Microfabricated elastomeric stencils for micropatterning cell cultures

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Abstract: Here we present an inexpensive method to fabricate microscopic cellular cultures, which does not require any surface modification of the substrate prior to cell seeding. The method utilizes a reusable elastomeric stencil (i.e., a membrane containing thru holes) which seals spontaneously against the surface. The stencil is applied to the cell-culture substrate before seeding. During seeding, the stencil prevents the substrate from being exposed to the cell suspension except on the hole areas. After cells are allowed to attach and the stencil is peeled off, cellular islands with a shape similar to the holes remain on the cell-culture substrate. This solvent-free method can be combined with a wide range of substrates (including biocompatible polymers, homogeneous or nonplanar surfaces, microelectronic chips, and gels), biomolecules, and virtually any adherent cell type. © 2000 John Wiley & Sons, Inc. J Biomed Mater Res, 52, 346–353, 2000.

Key words: cellular micropatterns; microfabricated stencil; microfluidic; PDMS; biocompatible materials

INTRODUCTION

In traditional cell culture, cell function is studied under homogeneous conditions across the substrate. However, cells respond to local concentrations of a variety of molecules present on the underlying surface (e.g., extracellular matrix proteins), dissolved in the extracellular environment (e.g., growth factors, oxygen, etc.), or anchored on the membrane of adjacent cells (e.g., membrane receptors). As a result, the analysis of homogeneous cell cultures is inevitably hindered by a distribution of confounding interactions. Microfabrication techniques offer great potential to design, on a micrometer scale, the biochemical composition of the substrate, the medium surrounding a given cell, as well as the type of cell contacting each cell. Previous work has demonstrated the micron-scale control of cell-substrate contact area,[1–5] cell attachment and growth,[6–13] and cell–cell interactions.[14–16] Usually, selective cell attachment is achieved indirectly by microfabricating a template to which cells adhere preferentially. The template may be made of metals,[1,2] self-assembled monolayers,[3,4,6,7,11,17] polymers,[5,8] extracellular matrix proteins,[13–16] cell-adhesive peptides,[9,10,12] or a combination thereof. This strategy has several drawbacks: (1) by the very presence of the template, cellular micropatterns on homogeneous surfaces are not possible; (2) for a given template, attachment selectivity varies broadly with cell type; (3) the composition of the template may be affected by proteins adsorbed from the seeding medium before the cells actually "see" the surface; and (4) in many cases, fabrication of the template requires experimental methods such as surface chemical modification, biochemical synthesis, and/or microfabrication, which are not readily available in most biological laboratories.

Alternatives to template-based patterning exist. We and others have recently been able to deliver cell suspensions onto selected regions of a substrate by means of microfluidic channels.[18,19] Although this method circumvents template-based patterning, it can be applied only to a few, metabolically slow cell types, because the flow (and, hence, the delivery of oxygen and nutrients) must be arrested, while the energy-con-

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suming processes of anchorage and spreading take place. Another alternative consists of using a stencil (i.e., a thin sheet containing holes of specified shapes and sizes). Metallic stencils have been micromachined by chemical etching and used for a long time to fabricate microelectrodes by shadow-evaporation of metal through the stencil holes. As early as 1967, Carter[1] used a nickel stencil to generate cellular micropatterns, but only to shadow-evaporate templates of cell-adherent palladium onto nonadherent acetate; the stencil was removed prior to cell seeding. Jimbo et al.[20] used a stainless-steel stencil (containing 130 μm-diameter holes) placed in contact with the cell-culture substrate during seeding to mask the attachment of neurons onto microelectrodes. However, metallic stencils suffer from two major drawbacks: (a) they do not seal against the substrate and, therefore, do not ensure that the cell suspension does not spread besides the hole areas; this necessarily limits its use to very dilute cell suspensions[20]; and (b) the fabrication of metallic stencils with hole diameters on the order of a single cell (~10–15 μm) is challenging.

Here we demonstrate the fabrication of a rubberlike stencil, which allows for making cellular micropatterns of virtually any adherent cell type on homogeneous (as well as heterogeneous) surfaces. The stencil is applied onto the cell-culture substrate during seeding and manually peeled off after seeding. Fabrication of the stencil is achieved by replica-molding poly(dimethylsiloxane) (PDMS) in a chamber containing columns. The height of the chamber and the shape of the columns are designed to match the desired thickness of the stencil and the shape of the holes, respectively. Since the replication procedure does not damage the master, the stencil can be replicated many times from the same mold, and access to a microfabrication facility is needed only once to fabricate the mold. Two equivalent processes for the microfabrication of the chamber are presented.

MATERIALS AND METHODS

Microfabrication of the master

The details for the microfabrication of the master have been reported elsewhere.[18] Briefly, a 50–300 μm layer of EPON-SU8 photoresist (Microchem Co., Newton, MA) is spun on a test-grade Si wafer coated with 1500 Å of Si_3N_4 (Microsystems Technology Laboratory, M.I.T.), dried at 95°C on a leveled hot plate, and exposed to collimated UV light through either a chrome mask (Advanced Reproductions, North Andover, MA) or a high-resolution transparency mask (Pageworks, Cambridge, MA) pressed against the photoresist layer. The unexposed areas are dissolved in propylene glycol methyl ether acetate (SU8 developer, Microchem Co.). Transparency masks are printed with a Hercules printer (Lino-type, Heidelberg, Germany) either at 3386 or 5000 dots per inch (~7.5 μm- or 5 μm-diameter dot, respectively). These transparency masks are inexpensive (~$20/ea.), quickly printed, and do not result in a loss of resolution for our application, because the cells are typically much larger (~35 μm) than the dot size. The features on the mask were designed to produce posts of various shapes and sizes on the master.

Preparation of PDMS prepolymer

PDMS prepolymer is prepared as a mixture of two commercially available components (Sylgard 184 kit, Dow Corning). They are mixed at 10:1 ratio by weight, as indicated by the manufacturer. The mixture is degassed to eliminate bubbles created during mixing by placing the PDMS prepolymer in a dedicated dessicator (Nalgene) at low (~30 Torr) vacuum. Breaking the vacuum periodically helps burst the bubbles from the surface of the mixture.

Fabrication of the chamber ("Microfluidic replication")

In this method, schematized in Fig. 1, the chamber is filled with PDMS prepolymer after it has been fabricated. Since our tissue culture substrates are circularly symmetric, we usually build circular chambers. The roof of the chamber is made with a thin adhesive film carefully applied by hand onto the master [Figs. 1(a) and (b)]. For sparsely spaced columns, where sagging of the film onto the wafer is likely to occur, pressure is applied through a solid surface such as a 1-mm-thick glass plate. The adhesive film is perforated with a small hole at its center. A simple connector between the hole and a syringe mounted with a Luer-lock fitting can be made with a flat piece of PDMS in which a piece of silicone tubing has been embedded, as described previously.[18] The flat PDMS piece is then manually positioned onto the adhesive film, so that the center of the silicone tubing coincides with the hole in the adhesive film, and the Luer-lock fitting (with the mounted syringe) is tight-fitted into the protruding end of the silicone tubing [Fig. 1(c)]. PDMS prepolymer is poured on the perimeter of the chamber (typically, a 2-in diameter disk), and it is introduced into the chamber by manually applying suction from the center hole [Fig. 1(d)]. When the chamber is full of PDMS, it is placed in an oven at 65–70°C for ~12 h (8 h overcuring to minimize bleaching of monomers, which could affect cell function). After curing is complete, the PDMS connector is removed, and the chamber is opened by carefully removing the roof (i.e., the adhesive film) with tweezers [Fig. 1(e)]. Subsequently, a thick (~3 mm) PDMS annulus slightly wet with PDMS at the bottom is placed on top of the opened chamber, and the assembly is cured again to cause the PDMS annulus to become bonded to the stencil. (Alternatively, the PDMS annulus can be bonded after separating the stencil from the master.) The stencil is then peeled off with

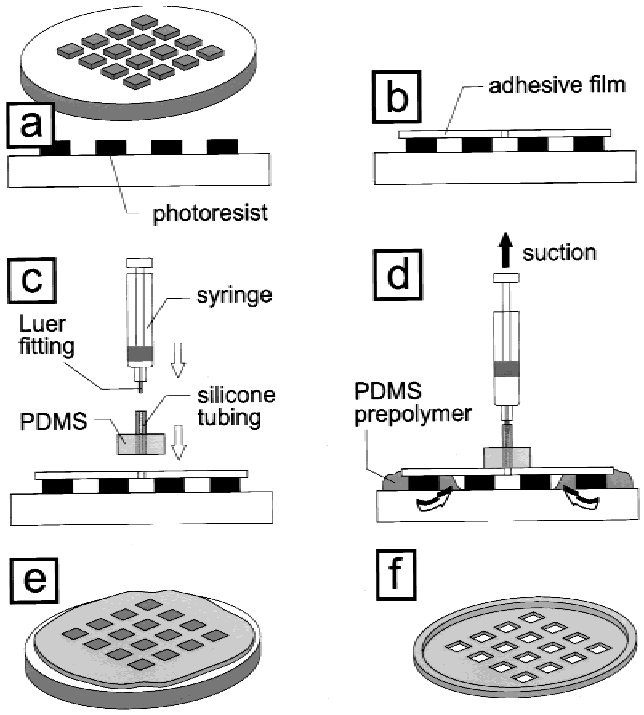

Figure 1. Schematic illustration of the stencil fabrication process based on microfluidic replication. (a) Master wafer containing photoresist posts; (b) master wafer covered by a piece of adhesive film perforated at the center, effectively forming a microfluidic chamber; (c) assembly of the outlet connector and syringe onto the microfluidic chamber; (d) filling of the chamber by aspirating PDMS prepolymer from the edges toward the center; (e) 3-dimensional representation of the master after peeling off the adhesive film; and (f) microfabricated stencil after separation from the master and bonding of a thick ring of PDMS.

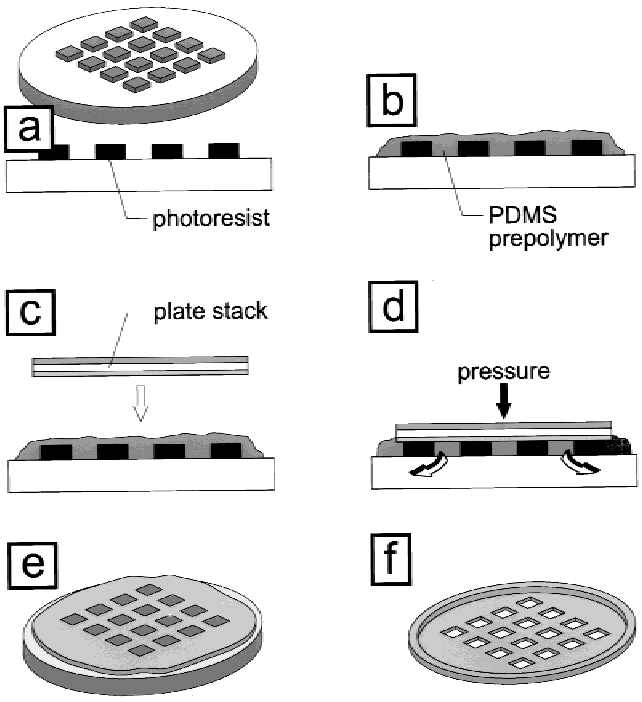

Figure 2. Schematic illustration of the stencil fabrication process based on applied pressure. (a) Master wafer containing photoresist posts; (b) master wafer covered with PDMS prepolymer; (c) application of a stack of plates (see Materials and Methods) onto the PDMS-covered master; (d) application of pressure to displace PDMS from the top of the microfabricated posts; (e) 3-dimensional representation of the master after removing the glass plate; and (f) microfabricated stencil after separation from the master and bonding of a thick ring of PDMS.

the help of tweezers under a dissection microscope to avoid tearing of small features [Fig. 1(f)].

Fabrication of the chamber ("Pressure-assisted replication")

Alternatively, one can use a compression-molding process to realize the stencils. In this method, schematized in Fig. 2, the master [Fig. 2(a)] is covered with prepolymer [Fig. 2(b)] and then a multilayer stack is used to form the mold chamber. First, a transparency film is carefully lowered onto the prepolymer, allowing surface tension to pull the transparency into intimate and continuous contact with the prepolymer mixture to prevent any bubbles from forming at the interface [Fig. 2(c)]. The flexibility of the film allows for easy removal from PDMS molds after curing. The master/prepolymer/transparency stack is then clamped within a sandwich that includes flat aluminum plates (top and bottom), a rigid Pyrex wafer and a rubber sheet (top only) [Fig. 2(d)]. The top and bottom aluminum plates provide uniform force onto the stack from both sides. The rubber sheet provides a mechanical buffer layer between the top aluminum plate and rigid Pyrex wafer, ensuring uniform pressure on the pyrex and preventing cracking from nonuniformities in the aluminum plate. The polished rigid Pyrex wafer produces a flat surface on top of the cured molds. The clamped PDMS prepolymer sandwich is cured for 3 h at 100°C on a hot plate. After curing, all layers are removed except the transparency. The flexible transparency is then easily removed [Fig. 2(e)] and the thin PDMS replicas are peeled off from the masters [Fig. 2(f)].

Conditioning of PDMS stencils

After separating it from the master, and after each use, the stencil is washed with acetone and ethanol, blow-dried, and gently pressed against the tissue culture surface of interest. If necessary (e.g., dead cells remain on the stencil surface) the stencil is cleaned in an O_2 plasma (200 W, 5 min) prior to applying it to the tissue-culture surface. The stencil/substrate assembly is then fully covered with deionized water either in air or in 100% CO_2. To deliver water under CO_2, the stencil-covered substrate is placed at the bottom of a tall (~25 cm) beaker that is continuously being flushed with CO_2. Since CO_2 is heavier than air, this ensures that the gas contacting the stencil is CO_2. After delivering the water, the stencil is brought out to air, which results in dissolution of

the bubbles into the water. This whole procedure can be performed inside a tissue-culture hood in sterile conditions. If the water is delivered in air, and although wetting is known to be greatly facilitated by the plasma oxidation step,[21,22] bubbles are almost inevitably trapped inside our small wells. To remove them, we squirt ethanol directly onto the stencil/substrate under running water, carefully avoiding static exposures to ethanol. The stencil/substrate assembly with fully wetted wells is sterilized by overnight UV light exposure in a tissue culture hood. However, this method of sterilization may not be suitable for long-term cultures, because PDMS is not transparent to UV light. Prior to seeding, we wash the stencil/substrate assembly twice with medium to fully substitute the water in the wells with medium.

Cell seeding and culture

Primary rat hepatocytes were isolated, as described by Dunn et al.,[23] and seeded and cultured in high-glucose Dulbecco-Modified Eagle's medium (DMEM) (Life Technologies, Gaithersburg, MD, Cat. No. 11995-065) supplemented with 0.5 Units/L insulin (Novo Nordisk A/S, Bagsuaerd, Denmark), 14 ng/mL glucagon (Eli Lilly and Co., Indianapolis, IN), 7.5 (g/mL hydrocortisone (Abbott Laboratories, North Chicago, IL), 20 ng/mL epidermal growth factor (Collaborative Biomedical Products, Bedford, MA), and 10% fetal bovine serum (Life Technologies, Cat. No. 16000-044).[24] Mouse 3T3-J2 fibroblasts (generous gift of Howard Green, Harvard Medical School) were cultured in high-glucose DMEM supplemented with 10% calf serum (Life Technologies, Cat No. 16170-078) and 2% penicillin/streptomycin mixture (Life Technologies, Cat. No. 15140-122). Serum was heat-inactivated at 56°C for 30 min before being added to DMEM. Before seeding, fibroblasts were detached from the tissue culture substrate with trypsin-EDTA (Life Techologies, Cat. No. 25200-056), centrifuged at 1000 rpm for 5 min and resuspended at 1 million cells/mL. Keratinocytes were isolated from human neonatal foreskins and cultured on a fibroblast feeder layer in keratinocyte basal medium (Clonetics) as described by Rheinwald and Green.[25] All cells were cultured in 60-mm tissue culture-grade polystyrene petri dishes (Falcon, Cat. No. 3002) and incubated at 37°C in a humidified mixture of 90% air and 10% CO_2. (Note: all percentages are by volume.)

Collagen gel preparation

Collagen type I was isolated from Lewis rat tail tendons and dissolved (~1.2 g/L) in HCl 0.1 mM (pH = 4 to prevent gel formation), as previously reported.[23] Our collagen gel "sandwich" cultures were based on Dunn et al.'s protocol.[23] For each 60-mm diameter petri dish, 2 mL of the gelling solution composed of 9:1 collagen type I and concentrated DMEM (Life Technologies, Cat. No. 12100-103) was added and incubated for 1 h at 37°C to cause gelling. After gelling, and before applying the PDMS stencil, any excess medium was carefully aspirated from the dish. The patterned hepatocyte cultures were covered with a second layer of 2 mL of freshly prepared gelling solution and incubated overnight. Finally, 2 mL of medium were added to each dish.

Direct patterning of cells with a PDMS stencil

After the PDMS stencil was applied to the tissue culture substrate, covered with deionized water, and bubbles were eliminated (see above), water was substituted twice by the seeding medium prior to seeding. After adding the cell suspension, the stencil/substrate assembly was incubated at 37°C for periods ranging 2–48 h (see text) to allow for cell attachment and spreading. After peeling off the stencil, the substrate was washed twice with seeding medium to remove unattached cells.

Patterning of cells on a curved surface

Glass cylinders (~6 mm diameter) were made by cleaving the top portion of an autoclave-sterilized Pasteur pipette. To prevent the cylindrical surfaces from rolling during manipulation, they were placed in a Petri dish filled with a ~1-mm-thick PDMS layer in which a ~2-mm-wide groove was cut with a razor blade. Thus, the portion of cylinder within the groove (~20%) was not available for seeding, but seeding onto undercut areas was still possible by tilting the whole Petri dish during ~1 h to allow for cell attachment.

RESULTS AND DISCUSSION

Replica-molding of microfabricated masters onto PDMS blocks has been used for a variety of lithographic techniques collectively known as "soft lithography" (for a review, see Ref. 26). Essentially, a mixture of two liquid components (PDMS prepolymer) is poured and allowed to thermally cure onto the master. Once solidified, the PDMS block is peeled off. Obviously, due to the lack of control during pouring, the master's microfabricated structures are fully covered with PDMS prepolymer and, thus, after curing, only the PDMS block surface facing the master contains replicated features. Recently, Jackman et al.[27] reported a procedure for creating PDMS stencils based on carefully spinning small quantities of PDMS prepolymer onto the master in such a way that the structures present on the master were not fully covered by PDMS prepolymer. However, spinning requires spin-coating equipment, is sensitive to particles present in the PDMS prepolymer, and is prone to failure due to wetting of the microfabricated structures by PDMS prepolymer.

Here we report an alternative strategy for micromolding PDMS stencils based on trapping PDMS in a microfluidic chamber containing columns. To build

the chamber, we first microfabricate a master wafer containing photoresist posts of specified shape and height. The posts become the supporting columns of a chamber when a capping surface or "roof" is added to the master. The posts are photolithographically defined on a Si_3N_4-coated silicon wafer with a commercially available high aspect-ratio photoresist, as previously reported[18] [Figs. 1(a) and 2(a)]. Our strategy yields two different methods, depending on whether the chamber is assembled before or after dispensing the PDMS prepolymer, as depicted schematically in Figs. 1 and 2, respectively. The close contact between the roof of the chamber and the chamber columns prevents the column tops from being covered by PDMS and results in the replication of the columns as thru holes. Since spinning is not necessary, our method can be straightforwardly implemented on the benchtop. More importantly, we demonstrate that the stencils are nontoxic for all cell types tested.

The choice of microchamber roof material depends on the replica-molding method. If we choose to assemble the chamber before dispensing PDMS prepolymer (Fig. 1), the roof consists of a circular piece of translucent adhesive film, which is perforated with a needle at its center [(Fig. 1(b)] to allow for a fluidic connection to a syringe [Fig. 1(c)]. PDMS prepolymer is then dispensed at the edges of the chamber, and suction is applied with the syringe to generate a radial flow of PDMS directed towards the center of the microchamber [Fig. 1(d)]. Hence, we call this a "microfluidic replication" method. After PDMS curing, the roof is first peeled off the master/PDMS [Fig. 1(e)]. We found that the pulling force exerted by the adhesive film onto the columns during peeling never overcomes the strength of adhesion between the columns and the silicon nitride-coated surface and that, surprisingly, the glue always stays on the adhesive film side without leaving visible remains on the photoresist. This is probably due to the inert polyether nature of the exposed photoresist. Finally, a PDMS annulus is bonded to the stencil, and the PDMS stencil is carefully separated from the master [Fig. 1(f)]. Without the PDMS annulus, the stencils are impractical to work with, because they fold and stick to themselves. The highly compliant nature of PDMS facilitates the release of the stencil. Since PDMS is in contact with adhesive during curing, we clean it by an acetone and ethanol wash followed by treatment in an oxygen plasma oven (March Instruments Inc.) (200 W, 5 min). We note that the stencils fabricated by microfluidic replication are not smooth on the side that contacted the glue. Therefore, they seal well on only one side.

Alternatively, the PDMS prepolymer may be dispensed before adding the roof [Fig. 2(b)]. In this case, the roof consists of a semi-rigid multilayer stack (transparency, glass, rubber) [Fig. 2(c)] and is pressed tightly against the master until PDMS is fully squeezed out from the top of the microfabricated structures, that is, until the master and the transparency come into intimate contact [Fig. 2(d)]. Separation of the roof [Fig. 2(e)], bonding of a PDMS annulus, and release of the PDMS stencil [Fig. 2(f)] are carried on analogously to the microfluidic method [Figs. 1(e) and (f), respectively]. Since this method requires a clamping setup, we dub it "pressure-assisted replication." When the applied pressure is insufficient, a proportion of the holes appears plugged with a thin PDMS membrane. Compared to the stencils produced by microfluidic replication, the stencils produced by clamping-assisted replication are smooth on both sides. Both replica-molding methods are essentially equivalent except for extreme conditions: in principle, microfluidic replication may fail in the fabrication of stencils with few holes per substrate (i.e., chambers containing few columns), because the roof is flexible; and clamping-assisted replication may fail in the fabrication of stencils with large hole-occupied areas (i.e., chambers with a large total surface area occupied by columns), because the required clamping force required to fully exclude PDMS risks breaking the master wafer. Experiments to test the limits of the patternable area are in progress.

The surface of PDMS is hydrophobic. As a result, when the PDMS stencil is applied onto a substrate and covered with water, a bubble is trapped in almost every stencil hole. When water is dispensed in ambient pure CO_2 (see Materials and Methods), then the trapped bubbles are constituted of CO_2 and, due to the high solubility of CO_2 in water, get immediately dissolved when the stencil/substrate assembly is brought to air, independent of the bubble size. On the other hand, dispensing water in air results in air bubbles that are harder to remove as the hole diameter decreases and the hole depth increases. Air bubbles trapped in holes with lateral dimensions of less than 100 μm proved particularly challenging to remove. We have explored combinations of the following procedures to facilitate the release of air bubbles from the PDMS stencil: (a) oxygen plasma-treatment of the PDMS surface so as to render it hydrophilic[22,28]; (b) placing the water-covered PDMS stencil in a low-vacuum (~15 mTorr) jar to cause the bubbles to expand and resurface; (c) adding ethanol to the water solution to lower the surface tension; (d) waiting (~24 h) for the air bubble to slowly dissolve in water; (e) facilitation of wetting by using a surfactant such as Tween or, equivalently, a protein solution (most polymer surfaces have high affinity for proteins). We start by oxidizing the PDMS stencil in an O_2 plasma, because the procedure also removes undesired contaminants. In general, plasma oxidation does not prevent bubbles from being trapped in our smallest (35-μm diameter) wells displaying large height-to-width ratios (~3:1) or even 100 μm-diameter squares with 1:1

aspect ratios. Vacuum degassing, on the other side, may cause the stencils to detach, if the hole density is high (due to the sum of upward buoyancy forces exerted by each bubble), or if bubbles are trapped under the PMDS (due to a speck of dust that prevented full contact at a particular spot). The fact that, in certain spots, bubbles reappear continuously makes us believe that bubbles also nucleate from air dissolved in PDMS or in water. We found the most practical procedure to be squirting of ethanol onto the stencil under running water. Running water is necessary to minimize the ethanol-PDMS contact time. (When pure ethanol is poured onto a dry stencil, it detaches within a few seconds, presumably because ethanol causes PDMS to swell.) After the ethanol squirting procedure is completed, if a small number of air bubbles remain, vacuum degassing may be safely used without risking detachment of the stencil. The wetted, stencil-covered surface may then be UV light-sterilized (see Materials and Methods). However, for long-term cultures, we prefer to dispense water under CO_2, because this procedure, unlike the ethanol-squirting method, can be performed easily under sterile conditions. We have observed that the wells never dewet once they are full, presumably due to water pinning at the edges[29] and/or to the higher hydrophilicity of the polystyrene bottom.

The sequence of micrographs in Fig. 3 illustrates step-by-step how the stencil is used for creating cellular micropatterns. In this case, the starting substrate was a tissue-culture-grade polystyrene petri dish and the cells were fibroblasts. After applying the stencil in dry conditions, water was added and bubbles were removed with one of the above-mentioned methods. Prior to seeding, water was substituted by medium [Fig. 3(a)]. Then, the cell suspension was added [Fig. 3(b)], and, after allowing 2 h for attachment and spreading [Fig. 3(c)], the stencil was removed [Fig. 3(d)]. As a toxicity test, we have waited as long as 48 h to remove stencils, with identical results. Since cells are known to make strong contacts in short periods of time and tend to be peeled off as a sheet rather than individually, it is surprising that removal of the stencil does not result in any apparent damage to the cells immediately adjacent to the PDMS walls, as assessed by their morphology and migration activity observed by time-lapse microscopy overnight after removal of the stencil (data not shown). This suggests that cells that start spreading on the floor of the well cannot spread upward the PDMS wall, and/or that cells crawling down the PDMS wall refuse to make contact with cells attached to the polystyrene substrate. We interpret this observation as a special case of "contact guidance"[30] — a phenomenological description of the behavior of cells in contact with topographic features that has been extensively reported in the litterature since the very first days of cell culture (for a review, see Ref. 31) — in which the sidewalls are perfectly vertical and dissimilar in composition to the bottom surface.

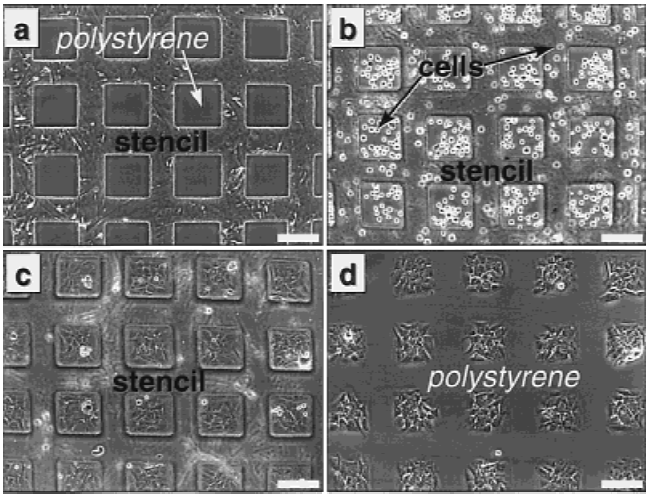

Figure 3. Sequence of phase-contrast micrographs depicting the creation of a cellular micropattern by means of a ~100-μm-thick stencil containing 140 μm-side squares separated by 100 μm. (a) The stencil is applied to a polystyrene surface and covered with seeding medium; (b) the cell suspension is dispensed; (c) cells after full attachment and spreading at the bottom of the holes (cells attached onto the stencil are out of focus); (d) cells after removal of the stencil. Scale bar = 100 μm.

The micropatterned fibroblast culture of Fig. 3(d) is unique for three reasons: (1) unless a specific surface is engineered to repel protein adsorption, a fibroblast pattern cannot be created by selective attachment onto an existing template, because fibroblasts will attach onto adsorbed extracellular matrix (ECM) protein, which they secrete in particularly large amounts; (2) patterning was done on an off-the-shelf substrate without the need of further chemical or physical processing; and (3) the fibroblasts are confined to an area of the same surface composition as its immediate surroundings, allowing for the study of migratory behavior, independent of changes in the biochemical composition of the substrate. More generally, since the method does not rely on the selective attachment of cells to a biochemical template, its success does not depend on the cell type, seeding density, or medium formulation, and, for the same reason, these variables may be changed for each application without compromising the patterning success. For example, nonconfluent islands or serum-free media may be desired for certain experiments, whereas multilayers of cells or serum-containing media may be required for others. Typically, we seed at 25,000–50,000 cells/cm^2 to obtain a confluent monolayer in each island. Two considerations make the method well suited for experiments, where cell numbers are precious: (a) during sedimentation of the cell suspension onto the stencil, shaking the dish displaces the cells at the top of the stencil but

not at the bottom of the wells, thus resulting in an effective higher cell density on the hole areas compared to the PDMS areas, as apparent in Fig. 3(b); and (b) cells may be seeded at very low densities and be allowed to grow until confluence to achieve a monolayer micropattern. Importantly, the areas that remain bare after removing the stencil are also apt for cell seeding of a second cell type such as hepatocytes (data not shown).

Heterogeneous surfaces can be patterned straightforwardly, precisely because the method is not based on chemical modification of the substrate. As shown in Fig. 4(a), 100-μm-side square cellular islands, each containing 5–7 cells, could be fabricated onto a gold/chromium microelectrode pattern on glass. The PDMS stencil was aligned by hand onto the gold electrodes with satisfactory angular accuracy. However, our attempts to register the stencil holes onto the electrodes were unsuccessful due to deformations of the stencil during its application. Although procedures to attach the stencils to more rigid structures such as metal stencils could, in principle, be devised (at the expense of sacrificing hole areas), we consider the flexibility of the stencil a fundamental limitation of patterning by means of elastomeric stencils.

On the other hand, by virtue of the fact that the stencil is flexible, it can be used to micropattern curved surfaces. The micropattern of fibroblasts shown in Fig. 4(b) was created on a ~6-mm diameter glass cylinder over more than 50% of its circumference. Note that only a few rows of the micropattern are in focus, due to the curvature of the surface.

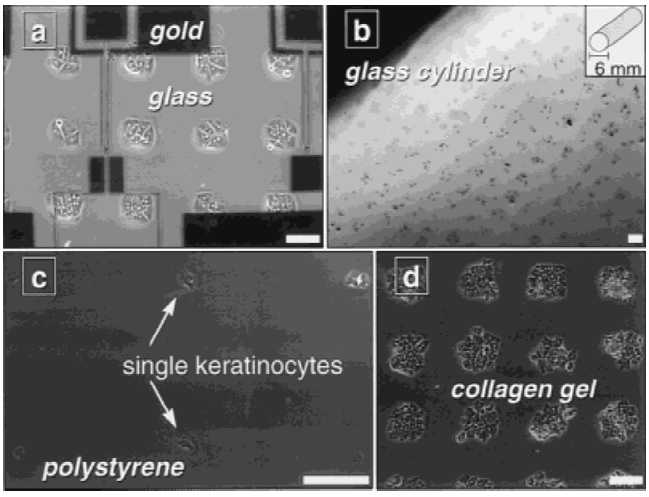

Figure 4. Optical micrographs of stencil-micropatterned cell cultures on various materials. (a) 100 μm-side square islands of hepatocytes separated by 150 μm spacings overlaying a gold microelectrode pattern on glass; (b) the surface of a ~6-mm diameter glass cylinder (see inset) containing 100 μm-side square islands of fibroblasts stained with hematoxilin and eosin; (c) micropattern of single keratinocytes created on polystyrene by means of a stencil containing round holes of 40 μm diameter; (d) micropattern of hepatocytes entrapped in a collagen gel "sandwich" created by means of the same stencil as in (a) (see text for details). Scale bar = 100 μm.

The resolution limits of stencil patterning have been explored. In practice, we found that when the hole diameter is 2–3 times as big as the diameter of the cell in suspension, it still fits only one cell at the bottom of the well, probably because, as the number of cells falling into a well increases during sedimentation, displacing fluid from the bottom of the well becomes increasingly difficult. The resolution depends on the height-to-width ratio of the holes and, therefore, on the thickness of the stencil. Thinner stencils have the advantages that the wells are easier to wet and to fill with cells, hole-to-hole spacing can be smaller, and master fabrication is easier; on the other hand, the stencils are more difficult to handle, and filling the chamber with PDMS or excluding PDMS from the column tops is more difficult as the thickness of the stencil decreases. Nevertheless, a ~100-μm-thick stencil containing rows with 40-μm diameter holes separated by 250 μm allowed for creating single-cell micropatterns of keratinocytes [see Fig. 4(c)]. A set of smaller (<35 μm diameter) holes on this same stencil (i.e., ~100 μm thick) failed to produce a cellular pattern, suggesting that the height-to-width ratio of a hole must be, approximately, >2.5.

Although the stencils seal best on dry surfaces, we have also been able to pattern humid surfaces such as collagen gels. Shown in Fig. 4(d) is a micropattern of hepatocytes created with the same stencil used for Fig. 3. Judging from the compliance of the cells to the (square) hole shape, it is apparent that the contact between the stencil and the collagen gel is tight enough to prevent the cell suspension from flowing into it, but loose enough for attached cells to send filopodia into it. In this case, the stencil was removed 24 h after seeding, and a second layer of collagen gel was added after the patterning step to produce the so-called "sandwich configuration."[23] Extracellular matrix protein gels are known to preserve increased levels of differentiation and the *in vivo* polarity that is generally lost in standard cultures on plastic.[23] Hence, we believe that the ability for patterning gels is of paramount importance for studying cell–cell and cell–ECM interactions in cell types that are highly polarity-dependent.

CONCLUSIONS

We have been able to microfabricate elastomeric stencils by replica-molding PDMS. The mold is made from a clean-room microfabricated master and consists of a chamber containing columns. Two benchtop methods to fill the chamber with PDMS have been demonstrated and compared. The molding process is

inexpensive and does not damage the masters; therefore, several copies of the same master are possible. The stencils are used to selectively block a substrate from contacting a cell suspension (or any other biological solution) only on the areas covered by PDMS, while leaving the substrate exposed on the stencil hole areas. We have not observed any adverse toxic effects of the stencils fabricated by either method for the seeding periods (48 h) and cell types tested in this work. The simplicity of the method allows for patterning, without chemical modification of the substrate, virtually any adherent cell type on a rich variety of materials, ranging from polystyrene petri dishes to a microelectrode circuit or a gel. We note that, while the method circumvents the need for creating a biochemical template, it may obviously be used to create one on the areas exposed through the stencil by, for example, adsorption of an ECM protein of interest or by chemical derivatization with self-assembled monolayers. The method is unique in its ability to create cellular micropatterns on homogeneous substrates and in the little expertise required to use the stencils. By virtue of its flexibility, the stencil shows great promise for patterning the curved surfaces of biomedical devices.

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