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Microcontact Printing of DNA Molecules

Sebastian A. Lange,[†,‡] Vladimir Benes,[‡] Dieter P. Kern,[§] J. K. Heinrich Hörber,[||] and André Bernard*[,†]

Indigon GmbH, Sindelfingerstrasse 3, 72070 Tübingen, Germany, European Molecular Biology Laboratory, Meyerhofstrasse 1, 69117 Heidelberg, Germany, University of Tübingen, Auf der Morgenstelle 10, 72076 Tübingen, Germany, and Department of Physiology, Wayne State University School of Medicine, 540 East Canfield Avenue, Detroit, Michigan 48201

The controlled placement of DNA molecules onto solid surfaces is the first step in the fabrication of DNA arrays. The sequential deposition of tiny drops containing the probe DNA fragments using arrays of spotting needles or ink jet nozzles has become a standard. However, a caveat of liquid spotting is the drying of the deposited drop because this creates the typical inhomogeneities, i.e., rims around the spot. Another drawback is that each DNA array is an original and has to be fabricated individually. Microcontact printing is a versatile technique to place proteins onto different target surfaces in uniformly patterned monolayers with high lateral resolution. Here, we show for the first time that DNA can also be printed with equally high resolution in the submicrometer range using an elastomeric stamp with chemically tailored surface. Two regimes for the transfer of the molecules were observed. Finally, microcontact printing of an array of DNA probes onto a solid support and its use in a subsequent hybridization assay was demonstrated.

Emerging microarray technology allows the expression of thousands of genes to be studied simultaneously. This has become possible by attaching DNA molecules (probes) to the surface of a microscope slide arranged in an array format. These arrays are applied broadly,[1−3] in particular in gene expression profiling,[4−7] single-nucleotide polymorphism detection,[8] and sequencing.[9]

A common way to fabricate DNA arrays[10] is to spot fluids containing the desired DNA fragment onto a microscope slide using metal pins[11] or microactuated nozzles.[12] A technologically more demanding way has been demonstrated by in situ synthesis of oligonucleotides (up to 25 bases) using light-activated chemistry combined with photolithographic techniques.[13,14] A major drawback of both production techniques is the inherent sequential nature of the process. Either one spot of oligonucleotides is deposited after another or one base is coupled to the previous one, with the consequence that each array is written de novo as an original. Thus, the speed of fabricating an entire DNA array is quite low (on the order of 30 min per array containing 10 000 features).

Analogously to conventional book printing, the standard techniques of DNA array production described above correspond to each letter being written individually, one after another. Hence, the idea to adapt concepts from the printing industry is obvious and calls for a "color printing drum" of DNA. A page of a book is printed in one step, so why should this not work for a complete array?

Microcontact printing (μCP) has been demonstrated as a technique for the parallel delivery of proteins as surface patterns onto a target substrate.[15,16] A stamp made of an elastomeric material such as poly(dimethylsiloxane) (PDMS) can be topographically structured by casting the prepolymer against a 3D master.[17,18] The stamp is inked with the molecules of interest, forming a more or less complete monolayer, rinsed with buffer, blown dry under a stream of nitrogen, and then printed onto the substrate surface.

Here we show that these concepts also apply to printing of DNA. Similar to printing proteins, μCP of DNA calls for carefully

* To whom correspondence should be addressed: (e-mail) a.bernard@indigon.de.

† Indigon GmbH.

‡ European Molecular Biology Laboratory.

§ University of Tübingen.

|| Wayne State University School of Medicine.

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tailored surface properties of the PDMS stamps. The surfaces have to be attractive enough to bind DNA molecules reversibly from solution. However, the binding forces must not be so strong that the release of the DNA is hindered when the stamp is removed from the target surface. Proteins, which are charged depending on their isoelectric point and on the pH in the surrounding medium (amphoteric), adsorb to uncharged surfaces such as PDMS mainly through hydrophobic interactions, which act over a very short subnanometer distance,[19−21] and form monolayers. This layer formation is in principle governed by a self-limiting deposition process, where the growing number of adsorbed proteins renders the surface hydrophilic and thus inhibits further adsorption of additional protein.

DNA on the other hand, owing to its phosphate backbone, is a highly negatively charged polymer or polyelectrolyte. Hence, electrostatic interactions play the major role for adsorption and transfer properties. Electrostatic forces, with their extended reach of a few nanometers, should facilitate a multilayered adsorption of DNA molecules to a point where the molecules' total charge compensates that of the surface. The impact of the surface charge on the adsorption has already been reported for adsorption kinetics of DNA fragments from solution to rigid substrates.[22,23] Thus, it becomes obvious that careful control of the surface properties, i.e., especially the charge density, was of great importance for the experiments performed.

EXPERIMENTAL SECTION

Fabrication of Stamps. PDMS stamps were fabricated by curing Sylgard 184 (Dow Corning, Midland, MI) on fluorinated silicon masters having etched 600-nm-deep features on their surface. For the generation of flat, unstructured stamps, PDMS was cured against polystyrene Petri dishes.

Surface Modification of Stamps. PDMS surfaces were treated in an oxygen plasma (pressure 0.8 Torr, load coil power 100 W; TePla) for 3 s. The hydrophilic stamps[24] were immediately immersed in an aqueous solution of 3% (aminopropyl)triethoxysilane (Fluka) adjusted to pH ∼6 with acetic acid. The solution was heated for 1 h to 80 °C under reflux conditions. Freshly silanized PDMS was exhaustively rinsed with deionized (DI) water. Silanized substrates can be stored several weeks if kept immersed in water.

Surface Chemistry of Substrates. Slide modifications were done as published.[25] Briefly, clean microscope glass slides (Menzel Gläser) were soaked overnight in 10% NaOH, washed with DI water, immersed in 10% HCl, and rinsed again with DI water. Subsequently, they were washed with methanol and immersed in (aminopropyl)trimethoxysilane solution (Fluka, 3% in methanol) for 15 min under sonication. The silanized slides were washed with methanol and then DI water, blown dry under a stream of nitrogen, and baked at 110 °C for 15 min. Such prepared slides were stored under dry and dust-free conditions and could be kept for several weeks.

Stamping Process. DNA samples were diluted 1:10 or 1:100 from stock (100 pmol/μL for oligonucleotides) in DI water (carbonated, pH 5.2) or any other nonamine-containing buffer with pH 5−7. Aminated stamps were then incubated with 10−40 μL of sample solution for 45 min, rinsed thoroughly with DI water, and then blown dry with nitrogen. The stamp was printed onto the substrate surface and left in place for an average contact time of 15 s.

Primers and DNA Probes. DNA oligonucleotides were purchased from various vendors (MWG Biotech, ROTH, and IBA GmbH, Göttingen, Germany). They all had lengths of between 18 and 25 bp and were HPLC purified and modified by the manufacturer, where applicable. Oligos used for coupling to the surface were labeled with a 5′-amino C6 linker. Fluorescent probes were internally either fluorescein isothiocyanate (FITC) or tetramethylrhodamine (TRITC) labeled. Probes used for immunolabeling were 5′-biotin labeled. To obtain DNA fragments of varying length, different sets of primers were defined and used in PCR reactions. PCR was performed according to standard procedures. It included 40 cycles of denaturing, annealing, and extension. The temperatures were calculated using the GC rule. The final products were purified with a QIAquick PCR purification kit (Qiagen). The desired fragment length was confirmed by gel electrophoresis.

Hybridization on Substrate Surfaces. Hybridization of probe DNA to the target DNA (immobilized DNA) was performed according to common protocols used in microarray technology.[26] Stamped targets were cross-linked to the glass surface with UV using a UV cross-linker (Stratalink) set to 650 mJ. Alternatively, target DNA, if aminated, was cross-linked using bis(sulfoxisuccinimidyl) subberate (BS3, Pierce). Slides were then postprocessed in a solution of succinic anhydride in methylpyrrolidinone and borate buffer to passivate the charged surface. They were prehybridized in a solution of 6× SSC (saline sodium citrate containing 1% bovine serum albumin, BSA, and 0.5% sodium dodecyl sulfate, SDS) and finally rinsed in DI water. Hybridization probes together with salmon sperm DNA were dissolved in hybridization buffer (5× SSC, 0.1% SDS, 1× Denhardts solution, and 50% dimethylformamide), denatured at 95 °C for 2 min, spread onto the slide, and covered with a coverslip to achieve a uniform hybridization reaction. Subsequently, the sealed slides were put in a hybridization oven for 12 h at 42−48 °C. After hybridization, slides were stringently washed in SSC buffers of decreasing ionic strength.

Atomic Force Microscopy (AFM). TheAFM scans were performed in air using a noncontact mode on a Dimension 3000/Nanoscope 3a (DI, Santa Barbara, CA) with silicon cantilevers (Ultrasharp Tips, Nanoprobes). Freshly cleaved mica was used as substrate for AFM. To ensure equivalent surface conditions as with amine-modified glass, the mica was also amino-modified using the same procedure as described above for substrates.

Microscopy. Fluorescent images were acquired with a microscope (Zeiss Axiovert 200 with 40× LD Achroplan objective), equipped with a charge-coupled (CCD) camera cooled to 0 °C

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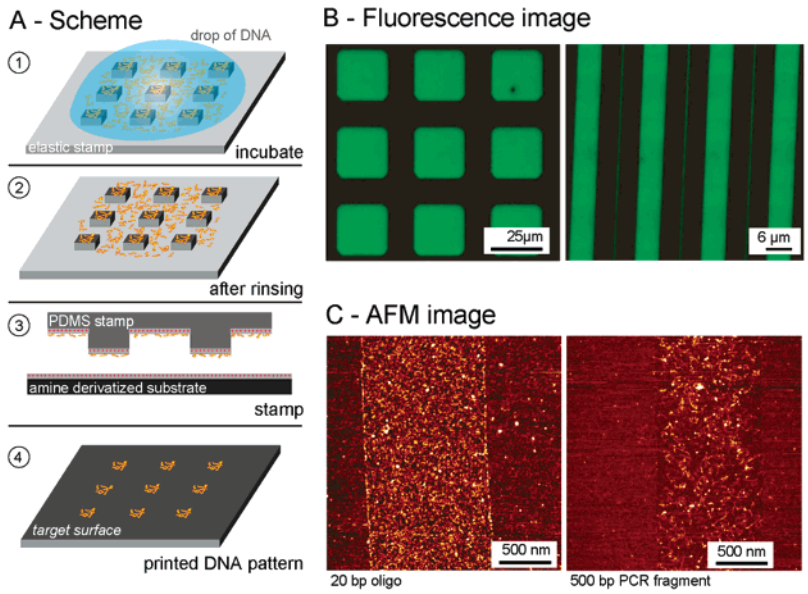

Figure 1. Microcontact printing of DNA molecules. (A) Scheme of DNA printing. The surface of the elastomeric material (PDMS) was modified such that it exposed positive charges on its surface. The stamp was incubated with target DNA molecules in a solution of low pH. The stamp was then rinsed, blown dry, and printed to deliver the DNA to the target surface. (B) Fluorescence images of patterned FITC-labeled oligonucleotides on a glass surface after printing. The pattern size is limited only by the ability to manufacture molds with the desired feature size. (C) AFM images revealing the printed DNA molecules deposited as patterns on mica substrates. AFM images (tapping mode in air) of stamped 1-μm lines of oligonucleotides (left, 20-bp oligos; right, 500-bp PCR fragments).

(Hamamatsu), captured, and analyzed using the SIMPLE PCI software package for this camera. Red- and green-labeled DNA were tagged by TRITC (λ_{ex}= 552 nm) and FITC (λ_{ex}= 520 nm), respectively. Dark-field images were acquired using a microscope (Nikon Eclipse) equipped with long-distance dark-field lenses. Images were captured using the same CCD camera.

Array Spotting and Stamping. Stamps, as well as microarray glass slides (Corning, GAPS slides), were spotted using a microarray spotter (sciFLEXXARRAYER, Scienion AG) equipped with one piezonozzle. PCR products were taken from the Amersham ScoreCard kit and diluted 1:10 in MES buffer (pH 7) to a concentration of 20 ng/μL, which amounts to roughly 0.075 pmol/μL. To concentrate enough DNA onto the surface of the stamp, an average of 30 single drops of 100 pL was deposited by the piezoneedle at every site. After spotting onto the stamps, they were printed onto a conventional microarray glass slide. Stamped slides and the directly spotted slides (control) were treated identically. The spotted probes were hybridized with the spike mix kit (Amersham) according to the manufacturer's protocols. The readout of the hybridized slides was done with a microarray scanner (GenePix 4000B, Axon Instruments) in two-color mode for Cy3 and Cy5.

Microfluidic Inking of Stamps. Microfluidic networks were used to deliver different probes of DNA onto the stamp surface as described elsewhere.[27] To facilitate the filling of the channels, DNA probes were diluted 1:100 in a 1:1 solution of ethanol and DI water.

Silver and Gold Enhancement. After blocking the slide surface with a BSA solution (1% in PBS) to prevent unspecific reactions, biotinylated oligonucleotides were incubated with an anti-biotin antibody conjugated to 5-nm colloidal gold particles (British BioCell). A standard silver enhancement solution (Sigma, SE−100) was used as recommended by the supplier to grow elemental silver particles. Alternatively, a gold enhancement kit (Nanoprobes, GoldEnhanceLM) was applied accordingly.

RESULTS AND DISCUSSION

In microcontact printing, the transfer of DNA from the stamp to the substrate surface takes place during a brief (a few seconds) contact between the two surfaces (Figure 1A). Fluorescence labels were used to follow the fate of DNA molecules after being adsorbed to the stamp and to visualize the printed and patterned DNA layer on a glass substrate. A precise and clear pattern was generated that lacks the typical inhomogeneities common to spotted arrays.[28] Feature sizes down to 1 μm were achieved easily (Figure 1B). To ensure tight binding of DNA on the stamp during rinsing, the adsorption and rinsing buffers must have a pH of between 5 and 9. If the pH rises above the pK_a of the surface amines at 9.2, the positively charged amine groups become deprotonated and hence neutral, allowing the negatively charged DNA molecule to detach from the stamp surface. To further corroborate the high uniformity of the printed films, AFM images were taken (Figure 1C). They revealed a homogeneous layer of the DNA at the surface. The edge definition of the surface pattern is very clean, and no excess of DNA was found at the rims of the structures (Figure 1C).

The density of DNA on the target surface after stamping was quantified by fluorescence measurements of labeled oligonucleotides. A reference curve was made by spotting defined amounts of labeled 20-base pair (bp) DNA oligonucleotides onto positively charged amine glass slides. This curve follows a typical saturation behavior, with saturation reached at 0.9×10^4 ($\pm 0.1 \times 10^4$) molecules/μm^2. Increasing the concentration of molecules in

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solution from this point on did not increase the measured intensity any further (data not shown). Calculating the "maximum density" of a tightly packed surface of molecules spanning 19 nm^2, assuming a length of 8.2 nm and a width of 2.3 nm for 20-bp oligos, resulted in $\sim 5 \times 10^4$ molecules/μm^2 and agrees well with the experimental value. The deviation of experimental versus calculated values may be explained by an incorrect approximation of the molecule geometry and dimension as well as by neglecting repulsive forces between molecules of the same charge. Especially the formation of an electrostatic double layer exerting repulsive forces on the incoming molecules can have significant impact on the surface density of molecules up to total suppression of adsorption, depending on buffer pH and ion strength.[29,30] In addition, the experimental value may also be subject to fluorescence quenching.

The influence of the buffer composition on the adsorption of DNA to the PDMS stamp was examined. We let a solution of 1 pmol/μL DNA, diluted in DI water, adsorb to an elastomeric stamp under various conditions and subsequently stamped it on the target surface. After rinsing but before stamping, the measured fluorescence indicated the formation of a dense layer on the stamp surface with an effective surface coverage of 8000 $\pm$ 500 molecules/μm^2. On the first print, we measured a surface density of $\sim$4000 $\pm$ 500 molecules/μm^2. Thus, the molecule density on the target surface amounts to only 50% of the initial density on the stamp. Different adsorption buffers decreased the initial surface coverage dramatically (30% of the maximum value for MES pH 7.0 buffer, 10% for PBS pH 7.2 buffer) and using carbonate buffer at pH 9.6 fully inhibits adsorption of DNA on the stamp (<1%) and its subsequent transfer. A high salt concentration as well as the presence of divalent ions such as Mg^{2+} in the PBS buffer did not change the coverage significantly (less than 13% of the maximum value). From these findings, we conclude that indeed the pH of the buffer is the key factor in adsorbing DNA to charged surfaces.

After the observed partial transfer of the DNA molecules, we analyzed the possibility of inking a stamp once and printing it several times. Evaluation of the fluorescence of multiply stamped 20-bp oligonucleotide patterns showed an exponentially decaying behavior (Figure 2A). Fitting a function of exponential decay (solid line in Figure 2) yielded a value close to 0.5 for the exponential basis, indicating a $\sim$50% chance for each molecule to be transferred. This transfer probability remained constant over several (at least five) stamping steps. Dashed lines represent the theoretical values for 25 and 75% transfer probability.

A significantly different transfer behavior was found for repeated stamping of longer molecules (500- or 1600-bp DNA). Here, the transferred amount of molecules was significantly higher than 50% for the first printing step, whereas the subsequent prints showed a dramatic drop in transfer rate (Figure 2B and C). Changing the concentration of the molecules in solution over 3 orders of magnitude (squares, circles, and triangles in Figure 2) did not influence the aforementioned behaviors. The transfer process is apparently governed by either of two considerably different regimes, depending on molecular size: Short DNA molecules appear to lie on the stamp surface without intersecting one another. In this case, stamping will transfer each molecule with a certain probability that solely depends on the net charge of the molecule. In contrast, the longer molecules adsorb onto

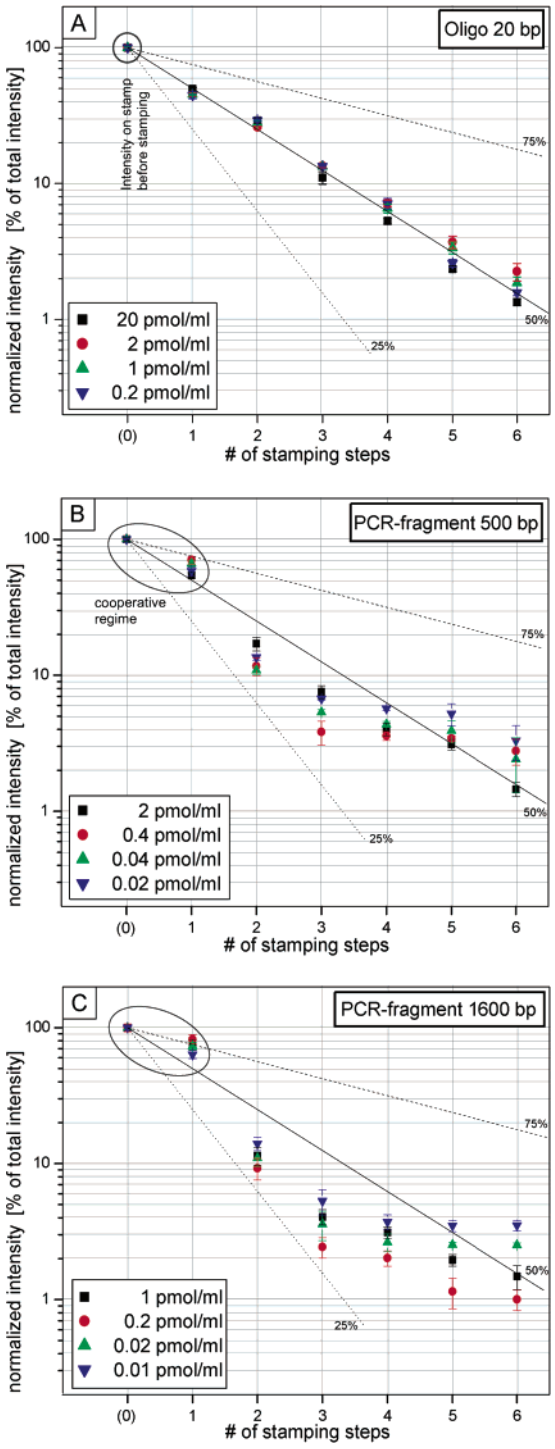

Figure 2. Transfer behavior of DNA for multiple successive stamping steps. Consecutive stamping without re-inking was performed using fluorescently labeled oligonucleotides of various lengths; (A) 20, (B) 500, and (C) 1600 bp. For each fragment length, four data sets with different initial molecule concentrations were recorded (symbols in plot). The amount of molecules transferred onto the target surface after every stamping step was quantified by measuring the fluorescence on the print. The intensity of each printing step was normalized to the initial intensity on the stamp and plotted on a logarithmic scale against the number of printing steps. Dashed lines are guidelines and mark the transfer rates of 25 and 75%, respectively.

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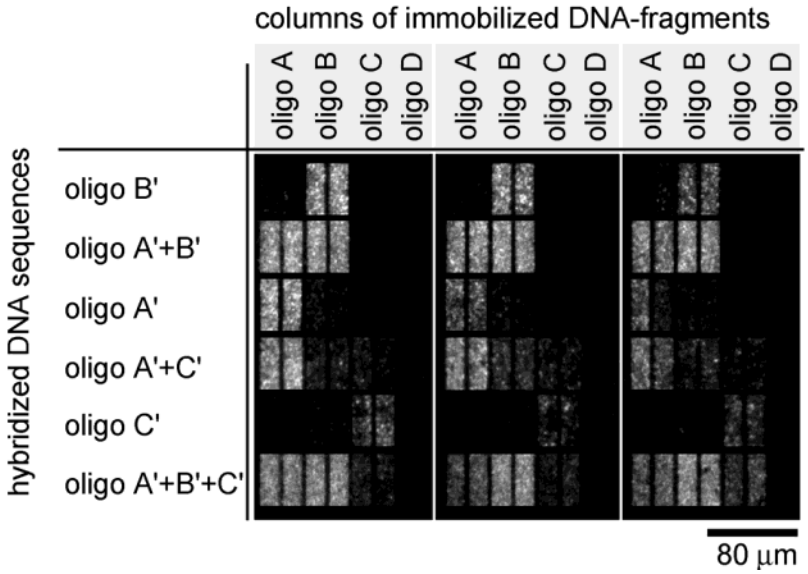

Figure 3. Parallel and simultaneous printing of various DNA molecules onto a target surface, visualized after hybridization using immunogold labeling and subsequent gold enhancement (autometallography). A 16-channel silicon microfluidic network (20-μm-wide channels with a 5-μm gap) has been used to ink a flat PDMS stamp with lines of different DNA molecules (A−D). After printing onto a glass substrate, spatially separated hybridization with probes specific to a subfraction of the arrayed molecules (A′−D′) was performed. The resulting line pattern (repeated twice to show reproducibility) reflects the specificity of the probe molecules used.

the stamp surface in such a way that they intersect each other and thus form a (multi-)layered and more or less entangled meshwork. The degree of entanglement may be related to both the density of molecules in the incubation solution and their size. During the stamping process, individual molecules are no longer transferred independently of each other: an entire layer of entangled molecules is transferred simultaneously. This "cooperative regime" seems to apply only to the first printing step (Figure 2B, C), whereas in the following steps the transfer rate dropped to 25%. This interpretation is corroborated by AFM images (data not shown) that show meshwork formation of the transferred long DNA fragments. It seems that, after removal of the DNA meshwork, the remaining molecules have reached a density where they can act independently of each other. For the third and subsequent printing stamp, an exponentially decreasing transfer behavior was again observed. We conclude that for long DNA fragments the surface concentration has to be even lower than we tested in order to circumvent the cooperative regime. For small molecules, it may have to be even higher to reach the regime of cooperative behavior. To pinpoint the transition between the two encountered regimes of DNA transfer, a much wider range of variations of molecule lengths and their concentration in the incubation solution has to be employed.

Having demonstrated that DNA can be successfully printed in multiple copies, yielding uniform surfaces with excellent edge definitions, we then showed the transfer of various molecules in a single parallel printing step. Furthermore, the generated pattern allowed for specific differential hybridization of labeled DNA from a complex mixture. To achieve this, spatially localized inking was performed by means of microfluidic networks.[27,31] Sixteen parallel microchannels, each loaded with a different sample, were brought in contact with a topographically flat stamp in order to generate arrays of several different DNA molecules.

A series of slides carrying the resulting line arrays of DNA probes were then hybridized with different combinations of complementary and noncomplementary DNA probes labeled with biotin. To visualize the hybridization, the biotin-labeled probes were treated with colloidal gold particles coated with an anti-biotin antibody. Bound complexes were visualized using a gold enhancement kit. Four different target DNA sequences were employed (labeled A−D). Probe molecules with the complementary sequences are labeled accordingly (A′−D′). The enhanced line pattern (Figure 3) reflected the specificity of the probe molecules; low intensities correlated with base pair mismatch; e.g., A′ binds to the target molecules B even though the sequence is not perfectly complementary (six mismatches overall). To avoid the detection of mismatched binding, a greater stringency of washing conditions could be employed.

To demonstrate the feasibility of microcontact printing of DNA further and to show its versatility, we used a conventionally spotted array of longer DNA probes (PCR products) on a PDMS stamp to print the entire array several times in parallel. Subsequent hybridization with fluorescent target DNA, prepared from a mixture of RNA sample material, produced results comparable to standard arrays (Figure 4, panels A−C, stamped; panel D, directly spotted). Three observations are noteworthy: First, the total amount of DNA adsorbed in one spot on the stamp surface—after drying the droplet—appears to be transferred layer by layer and in fractions to the target surface. Individual spots in panel A−C showed regions of higher and lower intensity, which add up to the initial intensity. This is in accordance with the hypothesis of layered stamping of DNA discussed above. Second, stamped and then hybridized microarrays appeared to be more sensitive to a low concentration of starting material of total RNA, as can be

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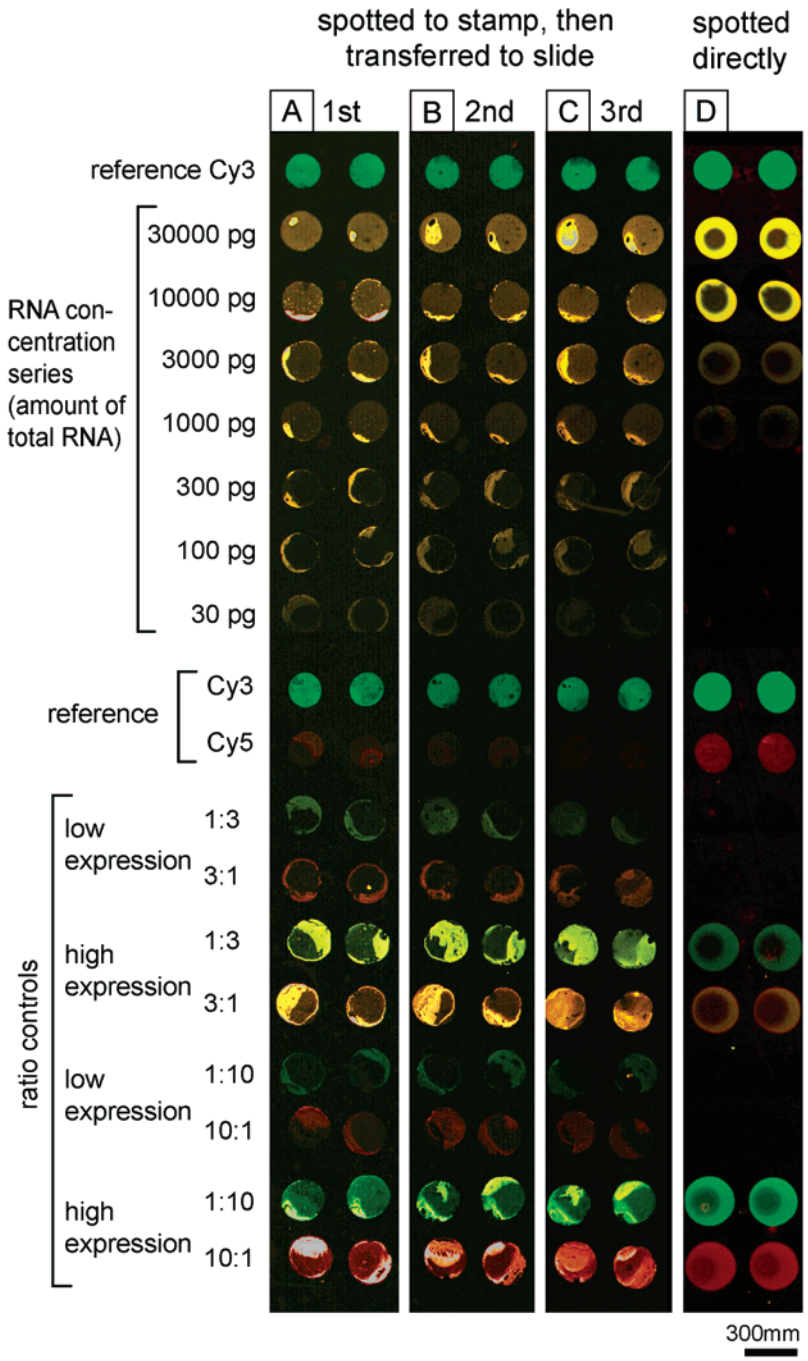

Figure 4. Parallel transfer of a complete DNA array and subsequent hybridization. Using a spotting device, a DNA array was generated on a PDMS stamp and was subsequently microcontact-printed three times in succession onto glass substrates. Standard hybridization with fluorescent target DNA reveals the binding pattern (panels A−C). As a control, a conventional microarray slide (aminosilane glass) was directly spotted (panel D). All slides were treated in accordance with common microarray protocols. The upper part of each slide represents a concentration series with different amounts of starting RNA material. The lower part is a ratio series, where relative intensities between the two channels Cy3 and Cy5 are compared. Direct labeled reference oligos with no match in the test material were spotted independently to control transfer properties. All the stamped and the directly spotted slides showed the expected values after scanning the slides with a confocal microarray scanner.

seen from the upper part of Figure 4. Compared to directly spotted microarrays, much lower concentrations of starting RNA were detected. DNA may be more accessible in stamped spots, thus allowing for a more efficient binding of DNA from hybridization solution. Third, and most important, multiple stamping of entire arrays is possible, as we found that at least three replicas of the array were fully functional (Figure 4, compare panels A, B, and C) and can be used independently and without loss of information in separate hybridization experiments.

CONCLUSION

We have shown that microcontact printing can be expanded to the use of DNA array generation. Electrostatic interactions seem to play the key role in the transfer behavior of DNA. Two distinct

regimes with different transfer properties were encountered and could be explained as a dependency on the length or the total net charge per molecule and the density (surface concentration) of the molecules adsorbed on the stamp surface. The major advantage of μCP of DNA is the capability of printing multiple arrays from one loaded stamp. This method could be developed to a potentially cost- and time-saving process, particularly for gene expression studies, where the ratio of bound to labeled molecules but not the total amount of material matters.

In the future, μCP of DNA—and analogously of other charged polymers or molecules—may help simplify, accelerate, and improve the fabrication of arrays. Gaining a full understanding of the transfer properties of DNA will enable researchers to adopt the technique of μCP for the production of precisely defined arrays with well-characterized molecule densities on a variety of substrates.

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