Lithography

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Two-Component Graded Deposition of Biomolecules with a Double-Barreled Nanopipette**

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There is currently great interest in depositing submicron features of biological molecules on surfaces for miniaturized assays[1–6] and to assemble new structures from biological building blocks.[7] Several methods that have been used to produce these features are based on scanning probe microscopy (SPM) and use either the tip of an atomic force microscope (AFM) or a nanopipette. Dip-pen lithography operates in air and uses an AFM tip to write features of sizes less than 100 nm.[1,5,8–14] Fountain pen lithography has been developed by using apertured AFM tips to produce sub-100-nm features[14] or nanopipettes[6] to produce features of around 300 nm. In both cases, the fountain pen lithography operates in air.

Nanopipettes have also been used for deposition in a buffer solution by using a method based on scanning ion-conductance microscopy (SICM).[15] In this case, voltage-driven deposition gives fine control for delivery at the single-molecule level; however, diffusion of molecules in solution and over the surface broadens the feature size when compared with deposition in air and results in features no smaller than 800 nm.[16–18] The nanopipette, however, has the advantage that it eliminates the need for conditioning of the tip (as is required by dip-pen nanolithography (DPN) for deposition) and provides a large reservoir of molecules. Registry is a common problem with all of these methods and makes it difficult to deposit a different type of molecule on top of or at a defined position relative to another feature. This is, however, a key requirement for the production of complex patterns on surfaces and the assembly of structures made from biological molecules. Currently, this difficulty has been addressed with DPN, by using nanofabricated surfaces with defined topographic features to give registry,[10] or through the use of a 'multiple-pen' array.[11] In our own two-component work, we have addressed this problem by using nanopipettes with fluorescent imaging to provide registry.[17] All of these solutions, however, require the use of a second tip to deposit an additional species.

Herein we present a methodology that eliminates the registry problem for the deposition of two different species on a surface. This is accomplished with a new form of scanning probe microscopy that we developed based on a double-barreled pipette. In this case, two species are independently delivered from each of the pipette barrels of a single tip. Furthermore, fine voltage-driven control of the deposition of two types of biomolecules can be achieved, in the absence of a bath, by creating a potential between the two barrels of the pipette. Operation in air provides smaller feature sizes than deposition in liquid as lateral diffusion is avoided. These advantages allow complex 'two-color' graded patterns to be written with biomolecules.

Double-barreled pipettes are fabricated from glass capillaries (diameter of 1.5 mm, with a septum down the center) by using a pipette puller (Sutter Instruments, Model P-2000). These pipette tips were imaged with scanning electron microscopy (SEM), and the barrels maintained their D-shape throughout pulling (see Figure 1 inset). The heights of the individual barrel openings (when oriented as shown in the Figure 1 inset) ranged from 140 to 185 nm and widths from 100 to 125 nm. As depicted in Figure 1, both barrels are filled with electrolyte solution and a potential (V) is created between the Ag/AgCl electrodes that are placed in the two barrels. When the pipette is held above a surface, a liquid droplet forms automatically at the tip (Figure 1 A). This allows the electrolyte to flow between the barrels and hence provides an ion current (I_{DC}) for control over the surface

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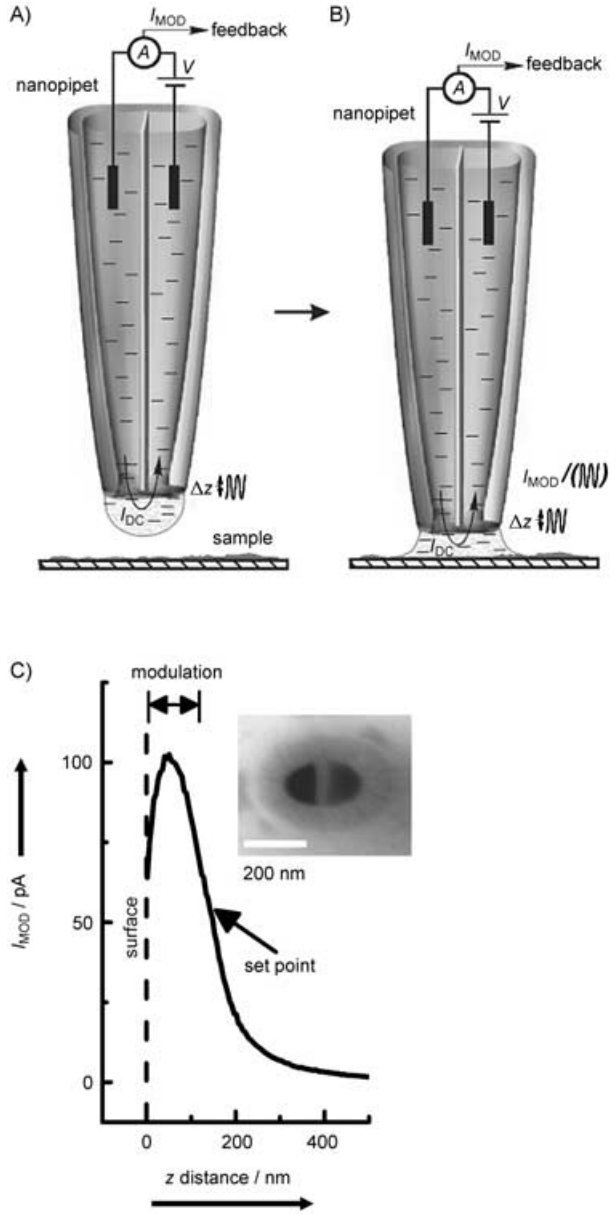

Figure 1. Schematic representation of the apparatus. A) A voltage (V) applied between Ag/AgCl electrodes in the two barrels of a glass-fabricated nanopipette creates an ion current (I_{DC}) through a droplet which forms at the tip. B) When the tip is brought into contact with a surface, modulation in the z direction creates a modulated ion current (I_{MOD}), which provides the feedback signal for control. C) Experimental approach curve for the double-barreled pipette obtained over a PDMS surface. Notice the steep dependence of I_{MOD} on the distance between the tip and the surface. The inset shows an SEM image of a gold-coated pipette tip.

without the need to insert the sample into a bath of conducting solution. As the pipette approaches the surface, a meniscus is formed between the tip and surface (Figure 1 B). I_{DC} is diminished as the tip moves closer to the surface.

To provide more robust distance control, the tip was modulated in the z direction (usually with an amplitude of ± 50 nm), and a modulated ion current (I_{MOD}) was detected with a lock-in amplifier (Model SR 830, Stanford Research Systems).[19] Generally, these pipettes are controlled at 75–125 nm above the sample, as determined by approach curves to the surface. Figure 1 C shows the steep dependence of the modulated ion current on the distance between the tip and the surface. The graph reaches a maximum when the tip just touches the surface at the peak of its modulation. Feedback control of the tip–surface distance is obtained by using a piezoelectric translation stage that adjusts the z position of the sample to maintain a constant value of I_{MOD} (indicated on the graph with 'Set Point'; usually around 50 pA, which corresponds to a tip–surface distance of 100 nm).

To test the feedback control of this system, a sample surface fabricated from polydimethylsiloxane (PDMS) and consisting of ridges of 2 μm in width and 1.2 μm in height was imaged with the double-barreled pipette and tapping-mode AFM in air.[20] Figure 2 shows the correspondence between the images obtained with the two techniques. Notably, the pipette cannot track up steep slopes or tight grooves quite as well as the AFM, and as such the ridges appear wider at their bases. This is understood to be a result of the differences in the size and geometry of the tips and has also been observed with single-barreled pipettes.[21] Nonetheless, the agreement in feature heights and spacing is good; in particular, the pipette successfully characterized the smaller peaks in the grooves. These results indicate that double-barreled pipettes can be reliably controlled over a surface while operating in air and allows not only topographical scanning but also deposition of biomolecules.

A droplet must form at the tip of the pipette to produce an ion current, and as such it is not clear whether a significant residue of the solution is left behind as the tip moves across the surface, or whether the surface tension causes the solution to dewet as the tip moves away. To investigate this, a fluorescent dye (alexa 488) was added to both barrels and excited with 488-nm laser light while the pipette was scanned across a glass surface. No fluorescent trail was observed (with a highly sensitive CCD camera) as the tip moved across the glass, which indicates that dewetting was occurring. To deposit materials on a surface, therefore, we must functionalize the surface to interact with the molecules to be deposited. To achieve this, the glass surface was coated with a positively charged polymer, polyethyleneimine (PEI), and both barrels were loaded with alexa 488-labeled rabbit immunoglobulin G (IgG, which is known to adhere to positively charged surfaces).[22] It was observed that the IgG migrated, due to electroosmotic flow, away from the positive electrode and towards the negative one[17] so that the protein was only seen to come out of one barrel at any time. Alteration in the direction of the applied voltage, however, would switch the barrel in which the protein was seen to exit (Figure 3 A). As expected, the IgG delivered from the pipette adhered to the PEI-modified glass surface and left fluorescent IgG molecules deposited on the surface as the pipette was moved across it.

As the double-barreled pipette operates in air, we expected a finer feature size than that obtained with single-barreled pipettes operating in solution. In solution, molecules will spread through three-dimensional diffusion to the surface or two-dimensional diffusion on the surface and thus broaden the feature size.[17] Previously, in solution we measured a spot

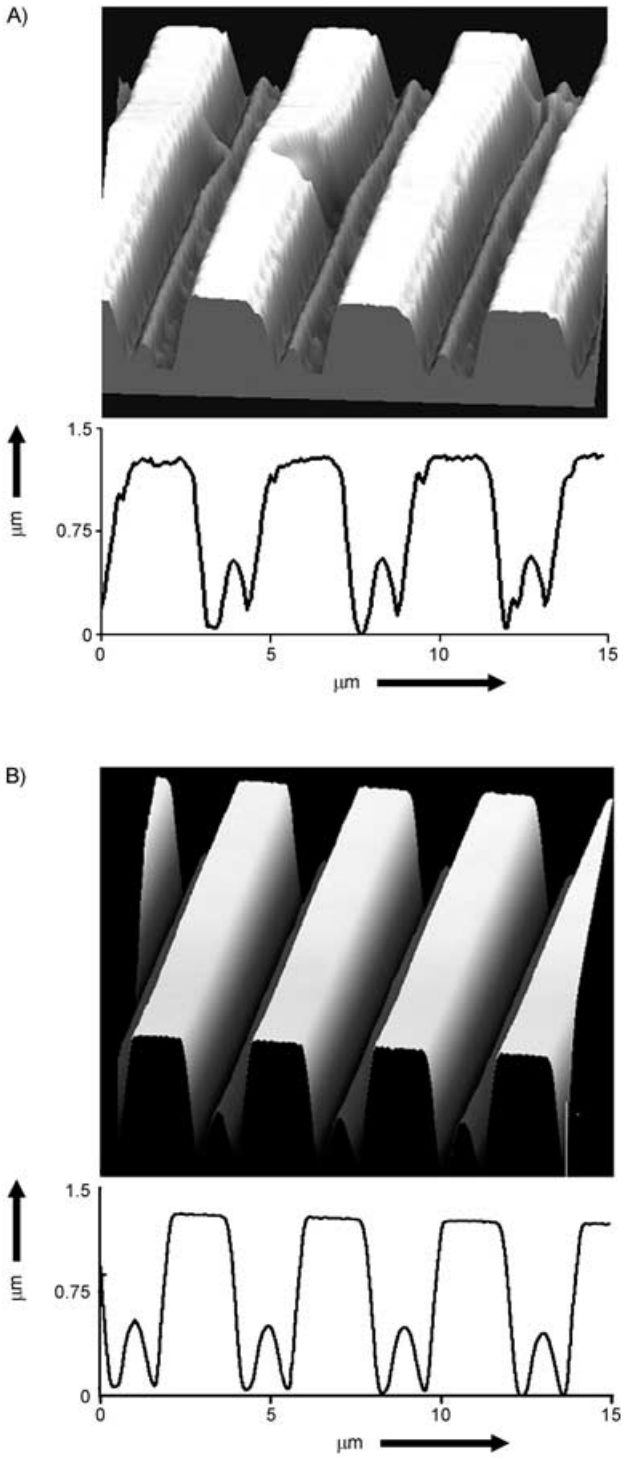

Figure 2. A) 3D image and cross-section of a PDMS stamp that was scanned in air with a double-barreled nanopipette. The image shown is a representation of 10 scans. The defect in the image is a physical defect in the stamp (control was maintained throughout taking this data). B) 3D Image and cross-section of the same stamp obtained with tapping-mode AFM in air.

size (full width at half-maximum, FWHM) of 1.3 μm with single-barreled pipettes and IgG (830 ± 80 nm for biotinylated DNA on a streptavidin-coated glass surface).[16] In these experiments, performed in air, the feature size deposited on

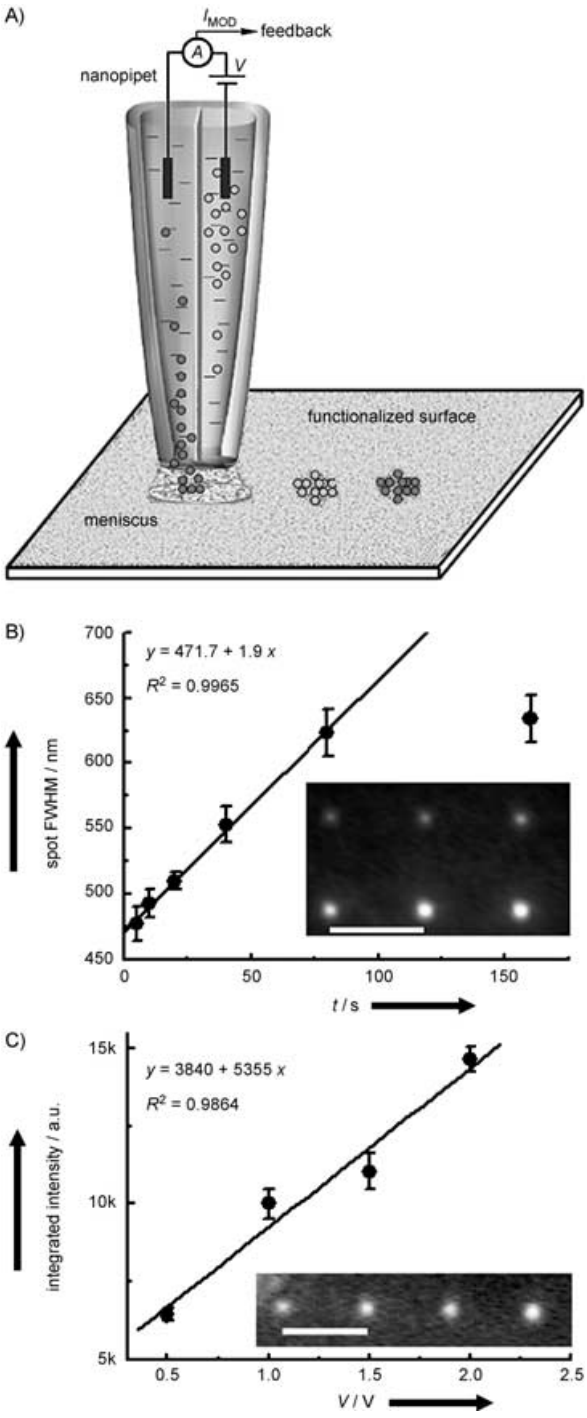

Figure 3. A) Principle of double-barreled pipette deposition. The two barrels of the nanopipette are filled with different fluorophore-labeled antibodies for deposition onto a PEI-coated glass surface. The voltage between the two barrels produces the distance–feedback control current and controls the molecular delivery. Note that antibodies are delivered only from one barrel at a time as they flow to the negative electrode. (B) & (C) show fluorescence images of alexa 488-labeled IgG spotted onto a PEI-coated glass surface. B) Spots at deposition times of 5, 10, 20, 40, 80, and 160 s and 2.0-V tip potential (inset) and linear fit to fwhm diameters of the spots. C) Spots deposited at 0.5-, 1.0-, 1.5-, and 2.0-V tip potentials for 10 s (inset) and linear fit to integrated intensities of the spots. Scale bars in both images are 5 μm. The error bars represent the error in Gaussian fits. Data are representative for an individual pipette; however, fit parameters can vary between pipettes.

the surface was found to depend on both the applied voltage and the deposition time. Dots spotted at a fixed voltage of 2.0 V on the surface with dwell times of 5, 10, 20, 40, 80, and 160 s are shown in the inset in Figure 3B. Gaussian fits of the profiles of these spots yielded detected feature sizes that ranged from 440 to 630 nm and were linearly distributed with the deposition time ($R^2 = 0.9965$, from 5 to 80 s) as shown in Figure 3B.[23]

A major advantage of the SICM-based writing method is the possibility for voltage control of the amount of material that is delivered. Voltage control of IgG delivery was tested through the application of 2.0, 1.5, 1.0, and 0.5 V across the two barrels with a deposition time of 10 s at each spot on the glass surface. At voltages lower than 500 mV, the ion current was too low to maintain control of the pipette and resulted in the pipette crashing into the surface. The inset in Figure 3C shows the fluorescent image from these dots. The integrated intensities of the Gaussian fits of the spot profiles were distributed linearly ($R^2 = 0.9864$, Figure 3C) with the applied voltage and shows that the amount of material being deposited can be controlled by variation of the voltage applied. The data shown in Figure 3B and 3C are representative of individual pipettes; the parameters were obtained by linear fits to these data and can vary between pipettes.[24]

The feature sizes observed here are significantly smaller than those previously obtained with single-barreled pipettes. This is because molecules deposited by a double-barreled pipette operating in air reach the surface through a small liquid meniscus and cannot diffuse laterally beyond its boundary while still in transit. With increased deposition time, the material has a greater opportunity to diffuse throughout the volume of the meniscus and reach the surface. However, as seen in the 160-s point in Figure 3B, the amount of material being deposited and the resultant feature size reaches a natural plateau as the material fills out the limited volume of the meniscus and saturates the surface of its footprint. At constant deposition time, increased voltage is understood to affect feature size by simply speeding up the rate at which molecules are driven towards the surface by electroosmotic flow.

More-complex features can be achieved by using biotinylated, fluorescently labeled DNA deposited onto a streptavidin-coated glass surface. Although we found that DNA gave comparable feature sizes to that of IgG (25 dots with a 5-s deposition time yielded an average spot size of 510 ± 40 nm, individual spot sizes ranged from 350 to 600 nm (data not shown)),[25] graded deposition was far more reliable with the DNA/streptavidin system. For instance, Figure 4A shows an example of a grayscale square of alexa 647-labeled DNA that was produced by writing several concentric squares of different sizes. Although graded depositions have been produced with a complex technique based on microfluidics,[26] to our knowledge the only previous report of this ability with an SPM method is our work with single-barreled pipettes.[16,17]

Furthermore, because double-barreled writing opens up the possibility for deposition of two separate species from the same tip, we explored this advantage by using DNA. The DNA was observed to migrate towards the positive electrode, only coming out of one barrel at a time, therefore allowing

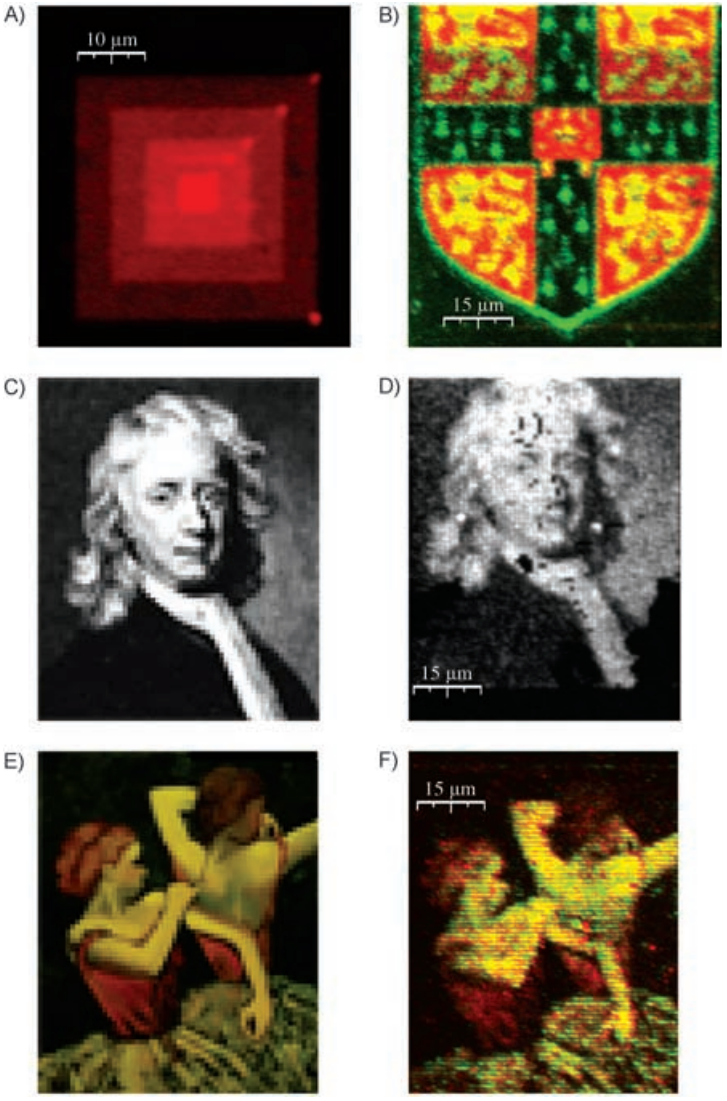

Figure 4. Fluorescence images of DNA deposited onto a streptavidin-coated glass surface. A) Gray-scale deposition of alexa 647-labeled DNA that was created by printing several squares on top of each other. B) Two DNA species, one labeled with alexa 647 and the other with rhodamine green were used to print a two-color image of the University of Cambridge crest. C) An image of Sir Isaac Newton downsized with Adobe Photoshop to 75×62 pixels. D) Double-barreled pipette-printed reproduction of the image shown in (C). The image was written with alexa 647-labeled DNA with 1-μm pixels (printed area is 75×62 μm^2). This image took 27 min to produce. E) An image of the painting "Degas Dancers" by Gina Candelori, downsized with Photoshop to 75×61 pixels and the blue channel removed. F) Double-barreled pipette-printed reproduction of the image in (E) with green and red channels written consecutively in rhodamine green and alexa 647-labeled DNA with 1-μm pixels (printed area is 75×61 μm^2). This image took 35 min to produce.

different DNA species to be driven from each barrel by a change in the sign of the applied voltage. Two different (noncomplementary) sequences of biotinylated DNA, one labeled with the fluorophore alexa 647 and the other with Rhodamine green, were loaded into the barrels and then delivered onto a streptavidin-coated glass surface. Figure 4B shows an example of two-color writing. In this case, the outline of the crest and lions were first drawn in green (Rhodamine green labeled DNA) and the red regions

(alexa 647-labeled DNA) then filled in after switching the sign of the tip potential. The yellow regions were produced where data from the two channels overlapped. The final image was created by measurement of the fluorescence on each channel individually (cross talk between the channels was measured as $<1\%$), scaling the data on a linear grayscale, and by using the two resulting images as 'red' and 'green' channels of an image in Photoshop (ver. 6.0.1, Adobe Systems Inc.) which overlays the two channels (with the third, the 'blue' channel, left blank). As Figure 4 indicates, it is easy to use this system to deliver different species to the same point on a surface. Registry problems are minimized because the tip location is always controlled by the *x* and *y* closed-loop piezos that can then be used to return the pipette to a given location after the voltage has been switched. This provides a significant advantage over our previous work with two-color deposition in which the switching of species was only possible by manually switching the pipettes and carefully placing the new tip in the same location.[17]

The many advantages of the double-barreled writing can be combined with lithography software to produce highly complex grayscale and two-color patterning of surfaces. The original and DNA-on-streptavidin reproduction of an image of Sir Isaac Newton are shown in Figure 4C and D, respectively (see the Experimental Section for details). Although several regions of pixels are missing in the image (particularly in the lower right-hand corner), the general fidelity to the original is quite high and the image is clearly recognizable. The missing pixels are likely due to nonuniform coating of streptavidin on the surface, which results in bare patches where the DNA cannot adsorb. The Degas Dancers, shown in Figure 4F, represents another example of a complex pattern written with biomolecules on the submicron scale (presented in the same way as Figure 4B). The images in Figure 4 demonstrate that the double-barreled pipette can produce complex graded patterns of biomolecules on the submicron scale.

The methods described herein significantly improve upon previously published nanopipette methods. We have shown that double-barreled pipettes, when operated in air, can be used for both topographical imaging and reliable deposition of DNA and protein onto modified surfaces. Through the minimization of lateral diffusion, we observed smaller feature sizes in these deposition experiments than could be obtained with a single-barreled pipette operating in solution. Furthermore, the creation of a potential between the two barrels of the pipette presents the opportunity to fill the barrels with different materials and write in two 'colors' by simply switching the direction of the voltage to change the color of the 'ink'. As noted above, this is particularly beneficial because such in situ switching minimizes the registry errors inherent in attempting to bring a new tip to the same place on the surface. Naturally, many of the advantages described herein could be expanded to systems with more than two barrels—pullers currently exist that are capable of creating tips with as many as seven barrels.[27] Such systems could use separate electrodes for control and delivery which would allow several species to be delivered simultaneously. The potential to deposit a larger number of species with the same pipette also greatly increases the complexity of patterns that can be produced. Furthermore, the possible application to nanoscale biological assays and combinatorial chemistry would allow advancement in bionanotechnology.

Experimental Section

The apparatus consists of an inverted fluorescence microscope in which nanopipettes can be mounted and controlled over the sample stage by using closed-loop piezos.[17] The PDMS stamp for topographical imaging was prepared by replicating a standard photolithography patterned photoresist master with sylgard 184 prepolymers (Dow Corning) through a literature procedure.[20] Surfaces for the IgG writing experiments were prepared by immersing a glass coverslip in an aqueous solution of polythyleneimine (PEI, 2.2 mg mL^{-1}) for approximately 15 min and then washing with deionized water. alexa 488-labeled rabbit IgG and streptavidin-coated glass surfaces were prepared as described previously.[16,17] Fluorophore-labeled DNA was purchased from MWG Biotech AG (Ebersberg, Germany) and the following sequences were used: biotin-5′-AGT CAA GCC ATT GTA GTC CCG CAA CAC ACT CGA GA-3′-alexa 647 and 5′-CTA TGC AGC CAT TGT AGT CC-3′-rhodamine green. IgG or DNA solutions were prepared in phosphate buffer solution (10 mM phosphate, 150 mM NaCl, 2 mM NaN_3, pH 7.2) and routinely delivered from the pipette by using a tip bias of 1 to 2 V.

The images shown in Figure 4C–F were prepared as follows. The original image (not shown) was reduced with Adobe Photoshop to 75 pixels in height and 61 pixels in width, Microcal Origin 7.0 was then used to convert the resulting bitmap into an 8-bit integer ASCII matrix. The relative intensity of each pixel was then scaled to a dwell time between 0 and 1 s over individual pixels. The ASCII data was converted into a lithography file for RHK Lithoedit (RHK Technologies; MI, USA) by using the 'find and replace' function in Microsoft Word to produce the code for 1-μm step sizes between pixels. For the image of Degas Dancers in Figure 4F the blue channel of the original (not shown) was removed with Adobe Photoshop. The red and green channels were processed separately as described above to produce two sets of lithography code that could be run individually to draw first one channel, then the other.

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[24] Spot sizes have been observed to vary by as much as 50% between tips. For instance, deposition of IgG at 2.0 V for 10 s yields spot sizes in the range of 500–750 nm depending on the pipette.
[25] Feature sizes reported herein are true spot sizes, deconvoluted with an optical resolution of 350 nm.
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