Fabrication of aligned microstructures with a single elastomeric stamp

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The fabrication of complex patterns of aligned microstructures has required the use of multiple applications of lithography. Here we describe an approach for microfabrication that encodes the two-dimensional spatial information of several photomasks onto a single elastomeric stamp by mapping each photomask onto distinct heights on the surface of the stamp. Pressing the stamp against a surface collapses the topography of the stamp such that each recessed layer contacts the surface in stepwise sequence; the greater the applied pressure, the larger the area of the stamp that contacts the surface. After contact of each new layer with the surface, we use techniques of soft lithography (microcontact printing, microfluidics, and patterning through membranes) to pattern the surfaces that contact the stamp and those that do not with inorganic, organic, or living materials. Microfabrication through the use of multilevel stamps provides a promising alternative to conventional lithography for the construction of multicomponent, aligned surfaces; these structures may find use as components of microfluidic devices or biological patterns.

Microlithography is a binary process. That is, lithography segregates a surface into regions that are exposed to a modification and regions that are masked from that modification. In photolithography, irradiation through a mask that consists of two regions (clear and opaque) defines a map of exposed and unexposed regions. Similarly, in soft lithography, placement of an elastomeric stamp that possesses two regions (recessed and nonrecessed) onto a surface defines regions that are stamped and those that are not (1, 2). Fabrication of complex, multilevel structures that contain more than two types of elements is required often for microelectronic, microfluidic, and microelectromechanical systems and necessitates multiple applications of lithography in which each step must be aligned spatially with previous ones. Alignment of patterning steps in the fabrication of organic, living, or soft structures has proven to be cumbersome for many reasons; the elastomeric stamps used in soft lithography are difficult to align over large areas, alignment of biological materials requires sterile working conditions throughout the fabrication process, and patterning onto devices that are not openly accessible (such as a sealed microfluidic device) is extremely challenging. Here, we describe an alternate approach toward the fabrication of complex structures that alleviates these difficulties by using lithographic patterning elements that are not binary. This approach relies on the use of a single multilevel, elastomeric stamp to generate a multicomponent, aligned structure.

Fig. 1 outlines our experimental approach. We use photolithography to define features of photoresist that possess different heights on a silicon wafer. Curing a prepolymer of PDMS against the photoresist master generates a PDMS stamp (1) or membrane (2) that has a surface relief with various depths. Placing this stamp on a substrate brings an initial set of regions into contact with the substrate. The application of pressure to the back of the stamp partially compresses the stamp such that slightly recessed features of the stamp come into contact with additional regions on the substrate. In principle, the larger the applied pressure, the deeper the levels of the stamp that contact the substrate and the greater the area of contact between the PDMS and the substrate. At each stage of compression of the stamp, the surface of the substrate can be patterned by applying soft lithographic techniques.

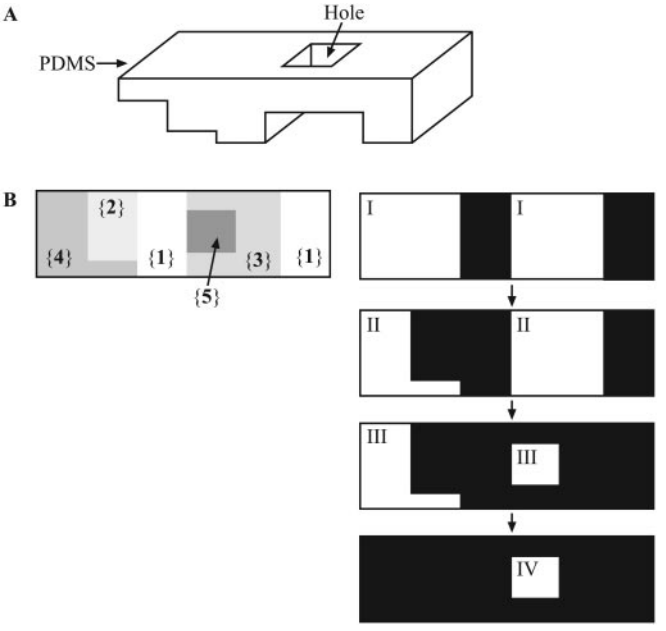

Fig. 1. Schematic outline of patterning with a five-level polydimethylsiloxane (PDMS) membrane. (*A*) The surface of the stamp consists of five distinct sets of regions, each located at a unique height. (*B*) In the top view of the stamp pattern, lighter shades indicate less recessed regions (*Left*). We label features that are not indented collectively as {**1**}, features that have the smallest indented depths as {2}, and so on. We denote the depths of the regions {**I**} by d_I ($d_1 = 0$). As in this example, holes in PDMS membranes are considered to have infinite depth and are denoted by {**N**} in an N-level membrane. (*Right*) In the series, black regions indicate contact with the substrate; when the stamp is placed on a surface and pressure is applied, the portions of the stamp that contact the surface are {**1**}, followed by {**2**}, and so on. The application of soft lithographic techniques (processes *I*, *II*, *III*, and *IV*) at each change in the masking pattern generates a distinct combination of processes experienced by each of the five regions on the substrate.

We have demonstrated the feasibility of using multilevel stamps with three soft lithographic techniques to generate aligned structures comprised of inorganic or organic materials: microcontact printing (μCP), micromolding in capillaries (3), and patterning through membranes (2). These results show that patterning with multilevel stamps adds functionality to soft lithography by enabling the fabrication of complex patterns of two or more organic or living materials.

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Abbreviations: PDMS, polydimethylsiloxane; μCP, microcontact printing; NRK, normal rat kidney epithelial; ECM, extracellular matrix.

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Materials and Methods

Chemicals and Proteins. All fluorescently labeled proteins were obtained (Molecular Probes) and used at a concentration of 10–50 μg/ml in PBS (GIBCO); red-, green-, and blue-labeled proteins were Alexa Fluor 594-, 488-, and 350-conjugated antibodies, respectively. The poly(ethylene oxide-propylene oxide) copolymer Pluronics F127 was obtained from BASF (Mount Olive, NJ). Malachite green and Congo red were obtained from Sigma. BSA (Intergen, Purchase, NY) was used as a 1% solution in PBS. Human fibronectin (BD Biosciences) was used at a concentration of 50 μg/ml in PBS. Evaporated films of silver were etched in an aqueous solution that consisted of 100 mM potassium thiosulfate, 10 mM potassium ferricyanide, and 1 mM potassium ferrocyanide (Aldrich) as described (4).

Fabrication and Application of Multilevel Stamps. Masters consisted of multiple patterned layers of irradiated SU-8 photoresist (MicroChem, Newton, MA). Each layer was fabricated by exposing and developing a layer of resist that was spun onto previously patterned layers; 100-nm-thick titanium microstructures made by lift-off served as alignment marks to aid in the registration of layers. Because SU-8 is a negative resist, patterned layers were resistant to the exposure or development of subsequent layers. Stamps of PDMS were made by treating the masters with a vapor of (tridecafluoro-1,1,2,2-tetrahydrooctyl)-1-trichlorosilane (United Chemical Technologies, Bristol, PA) for 30 min and then curing Sylgard 184 (Dow-Corning) for >1 h against the silanized master. Membranes of PDMS were fabricated by spin-coating Sylgard 184 on the master for ≈30 sec at 1,000–2,000 rpm (2). The thicknesses of stamps and membranes were ≈5 mm and 100–300 μm, respectively.

To compress a multilevel stamp, we placed it on a glass coverslip or polystyrene dish and applied light pressure to the stamp with tweezers until the desired levels in the stamp came into contact with the substrate. Regions in contact with the substrate appeared dark under diffuse illumination, whereas those not in contact appeared bright. For a given surface relief, thinner stamps required more pressure than thicker stamps did to collapse; pressures generated with tweezers could readily collapse features with depths of <20 μm for 5-mm-thick stamps. For μCP of proteins, the stamp on which proteins adsorbed was dried before printing. For fluidic delivery of proteins, both ends of the stamp were cut to provide access to channels, a drop of solution was placed at one end, and vacuum was applied at the other end (5). Channels between the stamp and substrate were not dried at any point. Because a layer of protein adsorbed onto both the stamp and substrate, collapse of the stamp did not transfer protein from one to the other. For both μCP and microfluidics, adsorption of proteins proceeded for >1 h, after which the stamps or channels were flushed extensively with PBS and water.

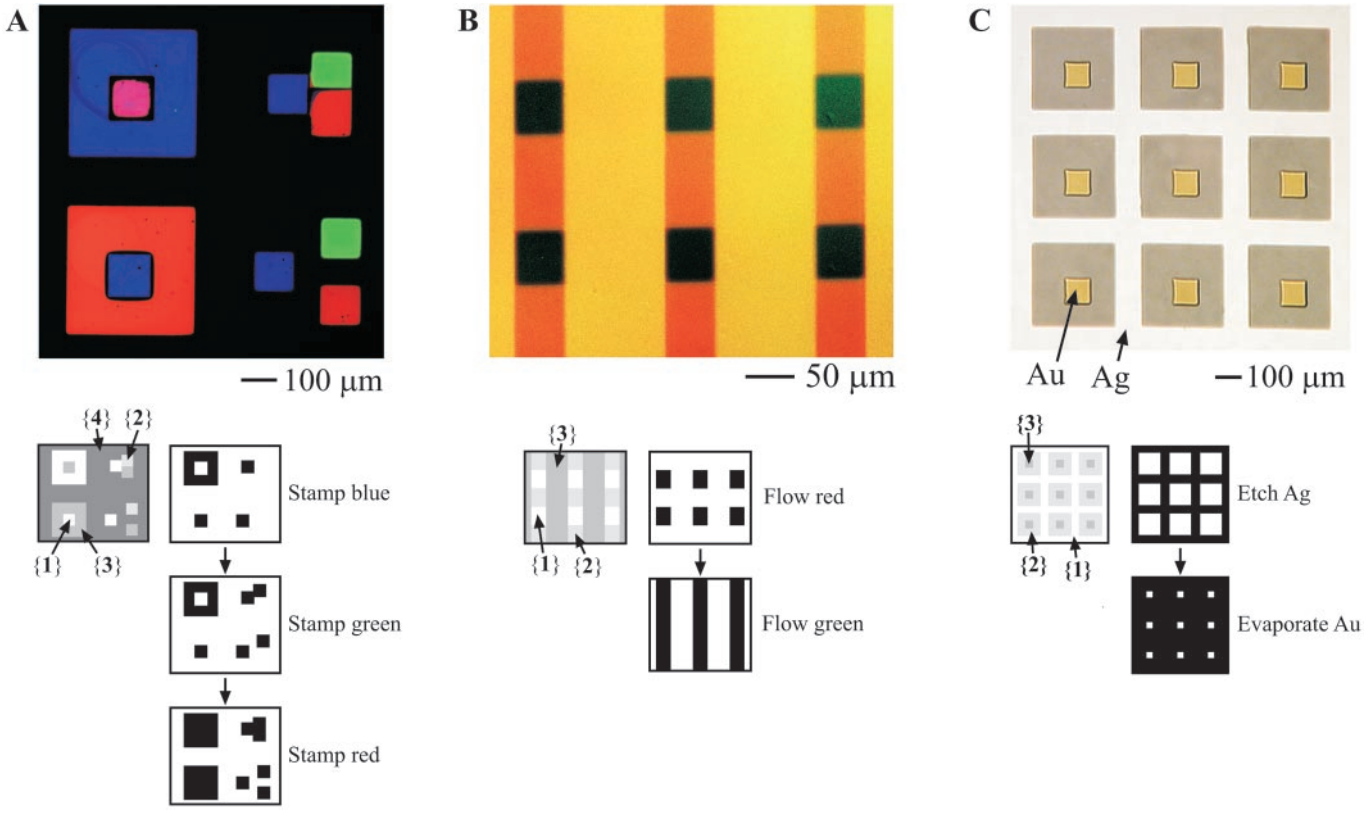

Fig. 2. Micrographs of aligned patterns. (*A*) Inking a four-level stamp (d_2 = 1 μm, d_3 = 2 μm, d_4 = 50 μm) with three fluorescently labeled proteins and stamping onto a glass slide generated a pattern of those proteins. Each stage of compression prints onto the substrate blue-, green-, and red-labeled proteins that had been adsorbed onto {**1**}, {**2**}, and {**3**}/{**4**}, respectively. (*B*) Flowing solutions of proteins in channels between a three-level stamp (d_2 = 2 μm, d_3 = 50 μm) and a glass slide created a grid of proteins. First, we flowed a solution of red-labeled protein between the stamp and substrate in the open channels of {**2**} and {**3**}; the channels consisted of a grid of lines. We then washed the channels with PBS, collapsed {**2**} onto the slide, and flowed a solution of green-labeled protein through {**3**}; the channels were a parallel set of lines. The second protein (a rabbit anti-mouse antibody) bound to the first protein (a goat anti-rabbit antibody), yielding a region that fluoresces yellow. In the schematics that accompany the image, channels are represented by white regions. (*C*) A combination of etching and evaporation through a three-level membrane (d_2 = 7 μm) generated a pattern of silver and gold on a glass slide. We placed a membrane (≈150 μm thick) on a ≈50-nm-thick film of silver on glass and etched the exposed silver on {**2**} and {**3**} with a ferricyanide solution (**4**). We then rinsed and dried the sample and evaporated ≈100 nm of gold through the holes in the membrane onto {**3**}.

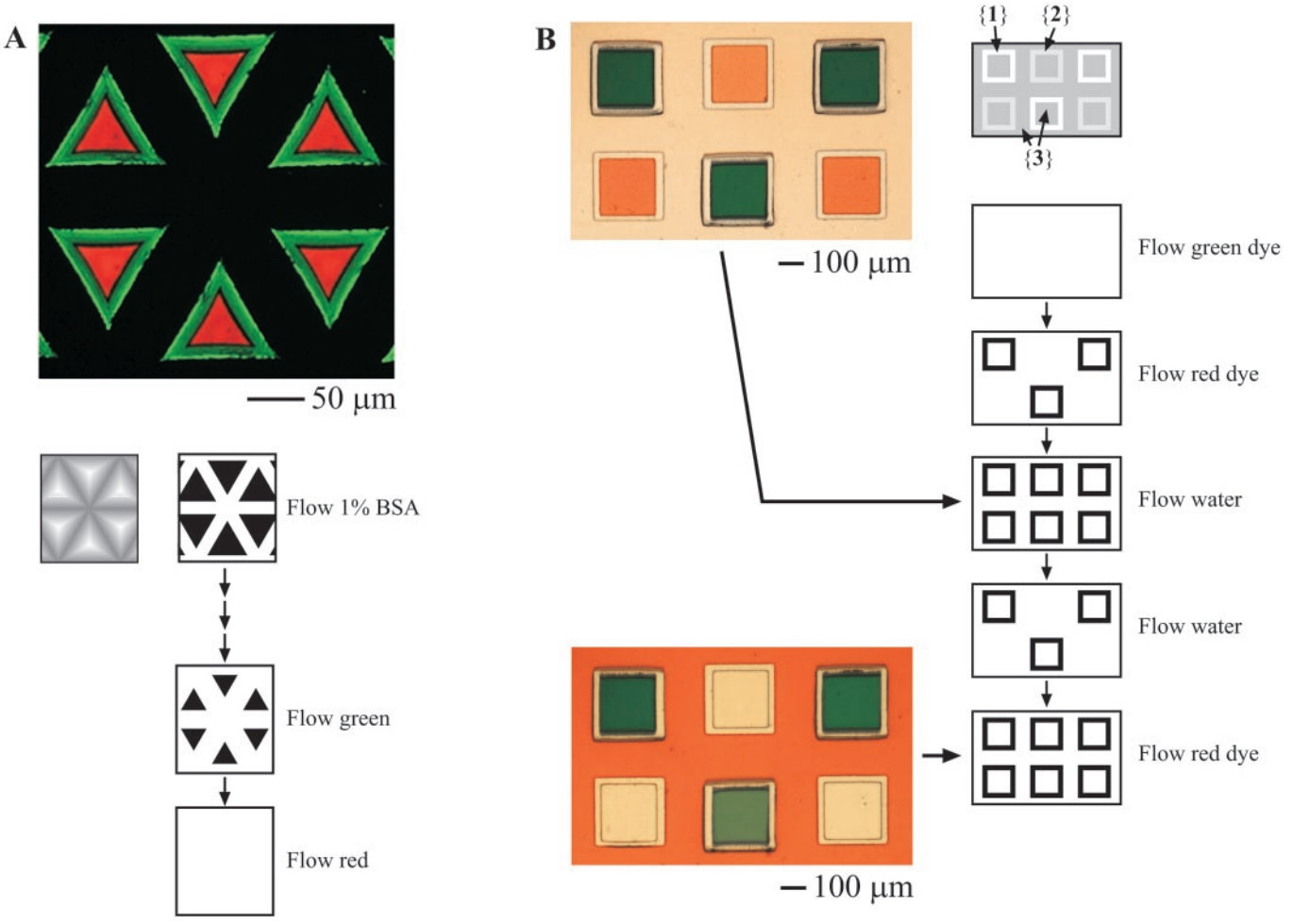

Fig. 3. Multilevel patterning in continuous and reversible formats. (*A*) Flowing solutions of proteins under a stamp with corner-cube topology resulted in deposition of an arbitrary gradient onto a Petri dish. We placed the stamp onto a dish, applied moderate pressure to the back of the stamp, and flowed in a solution of BSA. Reducing pressure to the stamp while flowing in successively more dilute solutions of green-labeled protein (from 50 to 10 μg/ml in PBS) generated a gradient of protein next to the BSA-coated regions. We then flowed in a solution of BSA to passivate the uncovered surface, flowed in a solution of red-labeled protein, and allowed the stamp to lift from the surface. (*B*) Fabrication of an addressable array of wells. Here $d_2 = 8$ μm and $d_3 = 50$ μm; an external fence prevents {**1**} from contacting the substrate without compression. A sequential combination of compressions and flows trapped liquid selectively in certain wells: after partial compression of the stamp, a solution of Malachite green dye was captured in the wells enclosed by {**1**}; the fluid in the channel was replaced by a solution of Congo red; and further compression of the stamp captured this solution into the second array of wells. The remaining solution was washed out with water, and an image was captured. A second image was taken after the wells holding the solution of Congo red were unsealed, washed with water, and resealed, and fresh solution of Congo red was introduced into the channel.

Cell Culture. Normal rat kidney epithelial (NRK) cells (ATCC CRL-1571) were cultured under a 5% CO_2 atmosphere in DMEM supplemented with 10% FCS, 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (GPS). NIH/3T3 mouse fibroblasts (ATCC CRL-1658) were cultured under a 5% CO_2 atmosphere in DMEM supplemented with 10% calf serum and GPS. Before experiments, NRK cells and fibroblasts were labeled with red- and green-labeled cell-permeant molecules (CellTracker orange and green used at a 1:1,000 dilution in medium, Molecular Probes), respectively. Cells then were detached with 0.25% trypsin/EDTA (GIBCO) and replated on patterned substrates by pipetting a suspension of the cells directly onto prewetted substrates. Unattached cells were removed by washing with fresh medium.

Image Acquisition. Fluorescence, bright-field, and phase-contrast micrographs were captured at ×4 or ×10 magnification with a Nikon Eclipse TE200 microscope that was equipped with a Spot RT charge-coupled device camera. All images were taken at full 1,520 × 1,080 resolution, and contrast and brightness were enhanced either with SPOT RT software or Adobe PHOTOSHOP.

Results and Discussion

Fig. 2*A* illustrates the result of μCP with a four-level stamp. Each level of the stamp was coated with a different protein; compressing the coated stamp against the substrate brings these levels sequentially into contact with the substrate and prints the pattern shown in Fig. 2*A*. We coated the stamp by a recursive procedure. We first exposed the stamp to a solution of red-labeled protein that adsorbed on {**1**}, {**2**}, {**3**}, and {**4**} and removed protein that coated {**1**} and {**2**} by stamping onto a glass slide under moderate pressure. When the stamp was exposed to a solution of green-labeled protein, the protein adsorbed selectively on the bare regions of the stamp, {**1**} and {**2**}, and not at all on {**3**} and {**4**}. Stamping onto a glass slide under light pressure and immersion in a solution of blue-labeled protein likewise replaced green-labeled protein with blue-labeled protein on {**1**}. Just before the final step, {**1**} was coated

with blue-labeled protein, {**2**} was coated with green-labeled protein, and {**3**} and {**4**} were coated with red-labeled protein. The depths d_I should be small compared with the lateral dimensions of the stamp to avoid substantial deformation of the stamp. The topology of the stamp is not constrained by multilevel μCP, because one can change the order of stamping by switching the depths of any two regions without altering the final stamped pattern.

Microfluidics and membrane-based lithography provide additional strategies for aligning complex structures using multilevel stamps (Fig. 2 *B* and *C*). These approaches require that features not in contact with the surface be connected at each stage of compression to be exposed to a passing fluid or gas. We have demonstrated this principle in Fig. 2*B* by using a three-level stamp to pattern a glass slide with flow. In general, for microfluidic patterning each region in {**I**} should be adjacent to at least one region in some {**J**}, where J > I. For instance, in Fig. 2*B* placing a fourth level in the stamp such that {**3**} and {**4**} are not adjacent (say, by putting regions in {**4**} inside the squares of {**2**}) does not achieve additional functionality in the patterning. For patterning through membranes, regions in {**I**} should be adjacent to regions in some {**J**}, where J > I; {**J**} can refer to solid regions of PDMS or to holes in the membrane (Fig. 2*C*).

The alignment of features using each of these techniques seems to be limited only by the alignment of the photolithographic master. As designed, regions that comprise a particular level all contact the surface before regions that comprise a deeper level do. For instance, in Fig. 2*A* regions of {**3**} never contact the substrate before all regions in {**2**} do. In μCP as illustrated in Fig. 2*A*, features that are adjacent on a stamp can appear slightly separated on the final substrate because of incomplete collapse of the walls separating the different layers of the stamp. By using stamps with shallower features ($d_2 = 0.5$ μm and $d_3 = 1$ μm) or with a deeper final trench ($d_4 = 100$–200 μm), we can collapse these walls completely to produce patterns of directly adjacent materials. Alternatively, true adjacency in features can be made by fluidic techniques; in Fig. 2*B*, no area separates regions coated with both red- and green-labeled proteins from those coated with only red-labeled proteins.

Aside from alignment, multilevel patterning adds functionality and versatility to traditional soft lithographic techniques. First, because every depth in the stamp corresponds to the deposition of a unique set of materials on a substrate, a graded or sloped feature on the stamp can generate a nearly infinite number of patterns of materials on a surface; increasing the compression of a sloped stamp brings a continuously larger set of regions into contact with the substrate. We have demonstrated the use of a graded stamp with a corner-cube topology to fabricate an arbitrary, graded pattern of proteins (Fig. 3*A*). Second, multilevel patterning creates contacts between the stamp and substrate that may be reversible. Fig. 2 *A–C* shows patterns that were fabricated by bringing the stamp into greater contact with the substrate with time. In contrast, Fig. 3*A* displays a pattern that was generated by allowing the stamp to reduce its contact with the substrate with time. Being able to compress or decompress to a particular level in the stamp affords flexibility in the patterning strategy. We illustrate this feature with an array of wells that are addressed by compression of the stamp (Fig. 3*B*). In this example, delivery and capture of liquid in the wells are separate events that are controlled by the compression of the stamp. Not only can these events be timed, but they are also reversible in that cycles of compression and decompression of the stamp may capture and release substances from the flow. This sort of device may find use as a set of addressable microreactors and in microanalytical systems that require sequential sampling of a changing flow.

We illustrated the utility of patterning with multilevel stamps in the construction of an addressable array of cellular cocultures

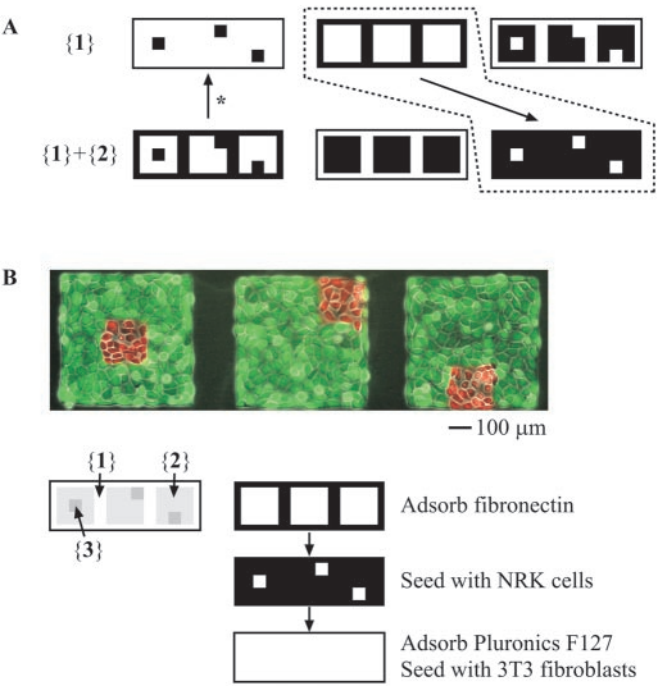

Fig. 4. Fabrication of patterned cellular cocultures. (*A*) Possible permutations of patterns mapped onto a multilevel stamp to build a coculture. The first row depicts a set of possible patterns generated by layer {**1**} placed against a substrate, and the second row depicts a set of possible patterns generated by compressing layer {**2**} onto the substrate. Each arrow indicates a choice of two patterns that could be used to generate a coculture. The dotted line delineates the patterns used in *B*. (*B*) Cocultures of labeled NRK cells and fibroblasts. The image combines fluorescence and phase-contrast views of NRK cells (red) and fibroblasts (green). The three-level membrane ($d_2 = 1$ μm, membrane thickness = $\approx$150 μm) was placed against a Petri dish, masking {**1**}. We coated regions corresponding to {**2**} and {**3**} with fibronectin, collapsed {**2**} of the membrane onto the dish, seeded NRK cells onto {**3**}, removed the membrane, coated {**1**} with 0.1% Pluronics F127 in DMEM to render it resistant to cell attachment, and seeded fibroblasts onto {**2**}. Removal of the membrane did not appear to disrupt the adsorbed layer of fibronectin, because protein does not print onto the hydrophobic stamp (6).

that aligns cells of different types spatially. Such a construct has wide applicability in the study of multicellular communication and organization. Fig. 4 illustrates an approach to organize cellular cocultures. Here, three distinct materials, extracellular matrix (ECM) protein and two cell types, must be delivered to three related sets of regions: (*i*) an array of ECM-coated areas, (*ii*) a subset of areas coated with ECM where cells of one type attach, and (*iii*) the remaining areas coated with ECM on which cells of another type attach. In theory, the levels in an N-level stamp can be ordered in *N*! ways that generate equivalent patterns on the substrate. This degeneracy often allows one to select the appropriate ordering that eases the fabrication of those patterns. In this case, there are 3! or 6 possible routes to build the surface. We demonstrate one of the routes in Fig. 4*B*, where coating with ECM precedes selective deposition of cells onto protein-coated regions. Other routes are equally feasible. One such route (denoted * in Fig. 4*A*) involves the deposition of protein and cells in one region followed by a nonadhesive block in the spaces between squares and then deposition of protein and cells in adjacent regions. This example illustrates the versatility of multilevel patterning in constructing complex features on a substrate.

The primary advantage of multilevel patterning is that a single stamp embodies all the spatial information required to fabricate a complex structure. No additional registration beyond that used

to create the initial master is needed; many stamps may be cast from the master. Compared with conventional lithography, multilevel patterning theoretically can require many more masks to generate an equivalent pattern. To distinguish N distinct sets of regions on a surface, multilevel patterning requires $N - 1$ masks, whereas lithography with binary masks may use as few as $\log_2 N$ masks. In practice, however, many degeneracies in pattern reduce the number of masks required for multilevel patterning. For instance, none of the patterns shown here can be made with fewer masks by conventional lithography (two for Figs. 2, *B* and *C*, and 4; three for Figs. 2*A* and 3*B*; Fig. 3*A* cannot be made by lithography with half-tone masks).

The techniques used here illustrate that the application of multilevel stamps is likely in areas where soft lithography already has shown to be advantageous such as in patterning of organic and living materials, patterning of flexible or curved substrates (7), and fabrication of microanalytical and fluidic devices (8, 9). The practical difficulty in aligning separate elastomeric stamps across large areas (10) has emerged as the primary obstacle toward the application of soft lithography in the fabrication of complex structures. The use of a single stamp as the patterning element removes this difficulty by shifting the need for alignment to the fabrication of the master, on which alignment between lithographic steps is well established. With manual application of pressure using forceps, we obtained successful patterning over several mm^2 in >90% of substrates; failure occurred when we did not accurately gauge by eye the extent of deformation. We anticipate that more uniform application of pressure to the stamps would extend this patterning to significantly larger areas.

In spirit, our approach is similar to the recent demonstrations of patterning through laminar flow (11) or with gray-scale masks (12, 13) and of increasing information storage with a multidepth disk (14). All these approaches rely on increasing the amount of information stored in the patterning element itself rather than on repeatedly using a half-tone mask. We have demonstrated that it is possible to replicate these information-rich patterning elements from a silicon master to elastomeric stamps such that repeated *de novo* fabrication of the original lithographic master is not necessary. We have reused stamps at least five times without loss of resolution by sonicating used stamps in 70% ethanol for 5 min to remove adsorbed proteins.

We have not ascertained the spatial limitations of multilevel patterning. All the soft lithographic procedures that were applied here may be used at a much smaller size scale than the 100-μm scale used in this work (15). We anticipate that reduction of feature sizes to micrometer or to submicrometer scales is feasible; the depths on the stamp will scale accordingly. At this size regime, manual application of pressure is probably too variable for reproducible results, and it may be necessary to use a translation stage to apply pressure. Barring these difficulties, we believe that patterning with multilevel stamps provides a viable alternative to conventional microlithography for the fabrication of complex, aligned microstructures.

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