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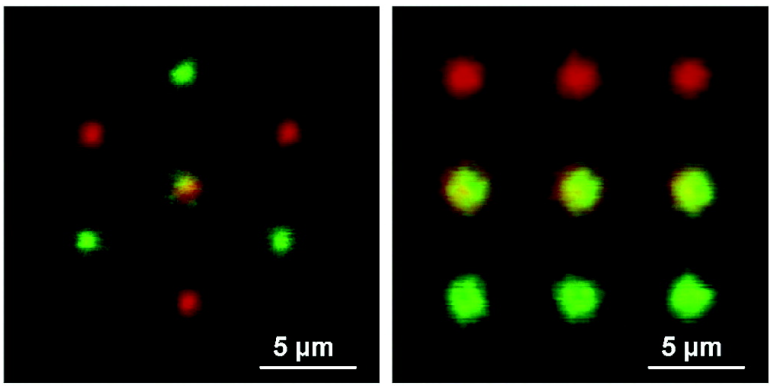

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Multicomponent Submicron Features of Biomolecules Created by Voltage Controlled Deposition from a Nanopipet

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Abstract: We have used a nanopipet as a nanopen to locally and controllably deposit complex biomolecules, including antibodies and DNA, onto a surface to create multicomponent and functional submicron features. Key advantages of this method are that the biomolecules are always in solution and the applied voltage provides fine control of the delivery down to the single molecule level. Two consecutive cycles of deposition, to produce spatially varying features with different biological properties, were demonstrated with fluorescently labeled antibodies or biotin. This approach combines "top-down" fabrication, using the nanopen for local application, and "bottom-up" fabrication, using molecular recognition for self-assembly at defined positions, and opens up new possibilities in nanotechnology.

Introduction

There is currently a great deal of interest in the controlled deposition of biological molecules onto surfaces to exploit biological recognition, to assemble novel structures,[1] to create miniaturized biological arrays, and to perform miniaturized assays.[2−4] For example, DNA and protein microarrays are now widely used to probe gene expression and protein levels in cells. The creation of submicron features potentially offers higher density arrays of molecules or structures, with consequent reduction in the amount of sample required and faster assay time. Therefore, methods that were developed on the micron scale,[5] such as microcontact printing,[6−11] microfluidics,[12,13] and ink-jet delivery,[14] are currently been extended to the submicron level.[9,11] Scanning probe microscopy, which involves the nanomanipulation of a fine probe over a surface, offers much higher resolution deposition down to the nanometer scale. In particular, the atomic force microscope has been utilized for both positive writing of DNA[15] and collagen[16] using so-called dip-pen lithography,[15−21] negative writing using the AFM tip to remove material,[22,23] and nanografting[24,25] to organize proteins on surfaces.

We are developing an alternative delivery system derived from scanning ion-conductance microscopy (SICM).[26] This technique is based on manipulation of a nanopipet over a surface using the ion current that flows between an electrode in the bath and an electrode in the pipet to maintain the sample to surface distance.[27] SICM has been used to image the surface of live cells with high topographic resolution.[28] This method is very reliable, enabling distance control to be maintained over a

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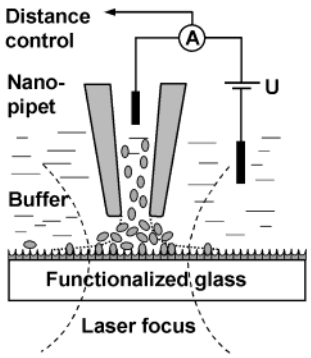

Figure 1. Schematic of the experiment. A voltage (*U*) is applied between two Ag/AgCl electrodes, one inserted into the nanopipet and the other inserted into the bath of buffer solution. The pipet is filled with biomolecules, and the ion current (*A*) is used as a fine control of the molecule delivery as well as for tip−surface distance control. Laser illumination and confocal detection are used to image the deposited features.

contracting heart cell[29] and the imaging of live cells for more than 24 h. The pipet has also been used to apply ions locally and measure their flow into the cell to detect the location of ion channels.[30]

To use the scanning ion-conductance microscope for nanowriting, the pipet is used as a reservoir for molecules[27,31,32] and hence serves as a nanopen. The deposition of molecules on the surface is then controlled by the size and sign of the voltage applied to the pipet.[33] The pipet can be used to deliver complex biomolecules onto a surface under aqueous buffer. Molecules diffuse from the tip of the pipet to the surface so that the feature size is larger than the pipet diameter, as shown in Figure 1. The control of delivery makes it possible to vary the density of biomolecules deposited to an area on the surface. This ability to produce a "gray scale" of the number of biomolecules on the surface has not been demonstrated for any of the scanning probe-based methods to our knowledge. However, control of the number of metal atoms deposited has been demonstrated by electrochemical STM.[34] We have previously shown that we can deliver short DNA oligonucleotides and a small protein onto a surface to create submicron features.[27] In this paper, we describe significant advances in the use of the nanopipet. First, a key issue for any deposition technology is that the biomolecules should be functional once they are delivered. We have approached this by showing that antibodies can be delivered and attached to a surface, while still retaining their recognition capability, and that DNA delivered from the nanopipet and attached to a surface is still capable of specific hybridization. We then go on to demonstrate consecutive deposition of biomolecules at the same point on the surface and hence create spatially varying fluorescent and biological properties. In doing these experiments, we have considerably extended the capability of the nanopen.

Experimental Section

Chemicals and Synthesis. 5-[([*N*-(biotinoyl)amino]hexanoyl)amino]pentylamine trifluoroacetate salt, *N*,*N*-dimethylformamide (DMF), rabbit immunoglobin G (IgG), human IgG, anhydrous pyridine, and other organic solvents were all purchased from Sigma-Aldrich Company. Fluorescently labeled protein and biotin molecules, Alexa Fluor 488-labeled biotin, Alexa Fluor 647-labeled goat anti-rabbit IgG antibody (H+L), and Alexa Fluor 647 *N*-hydroxylsuccinimide ester, as well as an Alexa Fluor 488 protein labeling kit, were all bought from Molecular Probes Europe BV (Leiden, The Netherlands).

The three single-stranded (ss) DNA were purchased from MWG Biotech AG (Ebersberg, Germany) with the following sequences: biotin-5′-AGT CAA GCC ATT GTA GTC CCG CAA CAC ACT CGA GA-3′-Alexa Fluor 647 (Alexa 647 DNA, 35 mer), 5′-TC TCG AGT GTG TTG CGG GAC TAC AAT GGC TTG-3′-Alexa Fluor 488 (Alexa 488 DNA, 32 mer), and biotin-5′-AGC TAA GGC AGT CAG TGG GA-3′ (blocking DNA, 20 mer). The first two fluorescently labeled DNA sequences are complementary to each other for the whole sequence of the Alexa 488 DNA. All working solutions were prepared with PBS buffer (10 mM phosphate, 150 mM NaCl, 2 mM NaN_3, pH 7.20) unless otherwise stated.

Preparation of Alexa Fluor 488-Labeled Rabbit IgG. The protocol in the Alexa Fluor 488 protein labeling kit provided by Molecular Probes was employed to label the rabbit IgG (see Supporting Information). The average labeling, determined by measuring the UV absorbance at 280 and 494 nm, was 1.5 Alexa 488 fluorophores per IgG molecule.

Preparation of Biotin Labeled with Alexa Fluor 647 Dye. To a vial of 1 mg of Alexa Fluor 647 *N*-hydroxylsuccinimide ester was added 400 μL of anhydrous DMF to fully dissolve the dye, and then 2.2 mg of 5-[([*N*-(biotinoyl)amino]hexanoyl)amino]pentylamine trifluoroacetate salt in 110 μL of anhydrous DMF was added, followed by 50 μL of anhydrous pyridine. The resulting reaction mixture was capped and stirred at room temperature for 18 h under a N_2 atmosphere. The solvent was then removed under reduced pressure, and the residue was dissolved in methanol. Purification was achieved by HPLC on a reverse phase analytical C_{18} column (XTerra Columns, Waters Corp., MA) by varying the organic component of the eluting solution between 5% and 95% acetonitrile (with 0.1% trifluoroacetic acid) in MilliQ water. The required compound was obtained as a dark solid, which formed a deep blue solution when dissolved in PBS buffer. No further spectroscopic characterization of the product was performed. However, as it showed strong binding ability to streptavidin, and exhibited a strong fluorescence upon excitation by red laser (633 nm), we concluded that the labeling was successful.

Glass Surfaces. Glass-bottomed dishes (WillCo, Wells B.V., The Netherlands) were coated with streptavidin by BioTeZ Berlin-Buch GmbH, Germany. The biotin binding capacity of these coatings was 280 fmol/mm^2 (68 pg/mm^2). Aminosilanated surfaces (positively charged at pH 7.2) and biotinynated and streptavidin-modified glass coverslips were prepared following literature procedures[35] (see Supporting Information). Rabbit IgG-coated surfaces were prepared by incubation of positively charged aminosilanated surfaces with a 0.1 mg/mL solution of rabbit IgG in PBS buffer for 30 min, followed by a thorough rinse with PBS buffer.

Nanopipets. Nanopipets were routinely fabricated from glass capillaries (borosilicate glass, inner diameter 0.58 mm, outer diameter 1.0 mm, Intracell, UK) using a laser-based pipet puller (P-2000, Sutter Instrument, CA). See Supporting Information for pulling parameters. Our 3 cm long pipets have a tapered region of about 5 mm length and a tip opening with 90−130 nm inner diameter and 240−280 nm outer diameter, as measured by scanning electron microscopy. For pipets with similar cone angles, the pipet resistance provides an in situ measurement of the inner pipet diameter (100 MΩ in 0.1 M NaCl). Pipets were backfilled with about 8 μL of solution of the fluorophore-labeled

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biomolecules at a concentration of 10 or 100 nM in PBS buffer using a microfiller (MF34G, World precision instruments, FL). An AgCl-coated Ag wire was inserted into the pipet to form an Ag/AgCl electrode.

Writing. Writing experiments were performed using an inverted microscope (Eclipse TE 200, Nikon) with an epi-fluorescence filter block holder. Two piezoelectric xyz-stages, one to move the pipet (Tritor 38, Piezosystem Jena, Germany) and one to scan the sample (P-517.3CL, modified for 20 μm z-resolution, Physik Instrumente, Germany), were attached to the microscope. A voltage in the range from -1 to 1 V was applied across the Ag/AgCl electrodes in the bath and the pipet, and the resulting ion current was amplified by a home-built preamplifier with 10^8 gain. The tip was modulated ± 50 nm at a frequency of 170 Hz, and the modulated ion current was recorded by a lockin amplifier (SR 830 Stanford Research Systems, CA). This signal was sent to a SPM controller (East Coast Scientific, Cambridge, UK or SPM 1000 RHK technology, MI) that produced the distance control feedback signal that was applied to the sample stage. From the measured approach curves, it was possible to show that the pipet-to-surface distance was controlled at about 120 nm during the deposition.[27] The deposition time was typically 10 s; see Supporting Information for further details. When two cycles of deposition with different molecules were performed, two different pipets were used and aligned relative to the laser focus with a precision of about 250 nm. Changes in the surface position during tip exchange could be measured by scanning a previously written feature.

Imaging. Images were recorded under 5 μW laser illumination at 488 nm (model 5400, Ion Laser Technology, Salt Lake City, UT) or 632.8 nm (LHP 925, Melles Griot, CA) on the same instrument used for writing. The fluorescence emission spectra of Alexa Fluor 488 and Alexa Fluor 647 are centered at 519 and 666 nm, respectively. The fluorescence signal was detected using an oil-immersion objective (Nikon Plan 100 DIC, 100×, NA = 1.25) and a photomultiplier tube (Photon Technology Surbiton, UK) or a photon counting avalanche photodiode (SPCM AQR-13, Perkin-Elmer) together with a multichannel scalar card (MCS pci, Ortec, TN). For the experiment with 488 nm excitation, we used a dichroic beam splitter (Omega Optical 505 DRLP), a long-pass filter (BA520, Nikon, Japan), and a 75 μm pinhole. For the two-color blue/red excitation experiments, we used a beam splitter and filter (FITC/Cy5, AHF Analyzentechnik AG, Germany) to separate the fluorescence signal, and a 100 μm pinhole to reject background signal and laser scattering. The emission filter blocks both laser wavelengths and lets the blue and red fluorescence pass, so that no filter change is necessary in our one detector setup. Emission from the red fluorophore under blue excitation was negligible. The optical resolution of the instrument and the alignment of the laser beams for the two-color experiment were determined by imaging 100 nm fluorescent microspheres (Tetraspeck, Molecular Probes, NL). The displacement of the piezo stage was calibrated using a hexagonal pattern formed by 2.9 μm beads and a micrometer scale bar (Pyser-SGI, UK). The typical integration time was 0.5 ms/pixel with image sizes of 512 × 512 or 256 × 256 pixels.

Results

The use of antibodies in numerous analytical techniques makes them a very important protein for surface immobilization studies. Their relatively large size (IgG has a MW of 145 000 and is 14.5 nm × 8.5 nm × 4.0 nm)[36] distinguishes them from protein G (MW 20 000), the only protein we had previously delivered using the nanopipet.

We found that we could deliver the goat anti-rabbit IgG antibody from the pipet by controlling the applied voltage. The flow of molecules from the pipet started at -0.5 V and increased linearly with applied voltage between -1.0 and -1.5 V (see Supporting Information, Figure S1).

We performed experiments to determine if antibodies and DNA deposited on the surface from the nanopipet maintained their functionality. First, we performed an experiment to compare the specificity of the anti-rabbit IgG delivered by the pipet for rabbit IgG against human IgG. A 2 × 2 array of Alexa Fluor 488-labeled rabbit IgG (green fluorescence) was deposited onto a positively charged glass surface. The surface was then treated with human IgG (0.1 mg/mL in PBS for 15 min) to cover the remaining area. Afterward, 100 nM Alexa Fluor 647-labeled goat anti-rabbit IgG (red fluorescence) was added for 30 min. The surface was washed with PBS buffer after each incubation step to remove excess IgG. Figure 2A and 2B shows the green fluorescent dots of rabbit IgG under blue laser excitation and the red fluorescence of the anti-rabbit IgG under red excitation. The cross talk between both channels is negligible. The anti-rabbit IgG has a higher affinity to the rabbit IgG than to the surrounding human IgG. This confirms that the deposited rabbit IgG maintains its biological function, although some background from anti-rabbit IgG adsorbed nonspecifically to human IgG is observed. This background signal is about 30% of the signal intensity of IgG bound specifically to anti-IgG, a comparable level to that observed by others.[7] In an immunoassay, one would use a different protein like BSA (bovine serum albumin) to block the surface and prevent unspecific adsorption. In a different experiment (data not shown), we found no adsorption of the anti-rabbit IgG delivered by the pipet onto a surface homogeneously covered with BSA and therefore would expect a much higher signal-to-background ratio when blocking the area between the spots with BSA.

We also performed a DNA hybridization experiment. Spots of Alexa 647-labeled biotinylated ssDNA (Alexa 647 DNA, 35 mer) were deposited from the nanopipet onto a streptavidin-coated glass surface as previously reported. The remainder of the surface was then coated with a different biotinylated ssDNA (blocking DNA, 20 mer). Finally, the coated surface was incubated under a solution of an oligonucleotide (Alexa 488 DNA, 32 mer) complementary in sequence to the initial ssDNA which had been delivered from the nanopipet. This complementary oligonucleotide was fluorescently labeled with Alexa 488. After incubation for 10 min, the surface was washed with PBS and imaged. Using red and blue laser excitation, we selectively excited the two different fluorophores (Figure 2D and 2E). It is seen that this second oligonucleotide hybridizes selectively to the initial oligonucleotide rather than the massively more abundant DNA used to fill the remainder of the surface.

To demonstrate that the nanopipet can deliver two molecular species to the same spot on a surface, four spots of Alexa 488-labeled biotin were deposited onto a streptavidin surface. A different pipet was then used to deliver four spots of Alexa 647-labeled biotin onto the same surface, with the center spot common to both depositions. Figure 3A and 3B shows images taken separately under blue and red laser illumination. A superposition of both images, where pixels that have intensities in both the red and the green channel are displayed in yellow, is shown in Figure 3C. This experiment demonstrates that small molecules can be delivered from the nanopipet and that they can be deposited and detected close together or colocalized. Figure 3D−F shows a similar experiment using IgG and anti-

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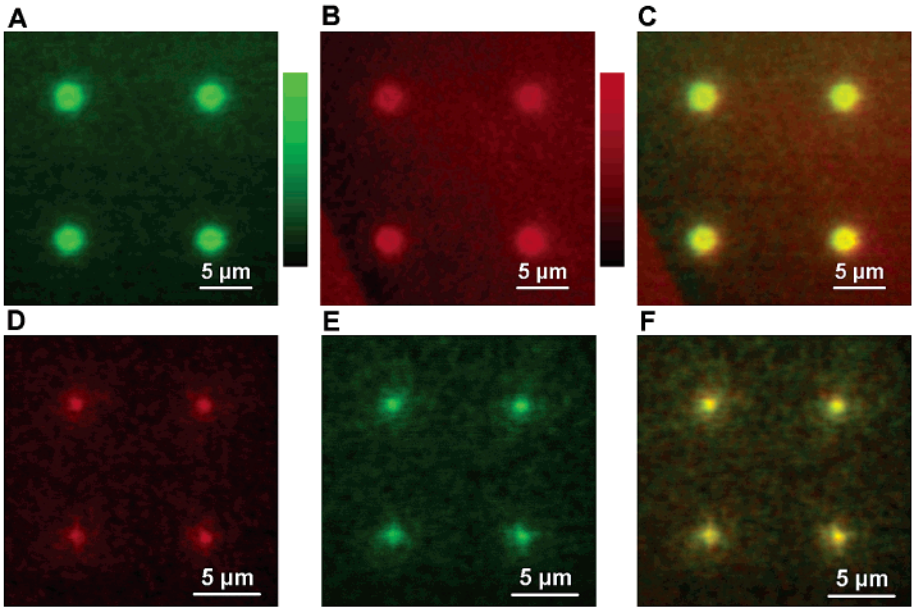

Figure 2. (Upper row) Spots of rabbit IgG (labeled with Alexa Fluor 488) delivered to a positively charged glass surface which was then treated with human IgG (unlabeled) and goat anti-rabbit IgG (labeled with Alexa Fluor 647): (A) blue excitation, (B) red excitation, (C) combined image. The anti-rabbit IgG has a higher affinity to the rabbit IgG than to the surrounding human IgG, showing that it is still functional. (Lower row) Spots of ssDNA labeled with biotin and Alexa Fluor 647 (Alexa 647 DNA, 35 mer) delivered by the nanopipet onto a streptavidin surface. The surface was filled with biotinylated DNA of a different sequence. DNA complementary to the spotted DNA and labeled with a different fluorophore (Alexa 488 DNA, 32 mer) was added in solution. Fluorescence images after washing with PBS buffer are shown: (D) red excitation, (E) blue excitation, (F) combined image. The second oligonucleotide hybridizes selectively to the initial spotted oligonucleotide.

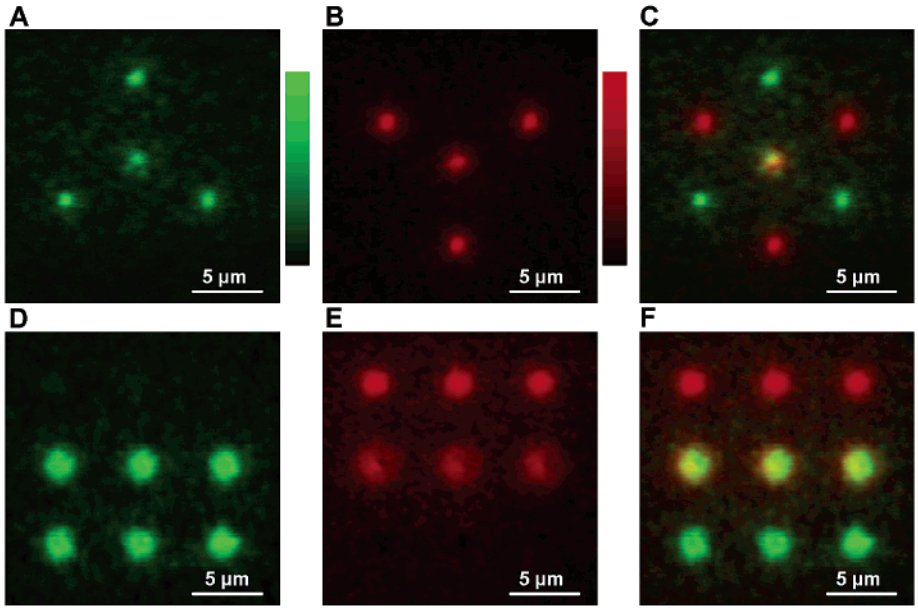

Figure 3. Fluorescence images. (Upper row) Consecutive deposition of Alexa 488-labeled biotin and Alexa 647-labeled biotin onto a streptavidin-coated glass surface: (A) image under blue excitation, (B) red excitation, (C) combined image. (Lower row) Consecutive delivery of Alexa 488-labeled IgG and Alexa 647-labeled anti-IgG: (D) blue excitation, (E) red excitation, (F) combined image.

IgG. First, Alexa Fluor 488-labeled rabbit IgG was spotted onto a positively charged glass surface to form the two lower rows, and then Alexa Fluor 647-labeled goat anti-rabbit IgG was spotted in the two upper rows. The middle row shows both IgG and anti-IgG bound to the surface or to each other. Both of these experiments show how the nanopipet can be used to colocalize mixtures of compounds on a submicron scale.

Because one of the potential advantages of the nanopipet is the ability to deliver individual molecules, we attempted to deliver a very low flux of anti-rabbit IgG onto a rabbit IgG surface using a constant applied potential. Each feature in Figure 4A is of the size of the laser focus (800 nm in this image), and the fluorescence intensity is consistent with it being an individual molecule (the anti-IgG molecules are on average labeled with 6 fluorophores and therefore do not show single-step photobleaching which would be one proof of single molecules). The center spot is 3 times as intense and therefore probably corresponds to three molecules. Despite trying to control the position of the molecules in the center of the image, the deposition appears quite random. Averaging over all 36 spots in this image gave a mean distance of 5.5 μm from the spot in the center.

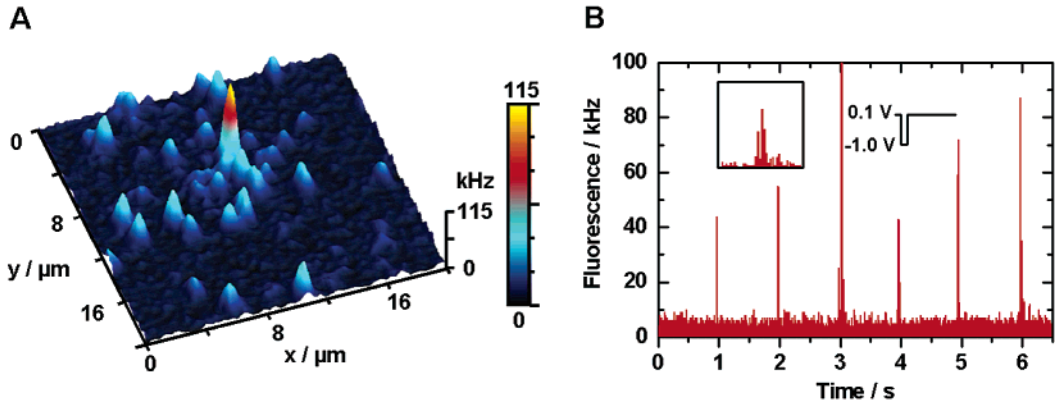

Figure 4. (A) Fluorescence image of individual anti-IgG molecules on an IgG-coated glass surface deposited with constant applied potential. (B) Pulsed delivery of fluorescently labeled rabbit anti-IgG from the nanopipet into solution. The fluorescence intensity under application of negative voltage pulses is shown. Right inset shows one voltage pulse; left inset shows one burst with 1 ms time resolution.

Figure 4B shows bursts of single or a few molecules of fluorescently labeled anti-rabbit IgG exiting the pipet on application of a negative voltage pulse. The fluorescence intensity of one molecule is about 40 counts ms^{-1}. The burst pattern (see inset) shows several bursts of 40 counts believed to be individual molecules. The diffusion time through the confocal volume is shorter that the time resolution of the experiment of 1 ms, so that two neighboring bursts of 40 counts were counted as two molecules. Based on this analysis, there were on average 3 molecules per pulse.

Discussion

In this work, we have shown that we can controllably deposit biotin and antibodies from the nanopipet. Together with our previously demonstrated deposition of DNA and protein G, this emphasizes the generality of this method. We have also demonstrated that these complex biomolecules are functional on the surface once deposited. This is a major advantage of this method of deposition because the molecules are always in solution and hence hydrated so denaturation does not appear to occur.

Delivery of fluorescently labeled biotin, IgG, and anti-IgG required a negative potential in the bath relative to the pipet. Electro-osmotic flow is caused by the mobile adlayer on the capillary wall moving in the electric field and dragging along the fluid inside the capillary.[37] Because the silica walls of the pipet are negatively charged and therefore the mobile adlayer is positively charged, the electro-osmotic flow is out of the pipet on application of a negative potential to the electrode in the bath. Modification of the molecular motion due to electrophoresis would be expected to be a smaller effect for these molecules, as seen in capillary electrophoresis, so that the overall flow is dominated by electro-osmosis. In contrast, the DNA requires a positive potential in the bath to exit the pipet. Electro-osmosis will be into the pipet in this case, so the overall flow cannot be dominated by electro-osmosis. We believe the flow is due to the highly negatively charged phosphate-ribose backbone of DNA which causes a much stronger electrophoretic effect. Furthermore, additional forces due to induced dipoles, as a result of the high electric field and field gradient in the tip, also known as dielectrophoresis, might also contribute to DNA delivery from the pipet.[38]

Delivery by a nanopipet involves subjecting the sample to a high electric field. It can be estimated that the electric field in the tip region on application of 500 mV is approximately 5000 V cm^{-1}.[33] This is higher than the field commonly used in capillary electrophoresis, typically 100 V cm^{-1}, although in the case of the nanopipet the high electric field only occurs over a length of a few micrometers. Despite this high field, the very small current, in the order of 10 nA, means that there is very little heating, no more than 1 K (see Supporting Information). The binding experiments with the anti-IgG and the hybridization experiments with DNA show that these conditions do not affect the deposited biomolecules and therefore functionality is maintained. This is an essential requirement for making diagnostic arrays or creating structures using nanopipet-deposited biomolecules as building blocks.

Once the molecules leave the tip of the nanopipet, there are two contributions to their motion toward the surface: the directed electro-osmotic flow from the pipet and diffusion in solution. Diffusion will broaden the features formed on the surface, as will any lateral 2D diffusion the molecules undergo once nonspecifically adsorbed onto the surface. Our simple modeling, based on a steady-state concentration profile,[39] predicts for a pipet of 100 nm diameter held 120 nm from the surface a bell-shaped intensity profile of the spot with a full width at half-maximum (fwhm) of about 450 nm, assuming no lateral diffusion on the surface. Convoluted with the optical resolution of 500 nm, we would expect the measured feature size to be 670 nm. For biotin and ssDNA,[27] we measured spot sizes (fwhm) in the range from 830 and 860 nm which is a little larger than predicted by simple diffusion out of the pipet, suggesting that there is possibly some lateral diffusion on the surface once the molecule adsorbs. In contrast to the above experiments where attachment was via a specific interaction, a significantly larger spot size (fwhm) of about 2 μm was observed for IgG deposition on charged glass where the interaction is nonspecific, suggesting that lateral diffusion is easier in this case.

It seems likely that there is also significant lateral interaction between the adsorbed molecules, which will lead to molecules binding at adjacent sites on the surface. Initial experiments to

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deposit single IgG molecules also support this picture, suggesting molecules can diffuse once on the surface for this particular interaction and are not stabilized by lateral interactions at this low coverage. Thus, for single molecule experiments, surfaces with high densities of binding sites or reactions with fast on rates may be needed to prevent surface diffusion. While smaller feature size will be desirable for some applications, our current resolution of about 800 nm is well-suited for optical read-out because the limit for optical resolution in the visible is about 250 nm.

We have demonstrated that it is possible to use consecutive delivery of both small molecules and large antibodies to the surface to produce a surface with spatially varying properties, in this case fluorescence. This "colored" surface is distinct from the effectively single tone surface features produced by AFM-based methods, or a gray scale using a single pipet. Extending the properties used to produce these "colored" surfaces, it might be possible in the future to set up combinations of molecules with varying binding, surface, or enzymatic properties.

In summary, we have demonstrated that delivery from a nanopen is a general and powerful method for depositing a controlled number of functional biomolecules at defined positions on surfaces with submicron resolution. We have shown that it is possible to produce surfaces with spatially varying properties by consecutive delivery of molecules to the same point on the surface. This opens up the possibility of producing surfaces with high molecular diversity and spatially varying properties by multiple cycles of deposition. It also becomes possible to combine in one method the two strategies used for nanotechnology: "top-down" fabrication, from the nanopen, and "bottom-up" fabrication, using specific interactions between molecules.[1] Local application of molecules to the surface so they can self-assemble at defined positions will produce a new and versatile variant of nanofabrication.

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Supporting Information Available: Additional experimental details, figure of IgG delivery, and discussion of electrical heating inside the pipet (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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