Direct Parallel Patterning of Multiplexed DNA and Protein Arrays

Sergey Rozhok, Nabil Amro, Joe Fragala, Tom Levesque, and Mike Nelson

NanoInk, Inc., Skokie, IL, USA, srozhok@nanoink.net

ABSTRACT

We report on recent technical advances in patterning DNA and protein patches with submicron dimensions through Dip-Pen Nanolithography® (DPN®) [1]. DPN is a method for nanoscale patterning of surfaces by the transfer of a material from the tip of a scanning probe microscope onto the surface. Since the first experimental work on direct patterning of DNA using DPN method [2], a few significant developments were implemented into the array fabrication routine. New commercially available DPN accessories like multiple pen arrays and inkwells [3] allow one to perform multiplexed DNA and protein patterning to form arrays on solid substrates with nanoscale registration. DNA and protein dot features as small as 200 nm in diameter can be routinely generated on glass or metal-coated substrates via DPN. The design of new accessories for ultra-high throughput printing and microfluidic ink delivery will be presented. DPN patterning of proteins and oligos, and screening for their biological activity, will be shown and discussed in detail.

Keywords: DNA/protein microarrays, patterning, nanoarrays, Dip-Pen Nanolithography

1 ULTRAHIGH-DENSITY NANOARRAYS

The development of the microarray technology has led to a new era of research when scientists are able to study thousands or even millions of different molecules at once, and collect the results in a systematic way. Since the invention of microarray technology in 1995 [4], a number of advanced technologies and methodologies have been developed and become available. Currently there are two principal ways of making DNA microarrays: the in-situ synthesis and the spotting methods. The smallest array features produced by these techniques are on the order of hundreds to tens of microns. However, even the highest-resolution arraying approaches that use optical lithography are not capable of producing features smaller than several microns. To meet the emerging needs of the pharmaceutical as well as medical research and diagnostics communities some High-Tech companies specializing in nanometer-scale manufacturing are developing techniques and methodology for patterning biological molecules on the nanoscale. One of those is NanoInk Inc.

NanoInk Inc. is a company that commercially develops a technology known as Dip-Pen Nanolithography (DPN). DPN is an on-surface fabrication process that uses an ultra sharp cantilever tip of an atomic force microscope (AFM) as a pen to write nanoscale patterns on substrates using various materials as "ink." The company was founded in 2002 and currently marketing its full industrial DPN system, NSCRIPTOR™ along with MEMS and software products. The company sees the future of DPN applications through enhancing DPN capabilities in order to build large-scale high-density solid state and biological arrays. In addition to commercial applications of DPN to produce chips with relatively large numbers of targets for genome screening, DPN technology offers the opportunity to fabricate custom microarrays that have a relatively small number of features depending on particular purposes and questions of the study. Currently, there are more than 500 publications presenting DPN applications in both fundamental and applied studies. The direct-write DPN process is suited to the deposition of biological molecules because it operates in ambient conditions, thus avoiding the use of photoresist chemistry or other harsh environmental conditions. Recent works have shown that nanometer-scale structures can be built with virtually any material, including DNA, antibodies and membrane-bound receptors. Using advantages of the AFM technology, which guarantees an unmatched flexibility and accuracy in material printing at the nanoscale, DPN enables the fabrication of chips with smaller and more closely spaced features. The DPN process allows preparing a million spots in the area occupied by one spot of a conventional array. For instance, a reduction in feature size from 200 μm to 50 nm would enable arrays with 100,000 spots to be generated in an area the size of a single feature in a typical robotically spotted microarray. Smaller feature sizes will require significantly smaller amounts of synthesis and labeling materials. Sample size is especially pertinent to drug targets that are expressed in vanishingly small quantities, such as membrane-associated proteins.

2 HIGH THROUGHPUT NEW DPN ACCESSORIES

As has been shown earlier [2], DPN is completely capable of patterning arrays of single and multiplexed oligos on chemically modified gold and glass surfaces. The throughput of the printing process is 100,000 spots in 100-x 100 μm^2 areas in less than an hour. However, the limitation of a single-pen print process makes it impossible to generate arrays of multiplexed targets over large areas similar to those prepared by standard photolithographic or robotic spotting methods. To extend the capability of the DPN technology, the microfabrication team at NanoInk has developed 1- and 2-D high-density pen array systems for

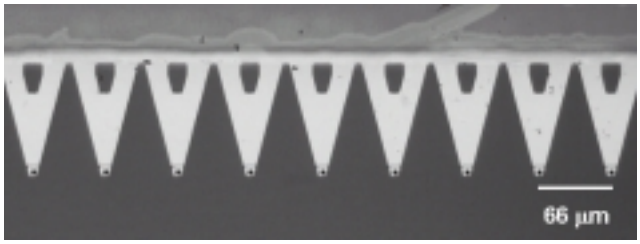

Figure 1. Optical image of a 1-D pen array tool

industrial-scale DPN-based manufacturing [3]. Such pen arrays incorporate multiple identical pens that can be brought simultaneously into contact with the surface during printing. Each pen can dispense picoliter droplets of desired biological material onto a substrate with nanoscale registration. Since the throughput of the DPN-arraying process scales linearly with the number of pens, this type of device could make DPN thousands of times faster, and thus easily produce nanoarrays with more than 1 million spots in seconds. Recently, a series of conceptual experiments on patterning multiplexed DNA and protein arrays was conducted using a 1-D pen system shown in Figure 1. Such an array has 12 pens in a row with tip-to-tip distance of 66 µm. The tip fabrication process and AFM probe information are presented elsewhere in [3]. Clearly a 1-D, 12 pen array tool can be used to pattern up to 12 different inks. To load inks onto the tips, NanoInk has developed a microfluidic ink delivery chip-based system called "inkwells". Inkwells can be integrated to the DPN instrument and used for simultaneous coating of multiple pens with single or multiple inks. Figure 2 (A and B) illustrates an inkwell chip and micowells, correspondingly. The microwells must match the pen array design to allow precise alignment of the tips to the channels and ink transport only to the bottom of the cantilever. Each reservoir of the inkwell chip can be filled with 0.3 µL of liquid ink, which reaches the microwells through the microchannels. Dipping the tips into the microwells (Figure 2C) results in simultaneous coating of 6 tips with inks on the bottom side of the cantilever. Such inking process avoids deposition of ink onto top of the cantilever and prevents cross-contamination between cantilevers within the array. Depending on the hydrophilicity of the cantilever surface and ink wettability, inks may form a large droplet or a thin film layer over the cantilever. To achieve deposition of nanoscale or even submicron features it is important that inks spread evenly over the cantilever surface in the form of a thin layer. The formulation of inks is a key issue for successful tip coating and further deposition of them onto a substrate. High-quality inks have to produce consistent spot size and morphology. The most common requirements for the formulation of inks used in printing technologies are based on actual need to spot a homogeneous solution, which would enabled easy ink flow and slow drying, and demonstrated specific affinity to the substrate. Figure 2d shows a result of coating tips with oligo inks. The amount of ink shown in Figure 2D, which is typically about a few

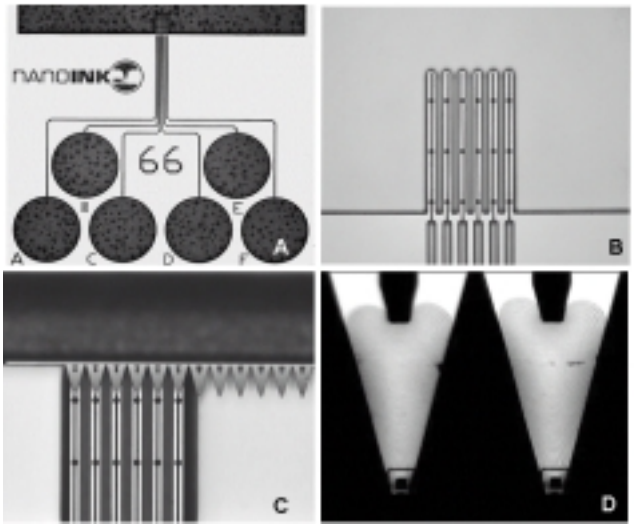

Figure 2. A) Overall layout of an inkwell chip and B) zoom-in view of the microwell array. The spacing between the channels is 66 mm. C) The “inking” process during which each pen is coated with particular ink. D) An appropriate ink composition makes a thin film ink distribution over the cantilever surface.

picoliters, is sufficient for deposition of more than 100,000 0.5 µm - diameter dot features.

2.1 Oligonucleotide Nanoarrays

Oligonucleotide arrays of amine and hexanethiol-modified oligos have been spotted directly onto chemically modified glass surface. Four types of target oligonucleotides and their complimentary (probe) sequences were purchased from Integrated DNA Technologies. They are unique 30- to 36-nucleotide sequences for (A) human immunodeficiency virus (HIV), (B) Ebola virus (EV), (C) variola virus (smallpox, VV), and (D) Bacillus anthracis (BA) protective antigen. The target oligos were spotted within a 50x50 μm^2 area with dot features ranging from 200 nm to several microns in size, Figure 3. The feature size of printed spots can be adjusted during the experiment by varying the humidity and tip-surface dwell time.

In a typical DPN DNA deposition experiment, Si_3N_4 AFM tips need to be modified with 3-aminopropyltrimethoxysilane (APTMS) to achieve uniform thin film ink coating over the cantilever. Then the silanized tips are coated with DNA inks, which were lyophilized oligos dissolved in a solution made of 90% dimethylformamide (DMF), 10% H_2O, 0.3 M $MgCl_2$ and 0.2% Tween20. The final oligo concentration was 1 mM. Printing of the oligo was done at 50% relative humidity and ambient temperature. As can be seen from Figure 3 and 4 the spot size, shape and emission intensity are extremely uniform within individual nanoscale features and from spot to spot. After printing, the substrate was left in a humidity

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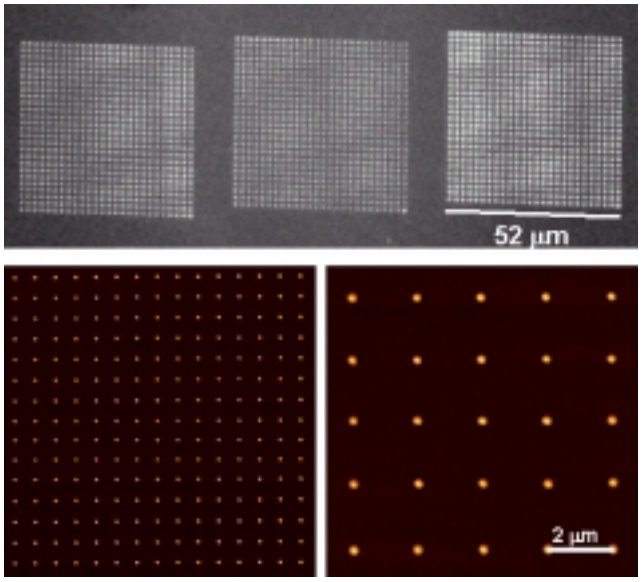

Figure 3. Top: Optical image of DNA arrays spotted on amine-modified silicon oxide surface. Each array has 676 features within 52 x 52 µm2 printed area. The spacing between the features is 2 µm. Bottom: Detailed AFM phase images of the arrays showing shape and size consistency within the array. The feature size is 210 ± 5 nm.

chamber (RH~75%) for 2 hours to allow binding of the oligos to the surface. Then, the substrate was passivated in 1 mM of 1-octadecanethiol (ODT) in ethanol for 10 sec to protect the unpatterned area against non-specific binding of the probe DNA. The hybridization process was performed in a humidity chamber at 37 °C for two hours adding a buffer solution with four complementary oligos labeled with fluorophore dyes. Conventional optical and atomic force microscopies were used to characterize DNA nanostructures. Figure 4 presents a fluorescent image of hybridized multiple probe arrays. The image was taken using an FITC filter set so that not all arrays are visible.

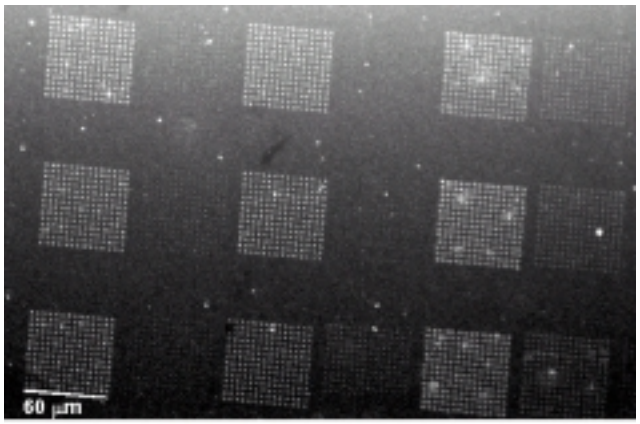

Figure 4. Hybridized HIV, EV, VV, and BA arrays spotted on amine - modified silicon oxide chip.

The distance between individual DNA dots is 4 µm, and the total number of features generated during the deposition time of 4 min was 2,700. In fact, the density of DPN generated features can be easily increased up to 10^5 per 100 μm^2.

2.2 Protein Nanoarrays

Due to the direct-write nature of the DPN process, DNA printing strategies can be adopted for patterning proteins, in particular, for their most interesting forms - antibodies and antigens. It has been shown that the DPN method is well suited for spotting protein solutions onto a solid surface. Several research groups using DPN technology at academic and government labs have succeeded with deposition of immuno-proteins, enzymes, and viruses [5-7]. As with DNA, the immobilization of proteins on surfaces requires special surface functionalization in order to provide binding along with preserving protein properties and retaining full activity. There are many different methods described in the literature on how to construct such a surface by applying different strategies of chemical surface modification, however protein arrays are still limited in their performance. The optimal slide surface would immobilize proteins via a specific and site-directed coupling to the surface with control of orientation to avoid a blocking of the binding site. The most straightforward method consists of immobilization of the unmodified protein by adsorption or by reaction of available protein groups with corresponding electrophilic groups on the slide surface. Recently, a similar approach was demonstrated in attaching viruses to antibodies immobilized on metal-ion-functionalized surfaces [8]. Such surfaces do not possess reactive groups but allow binding of large molecules via physical (electrostatic) adsorption. In our work we have tested metal ions as surface linking groups to immobilize antibodies on the surface in an active state. For characterization of the resulting protein nanostructures with regard to their activity, a specific binding between the Fab region of the immobilized antibody and the Fab-specific binding group of fluorophore-labeled antigen was used. Purified mouse and rabbit IgG, provided as lyophilized powder and anti-mouse IgG (Fab-specific)-FITC antibody and anti-rabbit IgG (F(ab)2-specific)-Cy3 antibody were purchased from Sigma-Aldrich and used without further purification. For patterning arrays of target antibodies on Zn^{2+}-modified silicon substrate a solution containing phosphate buffer, glycerol and protein was spotted on the surface at room temperature and 50% relative humidity via conventional DPN process. The use of additives such as glycerin in the protein ink solutions significantly enhances protein activity by inhibiting dehydration of deposited solution. The substrate with printed arrays was left for immobilization of the spotted antibodies for 2 hours in the humidity chamber (RH~75%). Then the substrate was immersed into 1% BSA solution in PBS for 30 min to block unpatterned areas. Finally, the sample was incubated in a

solution of fluorophore-labeled antigens and allowed to hybridize the proteins for 1 h at 37 °C in an incubation chamber. The fluorescence microscopy image of the hybridized arrays is shown in Figure 5. Based on the fact that the mouse/rabbit IgG and Fab fragment of the anti-mouse/rabbit IgG bind with high affinity to the Fab domain only, control of the effectiveness of antibody immobilization and proper orientation was achieved.

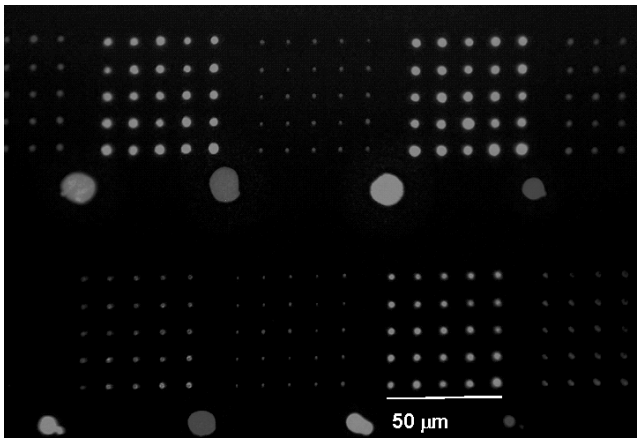

Figure 5. Hybridized mouse and rabbit IgG arrays spotted on Zn^{2+} modified silicon oxide chip.

2.3 Conclusion

Recent DPN works show that nanometer-scale structures can be built with virtually any material, including DNA, antibodies and viruses. Patterning via DPN can be highly controllable in terms of pattern size and shape, and the immobilized molecules remain functional and accessible. A large-scale integration of microfluidic technologies is still challenging. To meet the inking needs for 1- and 2-D pen systems, with an ultimate goal to deliver different ink to each pen in the device, NanoInk is developing chips with integrated inkwells. One of the consequences of miniaturization of biological assays to the nanoscale is to be able to distinguish hybridization signals. Monitoring these signals from nanoarrays will require high-resolution screening tools that can operate at the nanometer-length scale where conventional optical-based microarray readout methods are not capable.

2.4 References

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