

“Stamp-off” to Micropattern Sparse, Multicomponent Features

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Abstract

Spatially patterned subtractive de-inking, a process we term “stamp-off,” provides a simple method to generate sparse, multicomponent protein micropatterns. It has been applied to control cell adhesion, study adhesion biology, as well as to micropattern fragile surfaces. This technique can also readily be applied to study nanoscale interactions between cell membrane receptors and surface-immobilized ligands. It is based on conventional microcontact printing and as such requires the same reagents, including photolithographically defined masters, a spin-coater, poly(dimethyl siloxane) (PDMS), and conventional cell culture reagents such as glass coverslips and adhesive proteins. Stamp-off is conceptually simplified into three steps: (1) generation of an appropriate cell culture substrate, PDMS-coated glass, (2) micropatterning with stamp-off, and (3) cell deposition. After elaborating each of these three methods, we discuss limitations of the technique and its applications.

INTRODUCTION

Cell migration, proliferation, and differentiation are central to a variety of normal and pathophysiologic processes including embryonic development, tissue homeostasis, wound healing, and cancer progression. In each setting, it appears that there is an intimate and functional relationship between structure—of the cell and its interaction with the surrounding microenvironment—and function. Investigating how structure regulates function on the cellular scale ($\sim 0.1\text{--}10\ \mu\text{m}$) requires a technique to engineer structure while monitoring function at subcellular to cellular length scales ($\sim 0.1\text{--}10\ \mu\text{m}$). In the past decade, rapid advances in the ability to reliably and efficiently engineer surfaces with geometrically patterned regions presenting adhesive extracellular matrix surrounded by non-adhesive have led to major insights into how the structure of the cell, surrounding extracellular matrix, and cell–cell interactions drive cell functions such as cell life versus death, differentiation, intercellular communication, and migration (Bhatia, Balis, Yarmush, & Toner, 1998; Chen, Mrksich, Huang, Whitesides, & Ingber, 1997; Connelly et al., 2010; Desai, Gao, Raghavan, Liu, & Chen, 2009; Dupont et al., 2011; Gilbert et al., 2010; Jiang, Bruzewicz, Wong, Piel, & Whitesides, 2005; McBeath, Pirone, Nelson, Bhadriraju, & Chen, 2004; Nelson et al., 2005; Nelson, Vanduijn, Inman, Fletcher, & Bissell, 2006; They et al., 2006).

Methods to pattern adhesive surfaces have proliferated rapidly in the past decade, and many outstanding reviews cover approaches ranging from photopatterning to microfluidics (El-Ali, Sorger, & Jensen, 2006; Folch & Toner, 2000; Whitesides, 2006). One of the most widely applied micropatterning techniques is microcontact printing, originally developed by George Whitesides and colleagues over two decades ago (Xia & Whitesides, 1998). In this technique, an elastomeric stamp with bas-relief features is used to transfer an “inked” material onto a substrate. The elastomer is usually poly(dimethyl siloxane) (PDMS), which has several advantages: (1) it can readily be made to generate micron-scale features on a substrate of large area (a few cm^2) (Kane, Takayama, Ostuni, Ingber, & Whitesides, 1999), (2) PDMS has low surface energy, enabling it to be easily separated from the template during fabrication, so binds reversibly to the substance transferred during printing, and therefore permits easy removal of the stamp from the substrate after printing (Love, Estroff, Kriebel, Nuzzo, & Whitesides, 2005), and (3) it is relatively inert so does not react with many chemicals (Xia & Whitesides, 1998). Although microcontact printing was originally developed to pattern gold (Kumar & Whitesides, 1993), it was extended to directly micropattern proteins on biocompatible surfaces less than a decade after George Whitesides pioneered the technique (Bernard et al., 1998; James et al., 1998) and simplified to enable widespread adoption several years later (Tan, Liu, Nelson, Raghavan, & Chen, 2004). Thus, microcontact printing rapidly emerged as a technique of choice to pattern materials for biologic applications.

Despite its utility, conventional microcontact printing suffers from two major limitations. First, elastomeric stamps bearing small, sparse features are prone to deformation and collapse during printing, leading to undesired contact of the

inter-feature regions of the stamp with the underlying surface (Ruiz & Chen, 2007; Xia & Whitesides, 1998). Stamp collapse depends on the pressure applied during stamping (Hui, Jaogta, Lin, & Kramer, 2002). Investigators have addressed this issue by using PDMS stamps backed with glass (James et al., 1998), PDMS stamps coated with a rigid material (Odom, Love, Wolfe, Paul, & Whitesides, 2002), and stamps made from a material more rigid than conventional PDMS (Schmid & Michel, 2000). However, these strategies still suffer from an upper limit on the spacing between features, and as such they still place limits on feature design. In general via conventional microcontact printing methods, the non-adhesive space between patterned features cannot substantially exceed the size of the adhesive features. Thus, whether and how spacing between cells or the spacing of adhesions within cells affects function are challenging to address via conventional microcontact printing. Second, conventional microcontact printing was designed to pattern a single adhesive ligand, surrounded by a non-adhesive region; the substrates thus present “digital” adhesive cues to cells, and cannot pattern multiple ligands. Multiple ligands can be patterned simply by printing multiple times, but manual spatial registration between successive printing steps is not trivial (Rogers, Paul, & Whitesides, 1998). Thus, how cells integrate cues from multiple ligands remains obscure. Although powerful, conventional microcontact printing suffers from these significant drawbacks.

Here, we describe a method that is a simple extension of conventional microcontact printing to encode a surface with sparse, distinct patterns of multiple proteins. We describe our technique, called “stamp-off,” in detail and discuss applications as well as limitations.

1.1 METHOD

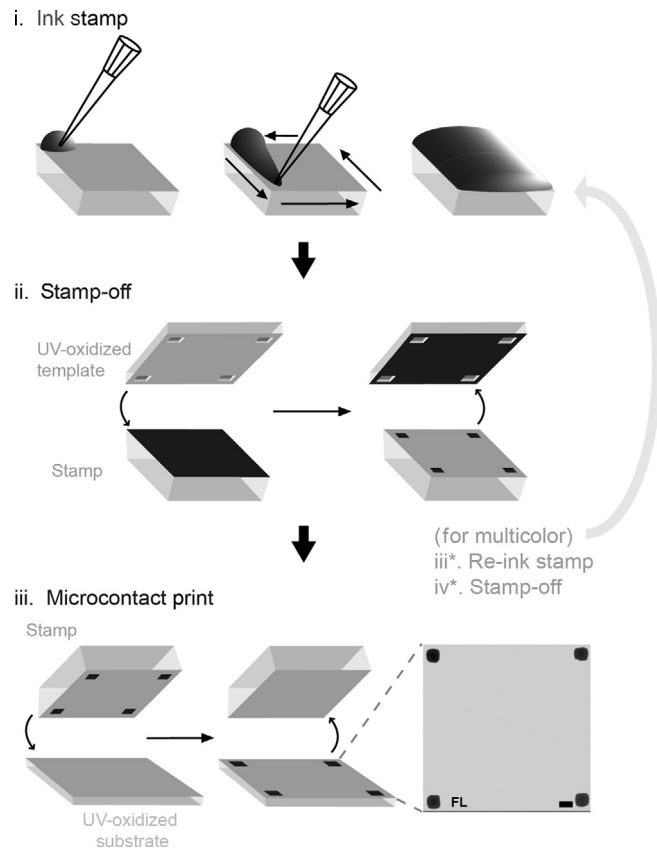
Stamp-off can be divided into three distinct sections: (1) preparing PDMS-coated glass, (2) patterning via stamp-off, and (3) seeding cells on the micropatterned surface. Below, we describe these three steps in detail.

I. Preparing PDMS-coated glass.

a. Materials

1. PDMS (Dow Corning, Sylgard 184. Contact Dow Corning for a local distributor and part number).
2. Glass coverslips. Choose a size and thickness that is compatible with your application. We typically use 22 mm × 22 mm square coverslips, number 1.5 thickness (Fisher No. 12-541B. These coverslips fit nicely in a 6-well plate).
3. Transfer pipette (Fisher No. 13-711-7M. Any pipette that will dispense viscous materials such as uncured PDMS will do. Precise metering of volume is *not* necessary).
4. Curved tweezers (EMS 0109-7-PO. Any tweezers that will handle coverslips will do; we find this particular one to be ergonomic and convenient).

5. 100% ethanol (Decon Labs, Inc. No. 2716. Need not be pure grade).
 6. milliQ water.
 7. Compressed nitrogen (e.g., Airgas No. UN1066) with a regulator (VWR No. 55850-474) and hose and spray nozzle (hose and spray nozzle: Teqcom No. TA-NS-2000; connector to regulator: Ryan Herco Fluid Flow Solutions No. 0161.202 5865445).
 8. Parafilm (Cole-Parmer No. PM996).
- b. Equipment**
1. Spin-coater with a chuck to accommodate coverslips (Laurell Technology Corporation, Model WS-400B-6NPP/LITE; the spin-coater should be placed in a fume hood or clean room to avoid dust particles during use).
 2. Heating oven (optional, only needed to expedite PDMS curing. For example, Fisher Scientific Isotemp Oven).
- c. Method**
1. Mix and degas the PDMS as per the manufacturer's recommendations. We find that a 10:1 ratio of base to cross linker (by weight) works well for microcontact printing.
 2. Remove a coverslip straight from the container, roughly center on the spin-coater and apply vacuum. Note that coverslips can be used straight out of the box without cleaning, but any dust or lint should be blown off with a stream of nitrogen. In the event coverslips need cleaning, you can sonicate them in 100% ethanol for 5 min, or shake in 1 N HCl for 15 min at room temperature. Each method of cleaning requires thorough rinsing with milliQ water after removing from ethanol or HCl.
 3. Use the transfer pipette to dispense roughly 100 μl of PDMS onto the middle of the coverslip. You can eyeball the volume since 100 μl is approximately pea-sized. It is better to drop too much rather than too little volume, since excess volume will simply spin off but too little volume will not coat the entire coverslip.
 4. Spin the coverslip at 6000 rpm for 60 s. This speed and time will give you a roughly 15 μm PDMS coating.
 5. Bake overnight at 60 $^{\circ}\text{C}$. Alternatively, let sit at room temperature for 48 h to cure.
 6. Sterilize
 1. Dip the coverslips in 100% ethanol for 5–10 s, careful to coat all regions of the coverslip with ethanol.
 2. Dip the coverslip in a dish filled with milliQ water, and hold for 5–10 s. Repeat three times.
 3. Thoroughly dry coverslip with a stream of N_2 .
 4. Place coverslip in a 6-well (or appropriate-size) plate.
 7. Seal plate with parafilm and store at room temperature until use. PDMS-coated coverslips can be stored indefinitely at room temperature.
- II. Stamp-off.** This step assumes that one has already made appropriate stamp-off templates. See [Fig. 1.1](#) for an example of stamp-off templates. Both the stamps

**FIGURE 1.1**

Stamp-off to generate sparse features. (i) First ink the stamp with protein. (ii) Then stamp-off onto a UV ozone-treated template. (iii) Finally, transfer the protein on the stamp to the cell culture substrate. The cell culture substrate (also PDMS) should be UV ozone-treated for 7 min. Fluorescent light (FL) micrograph shows an example of corresponding features.

Protein was bovine serum albumin tagged with AlexaFluor488. Scale bar, 20 μm . (iii*, iv*) Sequential re-inking and stamp-off can be performed to generate multicomponent patterns. See Fig. 1.2.

and stamp-off templates should be made fresh each time this protocol is performed.

a. Materials

1. Stamp (flat). This is most easily made by casting PDMS off a flat surface such as a polystyrene dish.
2. Stamp-off template (one for each pattern). This is made by casting PDMS off a photoresist pattern (a number of very good reviews cover generation of photoresist patterns, such as [Weibel, DiLuzio, and Whitesides \(2007\)](#)).

3. 100% ethanol (need not be pure; 70% ethanol will do).
 4. milliQ water.
 5. Curved tweezers (EMS 0109-7-PO. Any tweezers that will handle coverslips will do; we find this particular one to be ergonomic and convenient).
 6. Protein solutions (we typically use fibronectin (BD No. 356008) at 50 $\mu\text{g}/\text{ml}$ in milliQ water, although a variety of proteins, such as bovine serum albumin, antibodies, vitronectin, type-I collagen, work as well (Desai, Khan, Gopal, & Chen, 2011).
 7. 0.2% (w/v) Pluronic F127 (Sigma No. P2443) in cell culture grade H_2O .
- b. Equipment
1. Compressed nitrogen.
 2. Laminar Flow Hood.
 3. Ultraviolet ozone cleaner (Jelight No. 342. A discussion of a homemade deep ultraviolet ozone machine can be found in Azioune, Carpi, Tseng, Théry, and Piel (2010)).
- c. Method. For a visual explanation of conventional microcontact printing, see Desai, Yang, Sniadecki, Legant, and Chen (2007). Perform this method in a sterile field.
1. Clean stamps and stamp-off substrates by sonicating for 5 min in 100% ethanol, dipping in 100% ethanol, dipping in milliQ water, and blowing dry with a stream of nitrogen, and placing the stamp face-up in a sterile, dry polystyrene dish.
Note: Avoid touching the “face” of the substrates with tweezers, gloves, etc.
 2. “Ink” the stamp with the desired protein by covering the stamp face with the protein solution. As long as the stamp face is covered, the volume of solution does not matter.
Note: The PDMS surface is intrinsically hydrophobic, and this makes loading the stamp with aqueous solutions difficult. We perform two steps to address this: (1) pipette a series of approximately 50 μl droplets onto the four corners of the stamp face and let the drops sit undisturbed for 5–10 min (for illustrative purposes, only one drop is shown in Fig. 1.1i, left panel). During this time, protein should adsorb to the stamp face from the droplets, rendering the formerly hydrophobic surface hydrophilic at the drop–stamp interface. (2) Using a fresh pipette tip, “connect the dots” by holding the tip at an angle such that part of the tip touches the droplet and the other part of the tip touches the stamp edge. Move the pipette tip along the perimeter of the stamp, careful to maintain contact between the pipette tip, the droplet, and the stamp edge (see Fig. 1.1i, middle panel).
 3. Incubate the stamps with protein for 1 h at room temperature. Ensure that the solution does not evaporate.
Note: Depending on kinetics of adsorption, the protein solution may bead up during this time. If this happens, simply run a pipette tip along the

perimeter of the stamp while maintaining contact with the drop, as in step (2) above to ensure complete coverage of the stamp (see Fig. 1.1i, right panel).

4. Activate the stamp-off substrates by placing them in the ultraviolet ozone cleaner for 7 min at about 5 cm from the ultraviolet light source. This renders the PDMS hydrophilic for protein transfer. We have experienced that too little exposure time results in incomplete protein transfer from the stamp to the stamp-off substrate (see Figure 1 of [Desai et al. \(2011\)](#)). An exposure time of 7 min should compensate for fluctuations in bulb intensity, height, etc.

Note: Be sure to remove the lid of the vessel in which the stamp-off substrates are placed prior to cleaning with the ultraviolet ozone cleaner, since most conventional materials such as polystyrene and soda lime glass look transparent in the visible spectrum but in fact are opaque at deep ultraviolet wavelengths.

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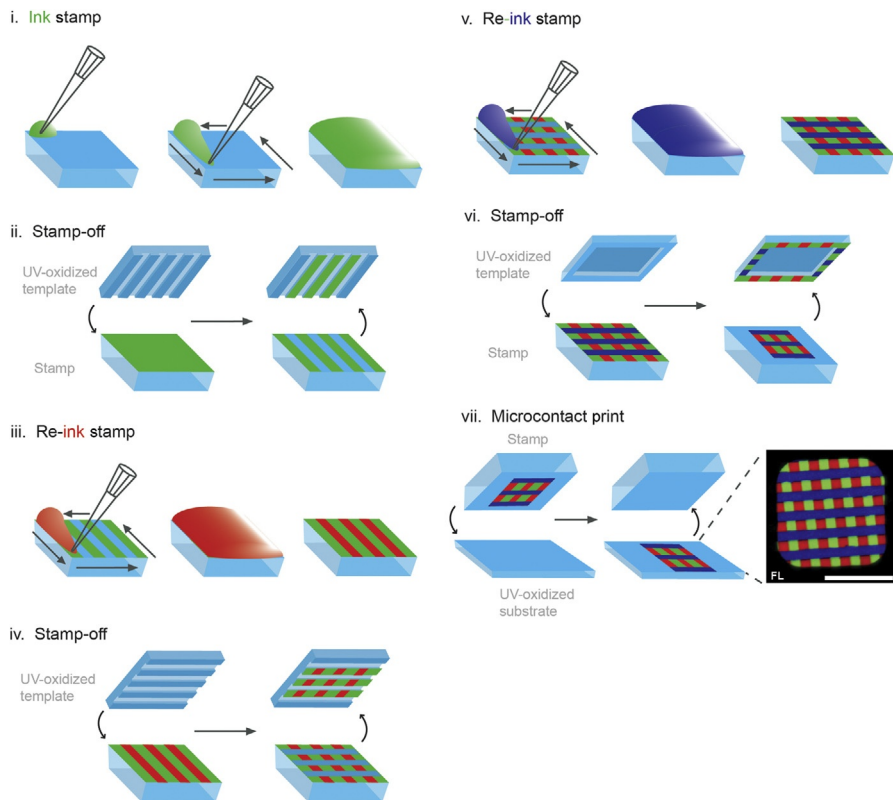
5. Rinse the stamps by: (1) pouring milliQ water into the dish containing the stamps, above the level of the stamps, (2) pick up the stamp and dip it for 5–10 s in a dish with fresh milliQ water, and (3) dry the stamp thoroughly with a stream of nitrogen.

Note: It is best to use the stamps immediately after drying, but we have found that they can be held dry for up to an hour without encountering problems.

6. Invert the activated stamp-off substrate onto the stamp (Fig. 1.1ii). One second of contact is sufficient for complete protein transfer.

Note: The stamp-off substrate must be used within 30 min of ozone treatment. If more than 30 min elapses, the substrate may be re-activated in the ultraviolet ozone cleaner.

7. Use tweezers to carefully lift the stamp-off substrate from the stamp. Lift in one smooth stroke to ensure clean pattern edges.
8. Repeat steps 2 (inking) through 7 (stamping) for each additional protein pattern (Fig. 1.1iii*, iv*). Rotate and/or translate the stamp relative to the stamp-off substrate as appropriate (see Fig. 1.2iv). Use a new, clean freshly-activated stamp-off substrate for each stamp-off step.
9. When the stamp bears the desired final pattern, activate the PDMS-coated coverslips (generated above) in the ultraviolet ozone cleaner for 7 min at about 5 cm from the ultraviolet light source.
10. Invert the stamp onto the activated PDMS-coated coverslip. Press firmly to ensure conformal contact. Leave in contact for at least 1 s for complete protein transfer (Fig. 1.1iii).

**FIGURE 1.2**

Stamp-off to generate adjacent, multicomponent features. (i) First ink the stamp with a protein (green). (ii) Then stamp-off onto a UV ozone-treated template. (iii) Re-ink the stamp with a second protein (red). The protein should be chosen such that it will adsorb to the bare PDMS but not to the first protein, see [Section 1.2](#). (iv) Then stamp-off to remove both the first and second protein. The stamp-off template should be UV ozone-treated for 7 min. (v) Re-ink the stamp with a third protein (blue). (vi) Stamp-off to remove the first, second, and third protein. (vii) Finally, transfer the protein on the stamp to the cell culture substrate. The cell culture substrate (also PDMS) should be UV ozone-treated for 7 min. Fluorescent light (FL) micrograph shows an example of adjacent, multicomponent features. Green, red, and blue proteins were bovine serum albumin tagged with AlexaFluor-488, -594, and -647, respectively. Scale bar, 20 μm .

Note: The stamp is topographically flat so there is no risk of stamp collapse. Press firmly as needed to ensure conformal contact.

11. Use tweezers to carefully lift the stamp-off substrate from the stamp. Lift in one smooth stroke to ensure clean pattern edges.
12. Incubate the coverslips in 0.2% Pluronic F127 for at least 1 h at room temperature.
13. Rinse the substrates three times with milliQ water.

III. Cell deposition.

a. Materials

1. Phosphate-buffered saline (PBS)
2. Appropriate cell culture medium (for example, DMEM + 10% fetal bovine serum)
3. Standard cell culture materials (pipettes, centrifuge tubes, vacuum source, laminar flow hood, cell culture incubator, etc.)

b. Method

1. Detach and resuspend cells as per normal. Adjust cell density to 1×10^6 cells/ml.
2. Rinse the micropatterned substrate with PBS, and replace the PBS with cell culture medium.
3. Add cell suspension to the micropatterned substrate at a density of 10,000 cells/cm². Shake plate in perpendicular directions to distribute cells in the medium.

Note: Shaking the plate by swirling it will *not* distribute the cells, and will instead force the cells to cluster in the center of the dish.

4. Incubate the micropatterned substrate in an environment appropriate to the culture conditions (e.g., a humidified incubator set to 37 °C and 5% CO₂ for many mammalian cell lines) for 20–60 min until cells have attached to the micropatterns and begun to spread (for instance, Normal Rat Kidney-52E's take about 20–30 min, whereas Human Umbilical Vein Endothelial Cells take 40–60 min to begin spreading).
5. Remove non-attached cells by very gently aspirating the medium. We recommend aspirating using a handheld pipet, as vacuum suction can easily disrupt cells from the micropattern.

Note: Be careful not to dewet the PDMS, as this will cause the Pluronic F127 to delaminate and/or the cells to dehydrate and therefore lead to pattern fouling and/or cell death. We find that simultaneously adding media with one hand while aspirating with the other hand works best to avoid dewetting of the PDMS.

6. Return the micropatterned substrate to the incubator and proceed with the experiment (e.g., imaging, lysing and harvesting protein, mRNA, etc.).

1.2 DISCUSSION

Stamp-off offers a number of advantages over conventional microcontact printing. The demonstrated advantages of employing stamp-off to biologic studies are several-fold. First, the use of multicolor surfaces has been used to interrogate the spatial and functional relationships of integrin receptors on the cell surface (Desai et al., 2011). Second, stamp-off has been used to generate sparse features without the risk of stamp collapse and pattern fouling that stamp collapse entails, since the stamp used in stamp-off is flat, in contrast to stamps used in conventional microcontact printing

(Desai et al., 2011). This permits investigation of whether and how spacing between cells or the spacing of adhesions within cells affects function. Third, stamp-off has been used to pattern fragile surfaces such as microfabricated post-array-detectors (mPADs), owing to the use of a topographically flat stamp (Han, Bielawski, Ting, Rodriguez, & Sniadecki, 2012; Sun, Weng, & Fu, 2012) (Fig. 1.3A). See Chapter 5, Vol. 121 for a detailed description of micropatterning mPADs with stamp-off. Fourth, when different types of cells bind to different surface coatings, stamp-off also has a potential application in patterned co-culture. For instance, hepatocytes and fibroblasts bind to different surfaces (type-I collagen and bare glass, respectively), and this has been exploited by the Bhatia group to generate patterned co-cultures of these cells. Micropatterned co-cultures were also engineered by the Chen group to control the juxtaposition of epithelial and mesenchymal cells (Tien, Nelson, & Chen, 2002). We have used stamp-off to pattern different cell types (Fig. 1.3B). Finally, cells interact with surfaces via molecular interactions in the nanoscale regime (Cavalcanti-Adam et al., 2007; Coyer et al., 2012; Paszek et al., 2012; Schwartzman et al., 2011). Stamp-off, but not conventional microcontact printing, can be used to pattern at the nanoscale to study such interactions (Coyer, Garcia, & Delamarche, 2007; Xia, Rogers, Paul, & Whitesides, 1999). Despite these advantages, stamp-off does have several limitations that should be considered when designing an experiment using stamp-off micropatterning.

First, attention must be paid to ensure accurate spatial alignment. Figure 1.2 illustrates that micropatterns with spatial alignment on the micrometer scale (the smallest features are $3\ \mu\text{m} \times 3\ \mu\text{m}$ in Fig. 1.2, and are positioned adjacently without overlap) can be easily generated, even though actual stamp placement is done by eye

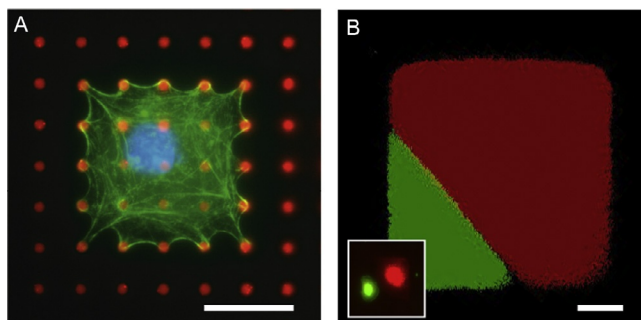


FIGURE 1.3

Applications of stamp-off. (A) A human pulmonary artery endothelial cell seeded on a micropatterned array of microposts. Blue: DNA, green: actin, red: microposts. Length scale bar, $20\ \mu\text{m}$. Adapted with permission from Han et al. (2012) and used with permission from Cell Press. (B) A three-color pattern, where black represents non-adhesive surface, red represents an adhesion protein, and green represents a different adhesion protein. *Inset*: cells, here expressing cell tracker red or green, segregate to different adhesion proteins. All scale bars, $20\ \mu\text{m}$.

and only alignment on the millimeter–centimeter scale is required (all alignments in Fig. 1.2 were done by eye). In contrast, to generate the pattern shown in Fig. 1.2 with conventional microcontact printing the resolution of stamp placement must be equal to that required for edge-to-edge spacing. That is, to achieve the same feature sizes and juxtapositions, one merely needs alignment by eye with stamp-off (1000–10,000 μm), but specialized equipment for alignment of the features themselves (typically 10 μm or less for micropatterns relevant to cell biology) with conventional microcontact printing.

Second, the inking steps should not use proteins that bind one another. For instance, if protein A binds protein B, then inking the stamp with protein B after A is already patterned on the surface would allow B to adsorb not only on the stamped-off (protein free) regions, but also directly to protein A. We have successfully micropatterned multicomponent surfaces presenting distinct micropatterns of fibronectin and protein G (unpublished), and collagen type-I with vitronectin (Desai et al., 2011) with stamp-off.

Lastly, a limitation to any microcontact printing-based technique such as stamp-off is that cells can potentially remodel the protein on the surface. This involves cellular digestion of surface-immobilized proteins and discretion and deposition of new proteins, and the timescale is a function of the proteins involved and cellular enzymatic activity, the latter of which depends on the soluble environment (Nelson, Raghavan, Tan, & Chen, 2003). Thus the timescale of remodeling is largely cell-type dependent. In contrast, degradation of the non-adhesive region used here (F127 Pluronics) is cell-type independent, has a half-life on the order of weeks (Nelson et al., 2003).

The power of micropatterning to illuminate fundamental cellular mechanisms is clear. Given the accessibility and advantages of stamp-off, we hope that the technique presented herein becomes a valuable tool for interrogating cell functions.

Acknowledgments

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