glass chamber on the silicon substrate to deliver neurons to etched wells, but clusters of neurons formed on each well (207). Jimbo et al (37) selectively seeded rat cortical neurons (from 18-day embryos) on transparent, indium-tin oxide microelectrodes on glass by using a stainless steel stencil containing 130-μm-diameter holes aligned in registration with the indium-tin oxide microelectrodes. Furthermore, the electrodes were recessed into the substrate (forming 20-μm-deep wells) and the wells were interconnected by 20-μm-wide grooves. Unfortunately, the stencil holes were too big and not all wells contained just one neuron. Connections between neurons in adjacent wells were thus established through the grooves. It was observed that once neurites grew into the grooves, they seldom grew over the walls. Intracellular-calcium imaging under low-Mg^{+2} conditions demonstrated periodic transients in the fluorescence of an intracellular calcium indicator, which were in synchrony with the electrical bursts sensed by the indium-tin oxide electrodes.

Toner's group (208) has introduced a method to micropattern cocultures of two cell types. Liver cocultures are usually created by mixing two cell types at random (Figure 6*a*, see color insert) at a certain cell-cell ratio and have been extensively used as an in vitro system to recreate cell-cell heterotypic interactions [review by Bhatia et al (209)]. However, owing to the random nature of the seeding process, many different interactions occur that confound data analysis and that do not necessarily correspond to the interactions so exquisitely structured in vivo. Microfabricated cocultures (Figure 6*b–d*, see color insert), compared to traditional random cocultures, represent the next step in the attempt to simulate in vivo interactions by allowing the researcher to specify cell density and the total length of contact ("heterotypic interface") between the two cell populations independently of cell-cell ratio (209). In this method, a micropattern of collagen (chemically immobilized on aminosilane SAM on glass) with a physisorbed albumin background is created by photolithography.

Bhatia et al (143) showed that primary hepatocytes from rat liver in serum-free medium attach selectively to the collagen areas and do not spread onto the albumin areas for the first 12–24 h. On the other hand, 3T3-J2 fibroblasts did not respect the collagen-albumin template: They attached nonpreferentially to all collagen- and albumin-coated areas (Figure 7, see color insert), presumably because fibroblasts can attach to their own abundant ECM protein secretions. Hence, a micropatterned coculture of hepatocytes and fibroblasts can be achieved by seeding fibroblasts after full attachment and spreading of the hepatocytes. It was found that peak albumin and urea levels increased with the length of the heterotypic interface [209, 210; graph in Figure 7 (see color insert)]. Intracellular albumin staining of microfabricated cocultures revealed that induction of hepatic

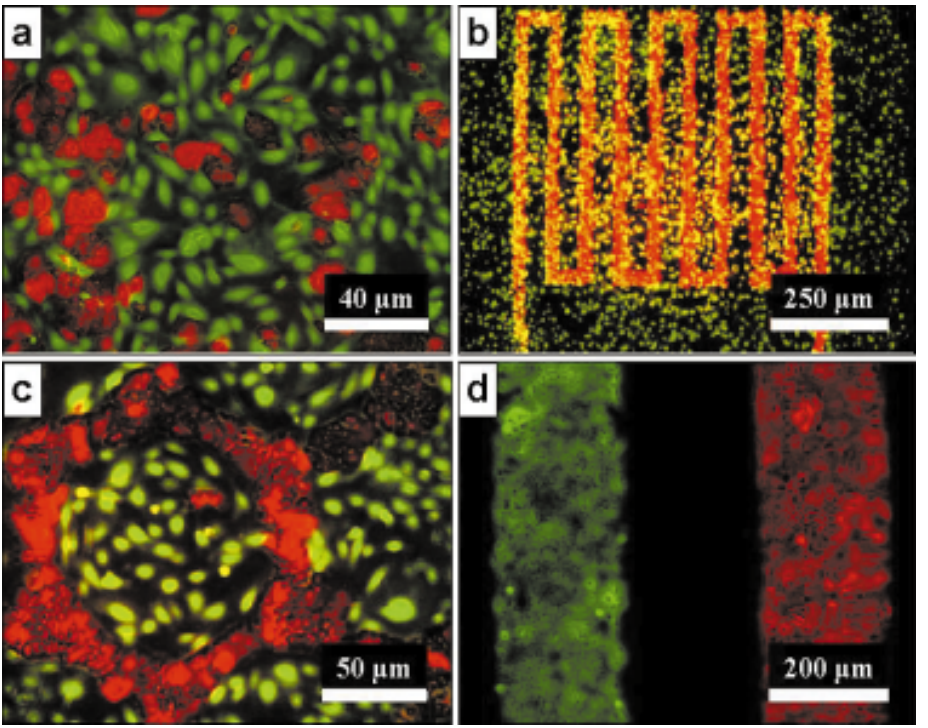

Figure 6 Comparison between random (*a*) and micropatterned (*b–d*) co-cultures. Compared with random co-cultures, microfabricated co-cultures allow for the design of the interface between different cell populations independently of the seeding density. Because the interface can be tailored with micrometer resolution, micropatterning allows for a more faithful in-vitro recreation of the cell-cell interactions found in vivo. (*a*) Random co-cultures of hepatocytes (*red*) and fibroblasts seeded at a 1:1 cell ratio on polystyrene homogeneously coated with collagen. (*b*) "Circuit" of hepatocytes on immobilized collagen with a background of fibroblasts on physisorbed albumin. (*c*) Hepatocytes (*red*) on physisorbed collagen patterns with a background of fibroblasts (*green*) on physisorbed albumin. (*d*) Fibroblast suspensions labeled with different dyes were delivered to selected areas of a homogeneous substrate by means of elastometric microchannels.

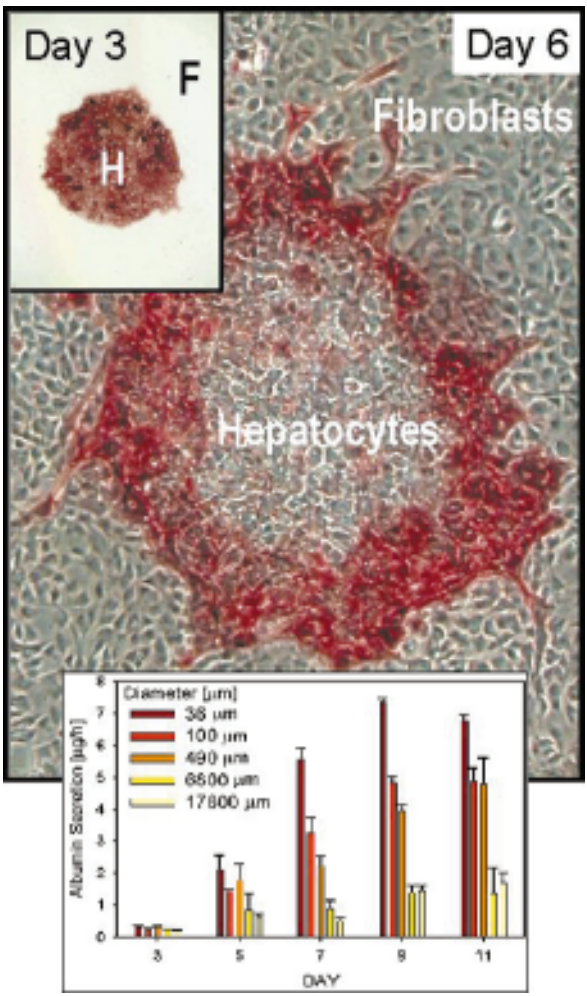

Figure 7 Micropatterned co-cultures of hepatocytes and fibroblasts. In 500 μm-diameter islands of rat hepatocytes surrounded by mouse 3T3-J2 fibroblasts, intracellular albumin (*red*) was present on all hepatocytes at day 3 of culture (*inset*) but was found to be limited to those hepatocytes only a few cell diameters away from the fibroblast interface by day 6. The graph shows that albumin secretion increases as the length of interaction (the island diameter) between the two cell populations increases (decreases). Adapted with permission from (210).

functions in hepatocytes occurred throughout all cell culture islands by day 3 [Figure 7, inset (see color insert)] but by day 6 was maintained only in the vicinity of fibroblasts, compared with hepatocytes far (more than ~5 cells diameters) from the heterotypic interface. It was also found that albumin production and urea synthesis, which are known to decay with time in random cocultures (211), are sustained for lengthy periods of time (~2 to 6 weeks) in certain microfabricated cocultures featuring the same cell-cell ratio as the random coculture. In addition, the response of hepatic function to changes in fibroblast number was distinct from that attributed to increased contact between hepatocytes and fibroblasts, suggesting that fibroblast number plays a role in modulation of hepatic function through homotypic fibroblast interactions (208).

MICROENGINEERING OF CELL-MEDIUM INTERACTIONS

In standard cultures, cells are covered by a homogeneous layer of medium. In vivo, however, the medium surrounding a given cell may differ drastically at distances ranging from a few millimeters to as little as one cell body. For example, cells of the epithelium feature two distinct membrane domains facing different fluids; endothelial cells lining the walls of capillaries are exposed to blood flow only on one side of the cell. Most cell types are, by virtue of their distance from the closest blood supply, living in a permanent yet dynamic gradient of nutrients and secreted factors that is often used by themselves and other cell types to guide their migration and growth. The behavior of cells under flow conditions in artificial chambers has been studied extensively (212), but the spatial heterogeneity of fluids found in vivo has not been addressed yet. Microfabrication technology offers the possibility of modulating the medium around cells on a cellular scale. Compared to the microfabrication techniques used to address cell-substrate and cell-cell interactions, however, the recreation of multifluid microfluidic environments is still in its infancy, and only prospective work can be reviewed here.

Folch et al (34) were able to deliver different cell suspensions to specific locations of the substrate by means of removable, biocompatible microfluidic channels made of PDMS [Figure 6*d* (see color insert)]. Each microchannel (~100 μm deep) could be filled simply by manual injection. After cell attachment (~1 h) under non-flow conditions, the microchannels may either be removed to produce a cellular micropattern or left over the cells for further studies under flow conditions. The technique can be used straightforwardly to micropattern several other cell types simultaneously. Takayama et al (36) have also used flow in PDMS microchannels to deliver cells and proteins. Importantly, they have been able to perfuse the same channel with various solutions simultaneously with little intermixing by operating under laminar-flow conditions. This method allows for perfusing different parts of the cell with different solutions. Micropatterns of proteins and cells (erythrocytes and *E. coli*) simultaneously introduced into the microchannels were demonstrated.

CONCLUSIONS AND FUTURE DIRECTIONS

As our knowledge of the interactions between a cell and its surroundings (the medium, the underlying substrate, or the neighboring cells) advances, more-sophisticated cell culture approaches are needed. Microfabrication techniques offer the potential to modulate, on a cellular level, the biochemical composition as well as the topography of the substrate, the type of cell neighboring each cell, and the medium surrounding each cell. Unfortunately, adapting existing micropatterning methods or conceiving altogether new ones has absorbed much of the research energy to this date. Beyond the obvious, albeit fascinating, exploration of selective attachment of proteins and cells, few scientific questions have been asked, much less answered, surely reflecting the limitations of the technology. In retrospect, many findings have been confounded by the physisorption of serum proteins, which are now known to play an important role in cell attachment, at the time of seeding.

Microfabrication technology has become an enabling technology for biologists in many areas. Important steps have been made toward building functional artificial neural networks. Micropatterns of PEG have been shown to inhibit protein physisorption, cell attachment, and cell spreading with micrometric control. It was thus shown that cell shape is a determining factor in cell function in culture. Micropatterned cocultures of two cell types were introduced to control cell-cell interactions on a micrometer scale, and it was found that the degree of contact between the two cell populations was correlated with cell function. Microfluidic devices that deliver a variety of biological solutions to different areas of a substrate are being developed. Yet the technology is entering the biomedicine field very slowly, partly because expertise in this area is still lacking in most biological laboratories. Several recent technologies that can be combined with tissue culture polymers and/or bypass chemical methods altogether should enable biologists to adopt microfabrication technology in their laboratories with minimal technological development.

Undoubtedly, much like DNA chips are emerging as a valuable tool for gene sequencing, cellular arrays will constitute the future "lab-on-a-chip," wherein hundreds or thousands of cellular islands will be cultured and monitored in parallel on a small area. These miniaturized cell cultures will allow the observation of single-cell dynamics with faster, less noisy assays by averaging their output by the hundreds or the thousands. Integrated within their own incubator substrates, they will occupy less bench space and consume smaller volumes of precious reagents. Compared to presently available cell-based biosensors, batch fabrication will reduce per-unit costs and allow for integration with microelectronics and/or micromechanical components. In sum, microfabricated surfaces are emerging as an indispensable tool for the new era of cell culture, especially in basic biology studies, tissue engineering, drug discovery, and biosensor applications.

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