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Micropatterned Multicolor Dynamically Adhesive Substrates to Control Cell Adhesion and Multicellular Organization

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ABSTRACT: We present a novel technique to examine cell–cell interactions and directed cell migration using micropatterned substrates of three distinct regions: an adhesive region, a nonadhesive region, and a dynamically adhesive region switched by addition of a soluble factor to the medium. Combining microcontact printing with avidin–biotin capture chemistry, we pattern nonadhesive regions of avidin that become adhesive through the capture of biotinylated fibronectin. Our strategy overcomes several limitations of current two-color dynamically adhesive substrates by incorporating a third, permanently nonadhesive region. Having three spatially and functionally distinct regions allows for the realization of more complex configurations of cellular cocultures as well as intricate interface geometries between two cell populations for diverse heterotypic cell–cell interaction studies. We can now achieve spatial control over the path and direction of migration in addition to temporal control of the onset of migration, enabling studies that better recapitulate coordinated multicellular migration and organization in vitro. We confirm that cellular behavior is unaltered on captured biotinylated fibronectin as compared to printed fibronectin by examining the cells’ ability to spread, form adhesions, and migrate. We demonstrate the versatility of this approach in studies of migration and cellular cocultures, and further highlight its utility by probing Notch–Delta juxtacrine signaling at a patterned interface.

INTRODUCTION

The ability to control the spatial localization and geometry of cells via surface engineering has contributed greatly to our understanding of how cell adhesion regulates a wide variety of cellular functions. Microcontact printing of adhesive proteins, a surface patterning tool based on soft lithography techniques developed by Whitesides and colleagues, restricts cell adhesion to specific regions and has enabled numerous studies illuminating mechanisms by which cell adhesion and shape impact cell survival, apoptosis, proliferation, differentiation, and migration. However, micropatterned surfaces generated via conventional microcontact printing are binary: one region permanently permits cell adhesion, and the remaining region permanently prevents cell adhesion. Thus, conventional microcontact printing is not well suited to pattern more than two regions and does not allow for the patterning of multiple cell types.

To overcome this limitation, subsequent patterning techniques allowed for the fabrication of multicolor substrates via sequential stamping with multiple proteins, multimask photolithography, photolithography, photoresist barriers and aminosilane-linked biomolecules, multilevel stamps, and stamp-off. These multicolor substrates comprised more than one type of adhesive region and have been used to spatially segregate different cell types or subcellular components by exploiting the preferential attachment of certain cell types or receptors to specific adhesive ligands. However, because these techniques depend on this preferential attachment, their applicability is restricted to a very narrow range of cell types that have unusual adhesion specificities. Most cell types adhere promiscuously to a wide range of shared adhesive ligands, preventing selective adhesion as a strategy for patterning multiple cell types. Additionally, these multicolor substrates do not allow for cells to be released from initial patterns and are thus not applicable to studies of cell migration or multicellular organization.

More recently, dynamically adhesive substrates have overcome many of these limitations by allowing for the nonadhesive region to be controllably induced to become adhesive via light exposure, electroactive or thermally responsive polymers, or physical masks. These dynamically adhesive substrates allow for robust coculture patterning where a first cell type is seeded on initial patterns and a second cell type is seeded immediately upon induced adhesiveness of the remaining nonadhesive regions. These dynamic substrates also allow for studies of cell migration where initially patterned, restricted cells are released from their patterns upon an induced change in

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the substrate, thus allowing for temporal control of the onset of cellular shape changes or unrestricted migration. Although these dynamic substrates facilitate a much wider range of applications than conventional micropatterned substrates, they are still limited by the fact that they are comprised of only two regions: the initially patterned region and the surrounding dynamically adhesive region. Thus, although they allow for cell migration following the adhesive switch, the subsequent surface is now essentially unpatterned so it no longer controls the path and direction of cell movement. Substrates comprised of only two regions also limit the complexity of coculture pattern geometries one can achieve, since only the first cell type geometry can be controlled and the second cell type would simply fill in the surrounding surface area. In order to realize configurations in which both cell types are patterned independently of one another, or where the pattern of cell movement once cells are released from initial patterns is controlled, a third permanently nonadhesive region becomes necessary.

Here, we present a simple strategy based on the avidin–biotin interaction to generate multicolor patterned substrates that allow for three spatially and functionally distinct regions: adhesive, dynamically adhesive, and nonadhesive. Incorporating this third, nonadhesive region enables control over the initial pattern geometry as well as the geometry of switched areas. In this paper, we describe two applications of this technique: migration and coculture. In migration studies, our technique now allows for spatial control over the path and direction of migration in addition to temporal control of the onset of migration. In coculture applications, our technique now allows for the patterning of both cell types independently, with control of the nonadhesive spacing, and the ability to generate a wide range of interface geometries between two cell populations for different kinds of heterotypic cell–cell interaction studies. This simple method will enable in vitro studies of complex cellular organization and coordinated multicellular migration that better recapitulate tissue microenvironments in vivo.

## EXPERIMENTAL SECTION

### Cell Culture and Reagents.

Human umbilical vein endothelial cells (HUVEC) and human mesenchymal stem cells (MSC) (Lonza, Walkersville, MD) were cultured as prescribed by the manufacturer. Chinese hamster ovary cells harboring Notch "Receiver" and Delta "Sender" transgenes, [receiver line: CHO-K1-TREx + UAS-H2B-Citrine + CMV-H2B-Cerulean + CMV-hNotchECDF-Gal4 clone F1; sender line: CHO-K1-TREx + TO-hDI1-mCherry] both graciously provided by Dr. Michael Elowitz (California Institute of Technology), were cultured as previously described. Human plasma fibronectin (BD Biosciences, Bedford, MA) was fluorescently labeled using Alexa Fluor 555 NHS ester (Invitrogen, Carlsbad, CA). Biotinylated fibronectin was obtained from Cytoskeleton, Inc. (Denver, CO) or made in-house using Biotin-X, SSE, 6-((biotinoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt (Sulfo-NHS-LC-Biotin), made in-house using Biotin-X, SSE, 6-((biotinoyl)amino)hexanoic acid, sulfosuccinimidyl ester, sodium salt (Sulfo-NHS-LC-Biotin), (Invitrogen, Carlsbad, CA), and fluorescently labeled using Alexa Fluor 647 NHS ester (Invitrogen, Carlsbad, CA). Neutravidin and Neutravidin–Oregon Green 488 conjugate were obtained from Invitrogen. Poly(dimethyl siloxane) (PDMS; Sygald 184, Dow Corning, Midland, MI) was used as previously described. Flat PDMS stamps were cast from a photosensitive silicon wafer, as previously described. Flat PDMS stamps were cast from a flat silicon wafer. For microcontact printing, PDMS stamps were inked by exposure to fibronectin or Neutravidin (50 μg/mL in PBS) for 1 h at room temperature and then thoroughly rinsed in sterile water and blown dry with a stream of compressed nitrogen. In parallel, the cell culture substrate (PDMS-coated glass coverslip) was activated in an ultraviolet ozone cleaner (Jelight Company, Irvine, CA) for 7 min. The fibronectin-inked stamp was then placed in conformal contact with the substrate for at least 1 s. Next, the Neutravidin-inked stamp was placed in conformal contact with the substrate for at least 1 s. For geometries that required precise alignment of the two stamps, stamp-off was used as previously described. F127 Pluronics was then adsorbed to the PDMS surfaces from a 0.2% (w/v) solution in sterile water for 1 h at room temperature to prevent protein adsorption to nonstamped portions of the PDMS, and then rinsed thoroughly (at least three times) with PBS to remove any residual Pluronics F127.

### Cell Seeding.

Cells were trypsinized and resuspended in serum-free culture media at an appropriate density for the pattern of interest (for sparsely patterned substrates like the cell pairs or single-track lines of 10–15 μm, seeding densities were kept low at ~5000 cells/cm² of total substrate area; for large multicellular patterns, seeding densities were higher at ~100 000 cells/cm²). Once cells spread to the extent of the fibronectin regions (2–24 h, depending on the cell type), a 10 μg/mL solution of biotinylated fibronectin in serum-free media was added to the substrates and incubated for 10 min at room temperature. Substrates were then rinsed twice with PBS to remove any uncaptured biotinylated fibronectin. For migration studies, substrates were immediately taken to an environmental chamber with temperature and CO₂ control for live microscopy (In Vivo Scientific, St. Louis, MO). For coculture patterning, the second cell type (HUVEC) was seeded immediately after addition and rinse of biotinylated fibronectin at an appropriate density for the pattern of interest in normal, serum-containing, growth media. Once cells spread to the extent of the Neutravidin regions, substrates were rinsed three times with PBS to remove any unattached cells and incubated in growth media at 37 °C, 5% CO₂.

### Immunofluorescence and Microscopy.

Substrates patterned with fluorescently labeled proteins were imaged on a Nikon TE200 or Nikon TE2000U microscope. For migration studies, cells were imaged using brightfield microscopy. To visually identify distinct cell types in patterned cocultures, cell types were labeled with the spectrally distinct fluorescent dyes, CellTracker Red CMTPX and Green CMFDA (Molecular Probes). For labeling, cells were incubated in 5 μM Cell Tracker dyes for 30 min in serum-free media. Cells were then rinsed and incubated in serum-containing media for at least 1 h.

### Quantification of Adhesions and Spreading.

To measure focal adhesions and cell spreading, we used the method used by Pirene et al. Briefly, cells were permeabilized with 0.5% Triton X-100 in cytoskeletal buffer, fixed in 4% paraformaldehyde in PBS, and immunolabeled for vinculin. Images were acquired with a 60× NA 1.4 objective on a TE2000U microscope with a Hamamatsu Orca CCD. Images were filtered and binarized to detect edges and remove background noise, and then segmented with a threshold of 0.25 μm² to detect focal adhesions. The cell outline was manually traced to measure cell spread area.

### Measurement of Migration Parameters.

Live cells were seeded on the appropriate substrate, allowed to spread and image via transmitted light, time-lapse microscopy every 15 min. Cells were manually tracked, and the relationship of mean square displacement (MSD) versus time was fit using a model that describes a persistent random walk: MSD = 2P(1 − P(t) − P(1 − expl−t/P)). Speed (S) and persistence time (P) were obtained from the curve fits and reported.

## RESULTS

### Fabrication of Dynamically Adhesive Substrates.

We developed an approach to generate multicolor substrates that comprise three distinct regions. Fibronectin is an adhesion protein that should always be adhesive to cells, but we reasoned that an alternative protein, Neutravidin, could be used as a dynamically adhesive coating, and Pluronics F127 should be permanently nonadhesive. We used soft lithography techniques, as previously described, to micropattern regions of fibronectin...
activated template to stamp off undesired regions of fibronectin (red) from a previously inked stamp. (2) Re-ink the stamp with Neutravidin (green). (3) Finally, transfer the fibronectin–Neutravidin pattern on the stamp to the cell culture substrate. The fluorescent light (FL) micrograph shows an example of corresponding features. (b) Switch mechanism. Neutravidin patterned regions are nonadhesive to cells but will capture biotinylated fibronectin in solution to then become adhesive. The fluorescent light (FL) micrograph shows an example of corresponding features from (a i, ii) where biotinylated fibronectin labeled with AlexaFluor-647 attaches specifically to the Neutravidin regions and not the fibronectin regions (red) or the nonadhesive regions (black). All scale bars, 100 μm.

We generated patterns in two ways that depended on the precision of micropatterning demanded by the experimental application: low precision (“forward printing”; Figure 1a, panel i) or high precision (“stamp-off”; Figure 1a, panel ii). For forward printing, we serially stamp fibronectin (illustrated in red in Figure 1a) and Neutravidin (illustrated in green in Figure 1a), manually rotating the stamps as needed (for example, by 90°) to generate orthogonal alignment (Figure 1a, panel I, steps 1–2). For experimental applications that demanded positioning of features at substantially higher spatial resolutions than achievable via manual stamp alignment (sub-millimeter scale), we used stamp-off (Figure 1a, panel ii). As an illustrative example in Figure 1a, panel ii, we patterned an array of 15 × 15 μm² fibronectin squares within 15 μm wide lines of Neutravidin. This was generated by first inking a stamp of 15 μm wide lines spaced 100 μm apart with fibronectin, then de-inking everything but the squares using a UV-ozone activated PDMS template (Figure 1a, panel ii, step 1), re-inking the same stamp with Neutravidin to fill in the gaps (step 2) (Neutravidin transfers only onto bare PDMS and not onto the previously printed fibronectin), and finally transferring the pattern to a cell culture substrate (step 3). The last step in both forward printing and stamp-off is to coat the remaining unstamped regions with Pluronics F127 to render them resistant to protein adsorption and therefore cell adhesion. Failure to add Pluronics F127 results in pattern fouling (see Figure S1, Supporting Information).

Neutravidin, a deglycosylated version of avidin, is non-adhesive to cells; however, the extremely high affinity between Neutravidin and biotin (Kₐ ~ 1 × 10⁻¹⁵ M)³⁷ allows for immediate capture of biotinylated ligands from solution. We reasoned that, by adding biotinylated fibronectin to the media, we could switch the Neutravidin region from cell nonadhesive to adhesive. The biotinylated fibronectin (labeled with AlexaFluor 647 for protein visualization) binds specifically to the Neutravidin region (Figure 1b, i and ii) but not to the originally printed fibronectin. In this way, we generate multicolor patterned substrates with three regions: adhesive (microcontact printed fibronectin), initially nonadhesive region (microcontact printed Neutravidin) that can be induced to become adhesive by the addition of biotinylated fibronectin, and nonadhesive (Pluronics F127).

It is important to note that, while Pluronics is established as a nonfouling agent that degrades in a cell-independent manner, it does have a finite lifespan that is likely limited by desorption.

Figure 1. Generating three-color dynamically adhesive substrates via two microcontact printing techniques. (a) (i) Forward printing. (1) Transfer the fibronectin (red) on a previously inked stamp to the cell culture substrate. (2) Then, transfer the Neutravidin (green) on a previously inked stamp to the same cell culture substrate by manually aligning features as needed. (3) Finally, incubate the substrate in 0.2% Pluronics F127 (w/v) in water for 1 h to render the remaining regions nonadhesive. The fluorescent light (FL) micrograph shows an example of corresponding features. (ii) Stamp-off. (1) Use a UV ozone-activated template to stamp off undesired regions of fibronectin (red) from a previously inked stamp. (2) Re-ink the stamp with Neutravidin (green). (3) Finally, transfer the fibronectin–Neutravidin pattern on the stamp to the cell culture substrate. The fluorescent light (FL) micrograph shows an example of corresponding features. (b) Switch mechanism. Neutravidin patterned regions are nonadhesive to cells but will capture biotinylated fibronectin in solution to then become adhesive. The fluorescent light (FL) micrograph shows an example of corresponding features from (a i, ii) where biotinylated fibronectin labeled with AlexaFluor-647 attaches specifically to the Neutravidin regions and not the fibronectin regions (red) or the nonadhesive regions (black). All scale bars, 100 μm.

Figure 1. continued
from the surface. The Pluronics is physisorbed onto the substrate and others have reported that the presence of serum proteins in the media will eventually displace the polymer from the surface, leading to eventual fouling of the nonadhesive area. Similarly, the Neutravidin region can indeed degrade likely due to cell proteases and remodeling. However, in combination, the Neutravidin−Pluronics surface coating is stable at least up to 2 days (Figure S2, Supporting Information), and Pluronics surfaces alone have been reported by our group to be stable for up to 5 days. Thus, while it is likely that the surface is remodeled over longer periods of time, we anticipate that this strategy can be used for shorter term experiments.

Characterization of the Substrates. Because the fibronectin is stamped onto the surface, whereas the “switched”, biotinylated fibronectin is captured from solution by Neutravidin, there was a possibility that cells would respond differently to printed versus captured fibronectin. To investigate this, we examined three cell responses to these different fibronectin coatings: spread area, adhesive area, and random motility parameters. We used endothelial cells as our model cell, and adsorbed fibronectin as a control, since most studies typically adsorb fibronectin onto a cell culture surface such as a glass coverslip. We first examined cell spread area on the surfaces by culturing cells in the presence of serum for 24 h, fixing them with 4% paraformaldehyde, staining them for F-actin with phalloidin, acquiring images of the phalloidin stains (Figure 2a) and finally processing the images to extract cell spread area (see the Experimental Section). Figure 2b shows that cell spreading was statistically identical across adsorbed, printed, and captured fibronectin.

Although cells spread to a similar extent, it was unclear whether their underlying adhesion to the various types of fibronectin was similar. To test this, we quantified the number of focal adhesions across the cell on the three surfaces. Cells cultivated for 24 h were permeabilized with 0.5% Triton-X and immunolabeled against mature focal adhesions with an antibody that recognizes the focal adhesion protein, vinculin. Results showed that cells adhered statistically identically to

Figure 2. Characterization of cellular behavior on dynamically adhesive substrates. (a) Cell spread area is shown and (b) computed from HUVECs seeded on the indicated matrix for 24 h, fixing and immunolabeling for F-actin. (c) Number of focal adhesions are shown and (d) computed from HUVECs seeded on the indicated matrix for 24 h, fixing and immunolabeling for vinculin. (e) HUVECs were followed via time-lapse phase microscopy on the indicated substrates for 2−4 h. Migration tracks, and mean squared displacement versus time was determined and fit to the persistent random walk model to describe cell migration. (f) The parameters speed and persistence time were computed from the model. Box and whisker plots are 5−95%. Scale bars, 25 μm.
printed and captured fibronectin, although they adhered statistically significantly more to adsorbed fibronectin than printed fibronectin (Figure 2c,d).

One output of cell adhesion is cell migration, so we next compared cell migration on the different surfaces. To compare cell migration, cells were seeded sparsely on each surface and tracked for a duration of 2−4 h, approximately 12 h after seeding. Trajectories of 10 illustrative cells are shown in Figure 2e. We confirmed that cells in this setting fit the persistent random walk model used to describe cell migration, consistent with prior expectations.40,41 This model relates the mean square displacement, MSD, to time, \( t \), as a function of cell speed, \( S \), and persistence, \( P \), and is of the form MSD = \( 2S^2P\left(t - P(1 - \exp\left(-t/P\right)) \right) \) . Although cell speed was statistically identical on all three surfaces (Figure 2f), persistence time (the average time between significant changes in direction) was substantially higher on adsorbed fibronectin compared to printed or captured fibronectin. Although we do not know what underlies this difference in persistence time, we suspect that it is related to the higher adhesive area observed for cells on adsorbed versus printed or captured fibronectin. Taken together, we conclude that cells behave statistically identically on printed and captured fibronectin, although some differences between these coatings compared to adsorbed fibronectin exist. Importantly, our technique here relies on printed and captured fibronectin only, and not adsorbed fibronectin. We therefore consider our micropatterned fibronectin and Neutravidin strategy effective for comparing the behavior of cells adhering to micropatterned fibronectin versus biotinylated fibronectin captured by Neutravidin.

**Patterning Cell Migration.** Since cells behave similarly on printed and captured fibronectin, we patterned these to make dynamic substrates, first to ask whether we could control both the onset and direction of migration of cells. To test how quickly cells would respond to the Neutravidin regions’ induced adhesivity, we confined cells to small, 35 × 35 μm² square islands (Figure 3a), and then switched the adhesivity of the surrounding Neutravidin region by adding biotinylated fibronectin to allow cells to begin migration. Ten cells in the field of view were tracked before and after the addition of biotinylated fibronectin. Plotting their trajectories before and after addition of biotinylated fibronectin (Figure 3b).
demonstrates that cells are initially confined to the square islands but become migratory after the addition of biotinylated fibronectin. Plotting displacement versus time (Figure 3c) shows that cells transition from stationary to migratory almost immediately after addition of biotinylated fibronectin. This highlights the rapidity with which we can induce the onset of single cell migration.

Previous methods have also shown the ability to temporally control the onset of migration through removal of physical constraints or electroactive, thermal, or photoactivated switching.18−23,25−33 Our approach can not only temporally control the onset of migration as above, but with three-color patterns, we can also constrain the path and direction of cell migration by patterning nonadhesive regions. To demonstrate control over both the onset and path of cell migration, single cells were seeded on an array of 15 × 15 μm² fibronectin squares embedded within 15 μm wide Neutavidin lines (Figure 3d, panels i, ii). Upon addition of biotinylated fibronectin to the
culture media, cells begin to migrate along the pattern Neutravidin lines but not the intervening space between the lines (panel iii). Cells were significantly more elongated after the addition of biotinylated fibronectin (panel iv), demonstrating that cells spread along the induced adhesive area. We can thus restrict cell migratory direction to predefined tracks, permitting ease of observation and analysis of cell migration. Additionally, the versatility of this technique in terms of pattern geometry allows for increasing pattern complexity allowing for the generation of systems relevant to in vivo coordinated multicellular migration by changing pattern shape.

**Patterning Cellular Cocultures.** How signals propagate throughout multicellular structures is another important area of investigation in developmental biology to which multicolor patterns could greatly contribute. Although prior approaches to dynamically adhesive substrates have permitted coculture patterning through the use of stencils, electroactive switching, and selective adhesion, these were limited to two-color patterns and thus were unable to realize configurations of complex interfacial geometries where both cell–cell contact and spacing between the different cell types could be controlled. In contrast, our three-color dynamic substrates allow us to micropattern much more complex configurations of cellular cocultures for diverse studies of heterotypic cell–cell interactions. To accomplish control over the patterning of two cell types on a three-color substrate, one population of cells was seeded and grown to confluence to fill the initial fibronectin pattern. Once the cells spread to the full extent of the fibronectin region, biotinylated fibronectin was added to the culture media and a second cell population was seeded, which quickly attached to the “switched” Neutravidin region (Figure 4a). We engineered a number of different geometrical interfaces between different cell types in large multicellular patterns as well as at single-cell resolution (Figure 4, panels b–c) and demonstrate that we are able to control the size, shape, and curvature of the interface in patterned cocultures. The simplicity of this technique also allows for much versatility in terms of being applicable to all or most cell types. Here, we have demonstrated patterning with human mesenchymal stem cells (Figure 4b), human umbilical vein endothelial cells (Figure 4d,e), and Chinese hamster ovary cells (Figure 4a,c). While higher resolution patterns consisting of fewer cells (Figure 4b,c) can be achieved very cleanly, larger multicellular patterns (Figure 4d,e) show a minor amount of crossover of the cell types due to any existing gaps in the first cell monolayer in which the second cell type is free to land upon subsequent seeding. While we can minimize this by seeding the first cell type at higher densities and waiting for complete confluence, there will always be some inherent noise in the patterning because these are living, biological systems that have processes we cannot control. Nonetheless, we are able to demonstrate patterning of large (millimeter-scale) multicellular structures with relatively clean heterotypic interfaces.

To illustrate the utility of such patterns of coculture, we examined an important question of interfacial juxtacrine signaling. Heterotypic cell–cell interactions occur at interfaces between two cell types and are commonly used in biological systems to orchestrate developmental processes such as proliferation, migration, differentiation, and tissue pattern formation. A receptor–ligand pair that mediates cell–cell interactions in a broad range of developmental patterning processes is the signaling pathway between the Notch receptor on one cell and the Delta ligand on an adjacent cell. Recent quantitative studies of the Notch–Delta interaction using genetically engineered cell lines to visualize the interaction in real time have shed considerable light on novel mechanisms of the interaction. To test whether the methods we have developed here could be used to further probe Notch–Delta interactions, we generated patterned cocultures of Notch receptor and Delta ligand expressing cells and confirmed activation of Notch at the interface between the two cell types (Figure 5). We micropatterned a coculture of tetracycline-inducible Delta expressing sender cells on the vertical fibronectin line, followed by Notch receptor cells with yellow fluorescent protein (YFP) reporters of Notch activity on the horizontal Neutravidin lines. Before addition of tetracycline (Figure 5a; t = 0), no sender cells express Delta and therefore no receiver cells harbor baseline Notch activity, as evidenced by baseline levels of YFP fluorescence. However, Delta was induced in sender cells upon addition of tetracycline, which then activates Notch signaling in neighboring receiver cells, visualized as YFP expression localized to the intersection of the vertical and horizontal lines approximately 24 h after addition of tetracycline. Average YFP pixel intensity profiles clearly indicate a peak of Notch activation at the interface between sender and receiver cells (Figure 5c). We conclude that our three-color dynamic substrates offer an
effective way to probe heterotypic interfacial juxtracrine signaling.

**DISCUSSION**

We developed a technique that combines microcontact printing with a simple dynamic attachment chemistry to achieve multicolor patterns with three distinct functional regions: adhesive (microcontact printed fibronectin), nonadhesive (Pluronics F127), and an initially nonadhesive region (microcontact printed Neutravidin) that can be induced to become adhesive by the capture of biotinylated fibronectin. We confirmed that cells spread, form adhesions, and exhibit motility to similar extents on captured biotinylated fibronectin as compared to printed fibronectin, thus making this an effective and powerful tool to examine cellular behavior. We then demonstrate the utility and versatility of this tool in studies of migration, cellular cocultures, and interfacial juxtracrine signaling.

Our technique offers several advantages over other current methods to generate dynamically adhesive substrates. Other methods include removal of physical constraints, electroactive switching, thermal- and photo-activatable polymers, and layer-by-layer deposition, but all of these have comprised only two regions (adhesive and dynamically adhesive). These substrates enable control over the initial pattern geometry, but the lack of a nonadhesive region prevents control over the dynamically adhesive region. Our multicolor substrates comprise three spatially and functionally distinct regions that allow for independent control over the initial adhesive geometry, as well as the dynamically adhesive region. In migration studies, adequately patterning the nonadhesive region allows for spatial control over the path and direction of migration in addition to temporal control of the onset of migration. For coculture applications, this three-color aspect now allows for the patterning of both cell types independently, with control of the nonadhesive spacing, and the ability to generate different interface geometries between two cell populations for diverse heterotypic cell–cell interaction studies. It is important to determine that the second cell type to be seeded will not undergo significant attachment to the first cell type as could be the case with some cell types. We present multiple cell types here (MSC, HUVEC, CHO) chosen for their biological significance in cell–cell interaction studies and did not see any significant attachment of one cell type onto another.

Other methods to pattern three distinct regions, such as Hui et al.’s patterned substrates of collagen, bare glass, and polyethylene glycol, allowed for cocultures of hepatocytes and fibroblasts in liver function studies; however, this depended on the rare selective adhesion of hepatocytes to collagen but not bare glass under serum-free conditions. Our technique overcomes this restriction of selective adhesion by combining this three-color approach with the dynamic capture of biotinylated fibronectin, making it applicable to most or all cell types. One study did demonstrate dynamically adhesive substrates in three-color, but this involved electrochemical switching to induce adhesiveity of the dynamic region. Electroactive switching requires the use of a voltage pulse to the substrate, potentially affecting cell behavior, and is experimentally more complex as it requires electrochemical instrumentation. This and many other dynamic substrate techniques, including physical membranes or stencils, are technically more challenging to implement than our method presented here, and may even cause physical damage to cells on the pattern edge. In contrast, our method allows for the induced adhesiveity of a patterned region via the simple addition of a soluble factor, biotinylated fibronectin, to the culture media, that exploits the very common avidin–biotin bond to allow for cell adhesion and does not otherwise affect cellular adhesion, spreading, or migration. With proper characterization as presented here in Figure 2, this technique can in principle be generalized to any solution capture method, via printed antibodies to capture a target protein, or Neutravidin and other biotinylated proteins. We believe that the simplicity of the method makes it extremely versatile and a promising approach in recapitulating the complexity of *in vivo* coordinated migration and cell–cell interactions.

**ASSOCIATED CONTENT**

Supporting Information

Additional figures addressing the nonadhesiveness and stability of the Neutravidin and Pluronics surface coatings are included. This material is available free of charge via the Internet at http://pubs.acs.org.

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*Notes* The authors declare no competing financial interest.

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