A General Method for Patterning Gradients of Biomolecules on Surfaces Using Microfluidic Networks

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This report outlines a general method for the fabrication of immobilized gradients of biomolecules on surfaces. This method utilizes a microfluidic network that generates a gradient of avidin in solution and immobilizes this protein on the surface of glass or poly(dimethylsiloxane) by physical adsorption. The immobilized gradient of avidin is then translated into gradients of biotinylated ligands (e.g., small molecules, oligomers of DNA, polysaccharides) using the specific interaction between biotin and avidin. This method can also generate immobilized gradients of certain proteins and artificial polymers by a direct transfer of gradients from solution onto the surface. The major advantage of this method is that almost any type of molecule can, in principle, be immobilized in a well-defined surface gradient of arbitrary shape with dimensions of a few micrometers to a few centimeters. It is possible to tailor the precise shapes of gradients on surfaces from gradients in solution, either kinetically or competitively. Kinetic methods rely on controlling the time that the surface is exposed to the gradient in solution: when a single protein adsorbs from solution, the amount that adsorbs depends both on its concentration in solution and on the time allowed for adsorption. Competitive methods rely on exposure of the surface to a complementary gradient of two proteins in solution (in these experiments, the sum of the concentrations of the proteins in solution is independent of positions although the concentration of each, individually, depends on the position. In this procedure, the relative amount of each protein, at saturation on the surface, depends only on its concentration.)

This paper describes a general method for generating gradients of immobilized molecules on surfaces by adsorption from gradients in solution. These gradients are generated using microfluidic devices described previously; the gradients can have dimensions from micrometers to centimeters. We (and others) have published several techniques that generate gradients in solution based on these devices.

Gradients of immobilized biomolecules on surfaces can influence the function and development of cells. While simple gradients of diffusible substances (in solutions) are experimentally accessible on scales down to micrometers by pipetting, the fabrication of immobilized gradients (that is, gradients on surfaces) has been a technical challenge. Techniques that generate immobilized gradients by retarded diffusion of substances, or electrochemical desorption of self-assembled monolayers (SAMs), lack the flexibility to fabricate gradients having complex shapes and are difficult to use at the micrometer scale (the scale of dimensions required for many biological processes). Phot-immobilization on SAMs gives gradients with high resolution, and can be used to fabricate gradients in arbitrary shapes, but usually requires organic synthesis and repeated use of the clean room, and is thus less useful to biologists than relatively simpler methods.

This paper describes a technique that can generate immobilized gradients of many kinds of biologically relevant molecules in various shapes, by using a two-step process: (i) patterning of immobilized gradients of avidin using microfluidic systems and (ii) recognition of derivatives of biotin by immobilized avidin. This method can also generate immobilized gradients of avidin. This method can also generate immobilized gradients of avidin.
avidin. The generation, in solution, of gradients in solution spanning dimensions of a few micrometers to a few centimeters essentially involves mixing two different solutions in a microfluidic network (µFN) and repeatedly splitting and recombining these streams according to well-defined designs, to generate gradients (as a series of steps in concentration).\(^{14}\) We can transfer this gradient in solution to a gradient on surfaces by adsorption. When one of the streams is a solution of avidin, and the other stream is either phosphate-buffered saline (PBS) or a second protein such as bovine serum albumin (BSA), this procedure generates a gradient of avidin immobilized by physical (noncovalent) adsorption on the surface. We can immobilize ligands that have been biotinylated, using irreversible recognition and binding of biotin by avidin, to generate the gradients of desired molecules.

We incorporated several chaotic advective mixers (CAMs) into the µFN.\(^{15}\) The combination of these CAMs, and a folded design of the µFN, made it possible to have a microfluidic device with small dimensions (~2 × 5 cm). Fabrication of a CAM, however, requires two levels of photolithography, and this level of technology may be inconvenient for some biochemists. The same results can be obtained by using a µFN that does not incorporate the CAM, a simpler design of network that requires only one level of photolithography.\(^{1-4}\)

**EXPERIMENTAL SECTION**

**Source of Materials.** All materials commercially obtained were used as received. Poly(dimethylsiloxane) (PDMS) prepolymer (Sylgard 184), SU-8, and silicon wafers (test grade) were purchased from Dow Corning (Midland, MI), MicroChem Corp. (Newton, MA), and Silicon Sense (Nashua, NH). Dextran–fluorescein–biotin conjugates were from Molecular Probes (www.probes.com). All other reagents were purchased from Sigma-Aldrich (www.sigmaaldrich.com).

**Fabrication of Masters by Photolithography.** Fabrication of the two-step features that contain the microchannels (first step) with the CAM (second step) followed a published protocol.\(^{15}\)

Designs for channel systems were generated in a CAD program (Freehand 10.0, Macromedia, San Francisco, CA). The designs for the microchannels and CAMs were printed on separate masks, with registration marks for alignment. High-resolution (5000 dpi) transparencies were produced by a commercial printer (Page-works Prepress Output, Cambridge, MA) from the CAD files.

Negative photoresist (SU-8 10) was spin-coated (~1500 rpm, 15 s) on a silicon wafer and baked to drive off solvent (105 °C, 5 min).\(^{16,17}\) We performed photolithography on the photoresist with the transparency film as the first photomask (carrying the feature alignment marks generated on the first layer of photoresist (the second mask, which carried the features of the CAM, with the alignment marks generated on the first layer of photoresist (the features on this layer became visible after the second step of baking) under a microscope attached to the mask aligner and performed photolithography. This procedure generated the CAMs on top of the microchannels. After postbaking (105 °C, 2 min), we developed the microfeatures on the silicon wafer in 1-methoxy-2-propanol acetate to remove the unexposed areas of the photoresist. The surface of the SU-8 master was exposed to a vapor of perfluoro-1,1,2,2-tetrahydrooctyltrichlorosilane (United Chemical Technologies, www.unitedchem.com) in a vacuum desiccator for ~2 h to prevent the adhesion of the PDMS to the silicon wafer.

The surfaces of the replica of PDMS with embossed µFN and a clean glass slide (both were sonicated in soapy water for 5 min, then washed with distilled water, and dried under a stream of nitrogen) were oxidized in an air plasma (~2 Torr, 60 s, 1000 W, Harrick Scientific, Ossining, NY) and brought into contact.\(^{18,19}\) It is important to thoroughly clean the surfaces of the PDMS before oxidation. In case the sealing repetitively fails, extraction of PDMS with organic solvents improves the rate of success in the sealing process.\(^{18}\) The two adhered to one another irreversibly to generate the closed channels. Once the closed channels had formed, we passed an aqueous solution of PBS through them immediately. When the entire assembly was under distilled water, the walls of the microchannels remained hydrophilic for a few days.

**Microfluidics.** The solutions of proteins were pumped through the µFN using syringe pumps or gravity. The rates of pumping were ~20 µL min\(^{-1}\) in most experiments. The concentration of all proteins (avidin, laminin, fibronectin) was 20 µg mL\(^{-1}\); polylysine was 20 µg mL\(^{-1}\). Immediately after the formation of the gradient of avidin on the surface, we incubated the µFN with BSA for 2 h at room temperature. We then incubated biotin or biotinylated reagents (oligo DNA and dextran, all used at 1 µg/mL) in the µFN for 1 h and visualized the sample under fluorescence microscopy with a Hamamatsu camera (ORCA ER). We fabricated gradients of polymers directly, the same way that we fabricated the gradient of avidin.

**Microcontact Printing.** We coated glass slides for microcontact printing with titanium (2 nm) and gold (20 nm) in an e-beam evaporator. We “inked” a PDMS stamp with desired features in a 2 mM solution of HS(CH\(_2\))\(_6\)CH\(_2\)OH in ethanol. The stamp was dried by a stream of compressed air and brought in contact with the gold surface to form the first SAM. The substrate of glass slide was then incubated with HS(CH\(_2\))\(_6\)(CH\(_2\)CH\(_2\))\(_3\)OH (2 mM, in ethanol) for 12 h. The opaque protective box (4 × 6 × 2 cm) used for protection of the SAMs in air plasma treatment was made by adding iron(II, III) oxide (~100 µm) in the prepolymer of PDMS.

**SIMULATIONS OF ADSORPTION OF PROTEINS**

We fabricated gradients of proteins on surfaces by transferring gradients in solution onto the surface by physical adsorption. There are two strategies for transferring gradients from solution to the surface: (i) The first uses a gradient of protein in a solution of noncompeting buffer (e.g., PBS). In this protocol, the amount of protein deposited on the surface depends on the concentration in solution and the time of contact between the solution and the surface (Figure 1). (ii) The second involves transferring proteins from a competing gradient of two proteins in solution in which

\(^{19}\) Ng, I. M. K.; Gitlin, I.; Stroock, A. D.; Whitesides, G. M. Electrophoresis 2002, 23, 3461–3473.
The sum of the concentrations of the proteins is constant. In this protocol, at saturation, the amount of protein deposited on the surface depends only on the relative concentrations of the two proteins in solution (assuming that adsorption of both proteins is irreversible, Figure 1). We use the nomenclature “(protein A ↔ PBS)” to denote the first type of gradient, where the gradient is simply made of decreasing concentrations of protein A in PBS. We use “(protein A ↔ protein B)” to denote this second type of gradient, in which decreasing concentrations of protein A correlate with increasing concentrations of B (Figure 1).

We simulated the surface gradients by first calculating the rate of adsorption for a protein on the surface ($k_i$, in M$^{-1}$ s$^{-1}$) from the first method of adsorption (“(protein A ↔ PBS)”). Once $k_i$ is known, it is possible to derive the amount of each protein adsorbed from decreasing concentrations (in steps) of proteins in solution. This procedure simulates the stepwise gradient we generate in experiments. We used a µFN with seven channels in these experiments. This µFN generated different concentrations of each protein in each channel; we will refer to these concentrations by the numbers between 1 and 7, channel 1 being the highest in concentration ($3.2 \times 10^{-7}$ M, the concentration used in the experiments) and 7 being the lowest (0 M).

**Adsorption of Avidin against PBS (protein A ↔ PBS).** We used an existing mathematical model to analyze the experimental results on the adsorption of avidin on the surface as a function of its concentration in solution, to generate the profile of gradient on the surface when it is transferred from (avidin ↔ PBS). The “well-mixed” model (a model that assumes that the rate of diffusion is faster than the rate of adsorption and that the concentration of proteins in the surface layer of each channel is uniform and equal to that in the flowing solution) is sufficient to describe the adsorption of proteins on solid surfaces. With two assumptions, using this model, we can solve the equations that describe the adsorption of proteins on surfaces analytically and generate the theoretical values of the surface densities of proteins. These assumptions are as follows: (i) The saturating coverage of the surface by the protein is one monolayer; (ii) the fluorescent intensities of the fluorescently labeled protein adsorbed on the surface accurately measure its surface density.

The well-mixed model starts from eq 1 to describe the adsorption of proteins on surfaces, where $c_i(t)$ and $c_{s, sat}$ are the surface density and the saturated (maximum possible) surface density of proteins, respectively (unit in mol m$^{-2}$), and $[c_{s, sat} - c_i(t)]$ refers to the surface sites available for binding.

$$\frac{dc_i(t)}{dt} = k_f [c_{s, sat} - c_i(t)] - k_r c_i(t)$$  \hspace{1cm} (1)

We used $c$ for the concentration of protein in solution (mol L$^{-1}$) and $k_f$ (M$^{-1}$ s$^{-1}$) and $k_r$ (s$^{-1}$) for the forward and reverse rate constants that describe adsorption and desorption of proteins on and from the surface. Solving for $c_i(t)$ yields eq 2. We assume

$$c_i(t) = \frac{k_f c_{s, sat} e^{-[k_f + k_r]t}}{k_f e^{-[k_f + k_r]t}}$$  \hspace{1cm} (2)

that adsorption of proteins on hydrophobic surfaces is irreversible: i.e., that $k_r = 0$. Once we have determined the value of $k_i$ by experiment, it is possible to use eq 2 to simulate the ratio between $c_i(t)$ and $c_{s, sat}$ to obtain the theoretical values of the fluorescence intensities across all channels. To solve for $k_i$, we simplify eq 2 as eq 3. For given values of $t$ and $c$, the ratio of $c_i(t)$ and $c_{s, sat}$ gives analytical solutions to the values of $k_i$ (eq 4).

$$c_i(t) = c_{s, sat} (1 - e^{-k_f t})$$  \hspace{1cm} (3)

$$k_f = -\frac{\ln[1 - c_i(t)/c_{s, sat}]}{ct}$$  \hspace{1cm} (4)

The solution concentration $c$ is $3.2 \times 10^{-7}$ M (the concentrations we used in the experiments) in channel 1 and decreases

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linearly (in steps) over seven channels. We know from experiments that the surfaces are not saturated at 5 min and mostly saturated at 50 min (see the Results and Discussion section, Figure 4). At 5 min, we cannot estimate the value of $c_{s, sat}$ (i.e., no channel gives saturating fluorescent intensities); at 50 min, there is no usable ratio between $c_s(t)$ and $c_{s, sat}$ (i.e., all channels but channel 0 are saturated). Neither of these conditions allows us to solve for $k_f$ analytically. At intermediate values of time (e.g., 15 min), some of the channels have unsaturated signals and some of the channels have saturated signals; this condition allows us to solve for $k_f$.

Thus, for each $c$ that gives rise to a $c_s(t)$ ($t = 15$ min, the values of fluorescence intensities of all channels after the immobilization of proteins) smaller than $c_{s, sat}$, there is a unique solution of $k_f$ that can be calculated from eq 4. For channel 5, $c = 1.07 \times 10^{-7}$ M, $k_f = 1.82 \times 10^4$ M$^{-1}$ s$^{-1}$; for channel 6, $c = 5.33 \times 10^{-8}$ M, $k_f = 1.60 \times 10^4$ M$^{-1}$ s$^{-1}$. These two values are reasonably close to each other; this result validates our assumptions in the calculations. We therefore use the average of the two, as the $k_f = 1.71 \times 10^4$ M$^{-1}$ s$^{-1}$.

Using values of $k_f$ obtained through this analytical procedure, we solved for the $c_s(t)$ as a function of any values of $c$ and $t$ and thus generated expected values of fluorescence intensities for each channel. Figure 1B shows simulated results generated using this procedure for each channel. The surface densities of proteins are clearly dependent on the concentration of the proteins in solution and on the time the solutions remain in contact with the surface. Control of the concentration, and the time allowed for adsorption, yields different profiles of gradients on the surface. For example, we can fabricate an approximately linear gradient if we maintain

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**Figure 2.** Design of the microfluidic channel that incorporates CAMs. (A) Diffusive mixing is enhanced by chaotic advection generated by an asymmetric herringbone pattern in the ceiling of the microchannels. Visualizations of the flow before entering the chaotic mixer, after the first cycle and after the fourth cycle of the mixer, are given on the right side of the figure. Cross-sectional images were taken with a confocal microscope. The last image (outlet) was compressed by 1/7 in the horizontal axis. The stretching and folding of the flow were visualized by injecting nonfluorescent and fluorescent solutions (avidin, labeled with fluorescein) in the network. A magnified figure that shows the design of the μFN is in Figure 3. (B) A photograph of a microfluidic network we used for the immobilization of protein gradients. We used blue and red ink to visualize the channels and illustrate the function of the network and its formation.
the gradient in solution in contact with the surface for 5 min (Figure 1B).

**Competitive Adsorption of Avidin against BSA (protein A ↔ protein B).** We next simulated the type of experiment in which a gradient of two proteins in solution—{avidin ↔ BSA}—generated a gradient immobilized on the surface. The total concentration of proteins in solution is the same in all channels (i.e., \[c_A(t) + c_B(t)\]). We assume that \(c_{s,sat}\) is a constant number, such that a site (an area) on the surface occupied by one adsorbed protein cannot adsorb another. We can describe the system of adsorption of two proteins (proteins A and B) on solid surfaces using eqs 5a and 5b.

\[
\frac{dc_{s,A}(t)}{dt} = k_{f,A}c_A\left(c_{s,sat} - c_{s,A}(t) - c_{s,B}(t)\right) - k_{r,A}c_{s,A}(t) \quad (5a)
\]

\[
\frac{dc_{s,B}(t)}{dt} = k_{f,B}c_B\left(c_{s,sat} - c_{s,A}(t) - c_{s,B}(t)\right) - k_{r,B}c_{s,B}(t) \quad (5b)
\]

For \(k_{r,A} = 0, k_{r,B} = 0\)

\[
\frac{dc_{s,A}(t)}{dt} = k_{f,A}c_A\left(c_{s,sat} - c_{s,A}(t) - c_{s,B}(t)\right) \quad (6a)
\]

\[
\frac{dc_{s,B}(t)}{dt} = k_{f,B}c_B\left(c_{s,sat} - c_{s,A}(t) - c_{s,B}(t)\right) \quad (6b)
\]

For simplicity, we used the same \(k_f\) for both avidin and BSA, \(k_{f,A} = k_{f,B} = 1.71 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) (calculated for adsorption of avidin in the previous section). We integrated eqs 6a and 6b in time using the Runge–Kutta scheme and solved for the values of \(c_{s,A}(t)\) and \(c_{s,B}(t)\). For each unique value of \(c_A\) and \(c_B\), there was a numerical solution for \(c_{s,A}(t)\) and \(c_{s,B}(t)\). These simulations show that the transfer of the gradient from solution to the surface is independent of time at times between 15 and 150 min (Figure 1C).

Although the predictions of our model agree with the results of the experiments, our model may not account for all cases of the adsorption of molecules on solid surfaces. We believe that the relative large molecular weights and similar avidities for the surface of the molecular species make several of our simplifications valid.

**RESULTS AND DISCUSSION**

**Design of the Gradient Generators.** Figure 2 shows the microfluidic network we used for fabricating linear gradients. Figure 2A illustrates the design of the generator of gradient and four repetitive cycles of mixing. We used blue and red dyes to trace the process of the mixing of fluids and the generation of

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the microfluidic network used in Figure 3A; hence, the gradients formed in the two designs described in Figure 3A and B are equivalent.

**Use of CAMs.** In the design with CAM, diffusional mixing in the mixing zones was enhanced by a mixer that stretches and folds the flows into each other and quickly reduces the distances between fluids of different compositions to values where diffusion alone can rapidly homogenize the fluid on a molecular scale.\(^\text{(15)}\) Figure 2A illustrates the patterned topography that generates appropriate transverse flows for chaotic mixing in microchannels. Four cycles of mixing, each using a set of asymmetric herringbone patterns, were sufficient homogenization of the solutions for the generation of gradient.\(^\text{(25)}\) The panels on the right of Figure 2A show confocal micrographs of the vertical cross section of the channel at three positions downstream in the mixing channel. The CAM and its performance are described elsewhere.\(^\text{(23)}\)

All gradients described in this paper utilized CAMs in the \(\mu\)FN. CAMs allow the mixing channels to be shorter and wider than designs that do not incorporate CAMs. Short channels that incorporate CAMs enable the gradient generator to make gradients of molecules having high molecular weights in relatively short channels using this design. The incorporation of CAMs in the \(\mu\)FN allows the use of higher flow rates than in the design without CAM; high flow rates shorten the time required to generate gradients (instead of hours, minutes). The performance of a design without the CAM is dependent on the flow rate, because it relies on keeping two laminar streams in the same channel for a sufficiently long time to complete mixing by diffusion.\(^\text{4}\) For example, the typical mixing time in the design without CAM is \(\sim20\) s for a protein (calculated for a channel of the width of \(\sim50\) \(\mu\)m, assuming the coefficient of diffusion of a protein is \(5 \times 10^{\text{11}}\) \(\text{m}^2\text{s}^{-1}\)).\(^\text{26}\) For streams traveling down \(\sim5\) mm of the mixing channel, the maximum rate of flow for complete mixing is 0.25 \(\text{mm s}^{-1}\) = 12 \(\text{mm min}^{-1}\). For a channel that has a cross section of \(50\text{ mm} \times 50\text{ mm}\), the maximum rate of flow is \(\sim1\) nL/min—a value much smaller than the rates that we use for the immobilization of protein on surfaces in the current design (typically \(\sim20\) \(\mu\)L/min).\(^\text{13}\) In addition, when the channels in the \(\mu\)FN are shorter and wider than those in the design without the CAM, the resistance is a value low enough that gravity alone can drive the fluids. Due to a larger cross section of this \(\mu\)FN than that in the design without the CAM, networks using CAMs are less prone to clogging and fouling caused by dust particles than networks using narrower channels described elsewhere.\(^\text{14}\) The major disadvantage of networks incorporating CAMs is that their fabrication is more complicated than that of networks that do not incorporate CAMs; for inexperienced users of photolithography, the \(\mu\)FN without the CAM still works efficiently for generating gradients.\(^\text{4}\)

**Generation of Molecular Gradients on the Surface.** We used two types of gradients in solution, (avidin ↔ PBS) and (avidin ↔ BSA), to generate gradients of avidin on surfaces of PDMS (Figure 4).\(^\text{27}\)

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**Figure 4.** Gradients of immobilized avidin on the surface. Immobilized gradients of avidin generated from gradients of (avidin ↔ PBS) in solution for (A) 5, (B) 15, or (C) 50 min. (D) Immobilized gradients of avidin generated from gradients of (avidin ↔ BSA) in solution for 50 min. (A'), (B'), (C'), and (D') indicate the corresponding measured profiles of intensities of fluorescence across the channels. The vertical columns indicate the theoretical fluorescent intensities (see the section Simulation of Adsorption of Proteins for details) in each channel. See text for details.

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To generate these two types of gradients, we passed a gradient of decreasing concentrations of avidin in PBS and increasing concentrations of BSA through the μFN, at ~20 μL/min in these experiments. The rapid rate of flow makes possible the generation of gradients in a few minutes. By using a high rate of flow, we also reduced the diffusion-mediated blurring of the profile of the gradient—the profile of the gradient can remain relatively well defined over 50 mm.

As discussed in the section entitled Simulation of Adsorption of Proteins, the interval of time over which the solution of protein is in contact with the surface, and during which adsorption takes place, is important when transferring a gradient of a single protein (avidin ↔ PBS) to the surface. Passing the solution through the μFN for 5 min at these concentrations yields an approximately linear gradient (Figure 4A), while passing the solution through for 50 min under the same conditions saturates the surface (Figure 4C). Passing the solution for 15 min under the same conditions saturates some of the channels (Figure 4B).

In principle, the second method is operationally more straightforward, but it does require two proteins. One of these proteins may be “inert” (e.g., BSA) and serves only to passivate the surface. The first method allows the fabrication with only a single protein but requires a more detailed understanding of the influence of the time of exposure of the surface to the solution on the relationship between the shapes of the gradients in solution and on the surface. It can generate immobilized gradients that would be complicated by the presence of a second protein (used in the second method). Generation of a gradient by competitive adsorption of two proteins (avidin ↔ BSA) is essentially independent of time, after the surface reaches saturation, provided the total concentration of protein in solution is constant across the gradient (Figure 4D, Figure 1).

The second procedure (avidin ↔ BSA) is therefore, in most circumstances, more straightforward to use, if we do not want to bother with kinetic control. It also has the advantage that it allows two (or more) proteins to be patterned in one step and that it can leave the entire surface covered by a protein that can resist the adsorption of additional proteins from solution. The advantage of the first method (avidin ↔ PBS) is that it is fast and does not require the use of a second blocking agent. A short period of time makes this technique convenient for the generation of gradients of molecules that are easily degraded (some biomolecules are sensitive to oxidation or thermal denaturation in ambient conditions). Once the gradient of avidin is generated (from either method), we can generate gradients of biotinylated molecule using the same procedure.

Since desorption of adsorbed proteins from the hydrophobic surface of PDMS is negligible over the course of a few days,28 these immobilized gradients are stable under conditions that do not allow for degradation of the protein. For example, the immobilized gradients can be stored for a long period of time at 4 °C.29 When used in cell culture, however, these immobilized gradients may decay within 2–3 days due to the presence of proteases in the culture media that degrade immobilized molecules.6

![Figure 5](image)

**Figure 5.** Gradients of various molecules on oxidized PDMS generated by using the avidin—biotin approach. (A) The avidin gradient; (B) a gradient of biotin—fluorescein; (C) a gradient of the 28-mer monobiotinylated DNA; (D) a gradient of biotinylated dextran. (E) A gradient of poly(L-lysine) is made by direct adsorption from a gradient of poly(L-lysine) in solutions. The profiles of the gradients are documented at the bottom of each graph. Light areas corresponds to high surface densities of immobilized molecules.

**Fabrication of Molecular Gradients Using the Recognition of Avidin by Biotin.** When fabricating a gradient using an (avidin ↔ PBS) protocol, we first adsorbed avidin on the surface and immediately passivated the surface by passing a solution of BSA through the μFN. Generation of gradients of other molecules involves simply incubating the gradient in immobilized avidin with the biotinylated molecules of interest (Figure 5B–D). As one demonstration, we generated gradients of a small molecule that can be easily visualized—biotinylated fluorescein (Figure 5B). Using the same procedure, we generated a linear gradient of a biotinylated 28-mer DNA sequence (5′/fluorescein/GAT TAC CGA TAC GGC ATT ACC GAT ACG G/biotin-3′) (Figure 5C) and of dextran (polymers of glucose, molecular mass ~3000 kDa, Figure 5D). Fabrication of immobilized gradients of these molecules using common biochemistry (e.g., formation of a gradient in avidin and immobilization to the gradient using biotin) simplified the procedure. In all cases, the linear gradient of avidin produced linear gradients of the molecules of interest on the surface. This methodology provides a general approach to fabricating gradients of many types of biomolecules, regardless of their propensity to adsorb on a surface or coefficients of diffusion. For molecules that

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denature when adsorbed, using the avidin–biotin approach (that has a layer of avidin as an initial layer on the surface) may provide a less denaturing alternative than direct adsorption.

Using avidin to generate the gradient has three advantages compared to other strategies for producing the gradients: (i) avidin is a protein that adsorbs onto the surface while retaining its ability to bind biotin. (ii) It has a relatively high molecular weight (molecular mass \( \approx 68 \text{ kDa} \), for the tetramer), and its rate of diffusion is relatively slow. The shape of the gradient in solution, and on the surface, can therefore be maintained for distances as long as a few centimeters. (iii) Since biotinylation of most biomolecules is available through commercially available kits, this method can generate immobilized gradients of many kinds of biomolecules. The strong interaction between avidin and biotin (\( K_D = 10^{-15} \text{ M} \)) allows essentially irreversible immobilization of biotinylated ligands to the surface of an avidin gradient.

**Fabrication of Gradients of Artificial Polymers.** We also fabricated gradients of several other types of polymers and proteins by direct adsorption from solution, since many types of polymers adsorb to solid surfaces. For example, we fabricated gradients of poly(l-lysine) (PL) on the surface of oxidized PDMS by direct physical adsorption from (PL ↔ PBS) (Figure 5E), since PL adsorbs strongly to anionic surfaces (e.g., oxidized PDMS or glass).

**Complex Gradients of Proteins.** We generated overlapping gradients of two immobilized extracellular matrix proteins by competitive adsorption from a solution gradient of (laminin ↔ fibronectin) (Figure 6). We could also produce gradients with complex shapes by combining several \( \mu \text{FNs}. \) We have shown previously that the gradient generator can produce gradients in a broad range of shapes, including periodic and nonmonotonic.

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**Figure 6.** Combination of overlapping gradients of laminin (Ln) and fibronectin (Fn) into complex contours: (A) illustrates the design of the \( \mu \text{FN}. \) Anti-Fn (mouse) and anti-Ln (rabbit) were used as primary antibodies, and anti-mouse-fluorescein and anti-rabbit-Texas red were used as secondary antibodies to visualize these gradients. Arrowheads point to the axis along which of fluorescence intensity was read. (B) Fluorescence intensities as functions of the distance across the channel.

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profiles, on the scale between 1 and 1000 μm. The smallest span of the gradient cannot be much less than 1 μm, because diffusion of molecules quickly obscures the patterns of laminar flows at this length scale. To illustrate the fabrication of a complex gradient, we combined two simple gradients and merged them together. In addition, we varied the width of the channel to add another level of complexity to the structure of the gradients (Figure 6). Figure 6A demonstrates the design of the network required for the generation of overlapping gradients of laminin (Ln) and fibronectin (Fn) by adsorption of these two proteins directly from solution. The contoured main channel overcomes the limitation that the direction of a gradient is always perpendicular to the direction of the flow of liquid inside straight channels: thus, the μFN generated gradients along two axes in the plane.

**Gradients in Confined Space.** We can also generate gradients of proteins confined in micropatterns. Combination of the generation of gradients with microcontact printing provided one route to gradients in confined space. Figure 7 outlines the strategy: we started with a gold film supported on a glass surface. We printed a thiol–HS(CH₂)₁₅CH₃ (C₁₆) onto the surface of gold film to form one SAM and incubated this printed surface of gold film with HS(CH₂)₁₁(CH₂CH₂O)₃OH (C₁₁EG₃) to form a second SAM in the regions not covered with C₁₆. The C₁₁EG₃-covered regions adsorb proteins; the area covered with C₁₁EG₃ resists adsorption of proteins.

Next, we generated the gradients on these patterned surfaces. Generation of gradients requires a μFN formed by sealing between the glass slide and a piece of PDMS carrying appropriate features. Since plasma oxidation and some wavelength of light destroy SAMs, we first protected the gold surface with an opaque “box” of PDMS (by adding iron oxide particles to the PDMS); this box acted as a physical barrier that prevented the oxygen plasma and any type of light generated in the plasma from reaching the patterned surfaces of SAMs. We oxidized (using an air plasma) the rest of the glass surface and a slab of PDMS embossed with the microchannel system. After oxidation, we removed the opaque PDMS box and brought the glass slide and the PDMS slab in contact (the surfaces of glass and PDMS bonded and sealed irreversibly) to form the microchannels that constituted the μFN. We generated a (Fn ↔ Ln) gradient using the μFN and transferred this gradient to the surface only in the regions patterned with C₁₆, since the C₁₁EG₃-covered SAMs resisted the adsorption of proteins (Figure 7B).

**CONCLUSION**

The methods described in this paper provide precise control over the compositions and shapes of immobilized gradients having

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micrometer-scale dimensions, either by direct transfer from solution or by indirect immobilization via the recognition of biotinylated ligands by avidin. It is applicable to many types of biomolecules (proteins, oligonucleotides, small molecules, artificial polymers), as long as they can be linked to biotin. Immobilized gradients of biomolecules such as oligomers of DNA, polysaccharides, and small molecules are difficult to generate, because, unlike proteins, they usually do not spontaneously and irreversibly adsorb onto solid surfaces such as glass; immobilization of these molecules on different types of substrates would normally require different types of chemistry.

This method has examined the transfer of two types of gradients in solution to the surface: {avidin ↔ PBS} and {avidin ↔ BSA}. The former is less time-consuming and can generate gradient on the surface of one single protein; but it requires a detailed understanding of the kinetics of adsorption of protein on solid surfaces to obtain the right profile of gradient on the surface. The latter is more useful when the precise shapes of the gradient on the surface must be controlled; but it requires the use of two proteins.

The incorporation of the CAM and the folded design of the μFN make the generation of gradient convenient and efficient. The fabrication of the master for the μFN that contains the CAM, however, requires some expertise in photolithography. The design of the μFN without the CAM is also applicable to all experiments described in this work.

This method is useful for cell biologists who are interested in phenotype-associated immobilized gradients, such as migration, formation of processes, and polarity of cells. We believe that this method is also useful for screening the activities of immobilized gradients of biomolecules that are known to affect chemotaxis in solution.\(^{1,2,4,5}\) It is simpler and more versatile than most other methods for generating gradients on surfaces.

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.

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