# Chemical nanoprinting: a novel method for fabricating DNA microchips

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## ABSTRACT

We have developed a novel cost-effective procedure, namely 'chemical nanoprinting', for oligonucleotide or cDNA chips manufacture. In this thermo-controlled process, the oligonucleotides, covalently attached to a highly loaded 'master-chip' through disulfide bonds, are chemically transferred to the acrylamide layer mounted on a 'print-chip'. It is demonstrated here that multiple identical print-chips can be produced from a single master-chip. This duplication process is a few hundreds of times faster than any existing methods and the speed of process and cost incurred are independent of the scale of the DNA chips.

#### INTRODUCTION

DNA microarrays have rapidly evolved to become one of the essential tools for high-throughput genetic analysis (1). In general, there are two types of technology of DNA chip manufacture: immobilization of prefabricated DNA or oligo-nucleotides onto microchips (2–4) and on-chip synthesis of DNA molecules (mostly short oligonucleotides) by either photolithographic synthesis or piezoelectric printing (5–7). The two methods enjoy different advantages over each other but they both suffer from a very low production speed, which in turn results in poor availability and high cost of the chip products. Here we report a novel method of DNA chip fabrication that can possibly markedly increase the current speed of manufacture.

#### MATERIALS AND METHODS

The reaction mixture containing 100  $\mu$ M of 5'-thiol-labeled oligonucleotides (Lac-thio: TCA TGG TCA TAG CTG TTT CC) and 3-mercaptopropyl-trimethoxysilane (added from a fresh 5 mM stock solution in sodium acetate buffer, 30 mM, pH 4.3) was prepared in the same buffer that was used to dilute the silane. The reaction was allowed to proceed for 1 h at room temperature. Then the reaction mixture was used directly for spotting on unmodified glass slides (Kebo-lab, Sweden) either manually (~120 nl/spot) or with an automated arrayer (Genetic Microsystems, Woburn, MA) (~1 nl/spot). After spotting, the glass slides were kept in a humidified chamber for 15 min at room temperature, dried at 50°C for 5 min and then used as the

master-chips. Immobilization of 5'-acrylic-labeled oligonucleotide (Lac-acrylic: TCA TGG TCA TAG CTG TTT CC) (Eurogenetec, Seraing, Belgium) via 3-mercaptopropyl-trimethoxysilane was done in an identical manner.

A stock solution of 3.75 ml 40% acrylamide (Pharmacia Biotech, Sweden), 3.75 ml 2% methylenebisacrylamide (Amersham Pharmacia Biotech, Uppsala, Sweden) and 400  $\mu$ l 10% ammonium persulfate (APS) (Pharmacia Biotech, Sweden) was prepared. 300  $\mu$ l of this stock was mixed with 0.75  $\mu$ l *N*,*N*,*N'*,*N'*-tetramethyl-ethylenediamine (TEMED) (Amersham Pharmacia Biotech) and immediately spread over the master-chip and covered with  $\gamma$ -methacryloxy-propyl-trimethoxysilane (Bindsilane) (Amersham Pharmacia Biotech) coated slides. The gel was allowed to polymerize at room temperature for 1 min and the sandwich was dipped in a preheated water bath.

For hybridization, 5'-Cy3-labeled oligonucleotide probe (Lac-sen-Cy3: GGA AAC AGC TAT GAC CAT GA) was diluted to 1  $\mu$ M in 5× SSC (750 mM NaCl, 125 mM sodium citrate, pH 7) with 0.1% Tween-20 and applied to the surface of the slides. A glass coverslip was mounted gently on top of the solution and the complex was transferred into a humidifier at 37°C for 45 min. The unhybridized probes were removed by rinsing with 5× SSC containing 0.1% Tween-20 (3 × 10 min) followed by 1× SSC containing 0.1% Tween-20 (3 × 15 min). After washing, the slides were dried with nitrogen gas and scanned on a GMS418 fluorescent scanner (Genetic Microsystems) to visualize hybridization signals. The fluorescence intensities of the spots were quantified with ImageQuant<sup>TM</sup> software (Molecular Dynamics, Sunnyvale, CA).

## **RESULTS AND DISCUSSION**

Synthetic 5'-thiol-labeled oligonucleotides were silanized and covalently attached onto the unmodified glass slides according to our recently published procedure (3). The DNA chips were referred to as 'master-chips'. A mixture of acrylamide, bisacrylamide, APS and TEMED was applied to the master-chip and covered with a glass slide pre-coated with Bindsilane. The gel mixture was allowed to polymerize at room temperature for 1 min and the slide 'sandwich' was heated at  $95^{\circ}$ C from 5 s to a few minutes. The two slides were then separated. The polyacrylamide layer was always found to be attached to the Bindsilane coated slide and the two components (the gel layer and the slide) collectively comprise the 'print-chip'. When hybridized with Cy3-labeled complementary oligonucleotides, such print-chips showed strong hybridization signals in a

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**Figure 1.** Oligonucleotides immobilized on a glass surface via a disulfide bond can be transferred to acrylamide-coated chips with great spatial precision. A hand-spotted master-chip was used to make prints on 10 acrylamide-coated chips at 95°C and then chips were hybridized to a complementary probe labeled with Cy3. The master-chip (after printing) was marked with a red frame.

spatial pattern that is strictly a mirror-image of that observed on the master-chip, suggesting that the 'printing' process outlined above can result in considerable transfer of oligonucleotides from the master-chip to the print-chip. The same procedure was repeated several times using the same masterchip and identical patterns were observed (Fig. 1).

We attempted to determine whether the observed oligonucleotide transfer is due to the stripping of non-covalently attached oligonucleotides in the presence of acrylamide molecules, or due to chemical detachment of the covalently attached oligonucleotides. First we spotted unlabeled oligonucleotides of the same sequence onto the 3-mercaptopropyl-trimethyoxysilane (thiol-silane) coated slides and performed the printing procedure as described above. It was found that almost all oligonucleotides were smeared into the applied gel layer without a defined spatial pattern on the first print and the amount of nucleotides was hardly detectable on subsequent print-chips (data not shown). This result suggested that none of those non-immobilized oligonucleotides in our initial printing system contribute to the strict spatial printing patterns (Fig. 1). We then tested non-covalently immobilized Cy3-labeled oligonucleotides on a 3-aminopropyl-trimethyoxysilane (amino-silane) coated glass surface. It is known that the oligonucleotide binding onto an amino surface is predominantly by electrostatic interaction (non-covalent). When we repeated the printing procedure on these chips, we did not observe any significant amount of oligonucleotide transfer onto the acrylamide gel (data not shown).

These results indicated that the significant nucleotide transfer from the master-chip to the print-chips in our printing system (Fig. 1) was not due to stripping of non-immobilized or non-covalently immobilized oligonucleotides. A possible explanation could be that a cross-reaction between disulfide bonds (by which oligonucleotides were attached on the master-chip) and the acrylamide molecules had resulted in the transfer of immobilized DNA from the master-chip to the print-chips.

To confirm that the acrylic groups have reactivity towards disulfide bonds, equal amounts of the 5'-acrylic-labeled oligonucleotides and 5'-thiol-labeled oligonucleotides (same sequence) were reacted separately with thiol-silane. The silanized oligonucleotides were spotted on the uncoated glass surface and hybridized with Cy3-labeled complementary sequence for comparison. It was found that acrylic-labeled oligonucleotides were covalently immobilized through thiolsilane to the same extent as thiol-labeled oligonucleotides,



Figure 2. Effect of temperature on the transfer efficiency of disulfide bond/ tethered oligonucleotides from a glass surface to an acrylamide surface. Master-chips were prepared by spotting 50, 5 and 2  $\mu$ M silanized Lac-thio solutions on the same glass slide. A separate master-chip was printed at room temperature, 75 and 95°C. After hybridizing with Lac-sen-Cy3, all slides were scanned at 25% laser power and 25% PMT gain. Signal intensities of the master-chips and their respective prints were summed and the percentage of oligonucleotides transferred to each print-chip was calculated accordingly.

suggesting that acrylic groups do have a reactivity towards disulfide bonds that is similar to thiol groups. Thus, attacking of the disulfide bonds by acrylic groups could be a reasonable mechanism of oligonucleotide transfer in the printing process.

We investigated the temperature dependence of the transfer of disulfide bond-tethered oligonucleotide from master-chip to print-chips. Separate master-chips were used for printing at room temperature, 70 and 95°C. Gel polymerization time and heating time were kept constant (1 min) for each print. All master-chips (after printing) and print-chips were hybridized with Cy3-labeled complementary oligonucleotides. Negligible amounts of oligonucleotide transfer (2-3%) were observed on print-chips made at room temperature. Oligonucleotide transfer was greatly improved by raising the printing temperature to 70°C whereas an increase in the printing temperature from 70 to 95°C resulted in ~100% increase in the transfer efficiency (Fig. 2). Different amounts of oligonucleotides immobilized on the master-chips had no influence on oligonucleotide transfer efficiency. In all subsequent printing experiments we used 95°C as the standard printing temperature.

We then tested the time dependence of the oligonucleotide transfer from the master-chip to the print-chips. Separate master-chips were used for printing at 95°C by varying the heating time. The gel polymerization time was fixed as 1 min at room temperature. As depicted in Figure 3, the efficiency of oligonucleotide transfer showed a close correlation with heating time.

Our goal was to make multiple print-chips from a single master-chip with equal levels of oligonucleotides transferred to all print-chips. We realized that every printing event will deplete a portion of the oligonucleotides from the master-chip surface, and accordingly the transfer efficiency has to be artificially increased in order to transfer equal amount of oligos from the master-chip onto the next print-chip. We used the following heating time scheme to adjust the transfer efficiency during 10 successive printing events: 3, 4, 5, 7, 8, 11, 16, 32 and 64 s and 5 min (Fig. 4). The heating time was kept short in order to limit the amount of oligonucleotides transferred to be <10% of the total amount of oligonucleotides on the master-chip in each step. Quantitative data derived from the hybridization

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Figure 3. Effect of heating time on the transfer of covalently-immobilized oligonucleotides (via disulfide bonds) from a glass surface to an acrylamide surface. Master-chips were prepared by spotting 100  $\mu$ M silanized Lac-thio solutions on the glass slides. Separate master-chips were printed at 95°C for 30 s and 1, 2, 3, 4, 5 and 6 min. After hybridization with the complimentary Lac-sen-Cy3, all slides were scanned at 25% laser power and 25% PMT gain. Signal intensities of the master-chips and their respective prints were summed and the percentage of oligonucleotides transferred to each print-chip was calculated accordingly.

signals of these print-chips indicate that almost equal amounts of oligonucleotide were transferred to each and every printchip (Fig. 4). The amounts of remaining oligonucleotides on the master-chip after 10 prints were still enough to make a few more print-chips. Good immobilization methods can generate oligonucleotide chips with a surface density of 50 pmol/cm<sup>2</sup>. The detection limit of the scanner we used is 0.05 pmol/cm<sup>2</sup> density of the fluorescent molecules. The surface density of 2 pmol/cm<sup>2</sup> of oligonucleotides per spot gives rise to a satisfactory hybridization signal in our hands. Accordingly, some 25 prints can be made from a master-chip made by currently available immobilization methods. The in situ synthesis can produce an array at a density of 100-200 pmol/cm<sup>2</sup> of oligonucleotides, theoretically enough to support the production of 50-100 print-chips. Using a fluorescent scanner with increased detection sensitivity, the print-chips with lower level of oligonucleotide could potentially also be used. Such a twist would make it possible to produce more (some 200-500) print-chips from a single master-chip.

We then checked whether the printing procedure described above could be applied to high-density DNA chips. Masterchips were prepared using an automated arrayer and were printed 10 times using a similar heating scheme. A typical set of high-density print-chips is depicted in Figure 5A, showing that high-density chips can also be produced using our method.

We then further analyzed the quality of the print-chips by examining the 'resolution' (spot size) changes on successive print-chips. We defined the size of the spot as pixels above background. It was found that the average spot size on the print-chip is 15% larger than that of the master-chip, suggesting an average increase of <8% in spot diameter. It is possible that this minor 'resolution' loss is due to the locomotion of the polyacrylamide gel during the drying process. This could potentially become a problem in printing high-density chips where the distance between spots is approaching this threshold, but for most high-density chips such a level of 'resolution' change would be well acceptable. Improvement in the polymerization of acrylamide or introduction of other polymers may also improve or eliminate such 'resolution' losses. Nucleic Acids Research, 2001, Vol. 29, No. 2 e2



**Figure 4.** Multiple print-chips obtained from a single master-chip at  $95^{\circ}$ C. The even transfer of oligonucleotides was regulated by varying the heating time (3, 4, 5, 7, 8, 11, 16, 32, 64 s and 5 min, respectively) from prints 1 to 10. After hybridization with the complimentary Lac-sen-Cy3, all slides were scanned at 25% laser power and 25% PMT gain. Signal intensities of the master-chip and all prints (P1–P10) were summed (M) and the percentage of oligonucleotides transferred to the print-chips were calculated accordingly and plotted.

Chip-to-chip and spot-to-spot variations in the amount of deposited DNA have been a major problem for spotted arrays. Such variation can be from 3- to 10-fold. One rationale for developing our chip-printing scheme has been that the printchips could be made identical or directly comparable with each other by this method. To test this hypothesis, chip-to-chip oligonucleotide transfer variation was also analyzed using the high-density print-chips. For each spot, the signal intensity was first normalized against the average signal intensity of the same chip. Then the average value of the same spot from all 10 print-chips was set as 1 for plotting. The normalization process will discount the inherited drawbacks of spot-to-spot variation during the master-chip fabrication. It also allows the slightly different printing efficiency for each print-chip to be accounted for in the comparison. As depicted in Figure 5B, the normalized intensity of each spot was virtually identical between all 10 print-chips. This property of the printing process will make the data from different chips printed from the same master-chip directly comparable after proper normalization, even if the variation between different master-chips cannot be eliminated.

In summary, we have demonstrated that chemical nanoprinting is a feasible way of rapid mass production of DNA chips. The fabrication speed reported here (on average 45 s/chip) is hundreds of times faster than any existing technology. Importantly, the printing speed is, in theory, independent of the scale of the chips and this property would make this method even more attractive in making ultra-high density chips. Microstamping has been used for making surface patterns with different biological properties but its application in DNA chip fabrication has never been attempted (8). Duplication of PCR colonies formed within polyacrylamide have been elegantly demonstrated recently (9). It is unlikely, however, that this method could be employed in normal DNA chip fabrication simply because it requires all entities on the chip to be uniformly amplified whereas all oligonucleotide chips and most other DNA chips would not accommodate this requirement. Thus our chemical nanoprinting method demonstrated for the first time that a printing mechanism can be amended to become a new dimension of the manufacture of normal DNA chips.



**Figure 5.** Chemical nanoprinting of high-density DNA chips. (**A**) A silanized Lac-thio solution  $(100 \,\mu\text{M})$  was arrayed at 1000 spots/cm<sup>2</sup> density on the glass slides. The master-chip was used to make 10 print-chips. The master-chip (after printing) was marked with a red frame. (**B**) The normalized intensity of 20 randomly selected spots was compared across all 10 print-chips. The average normalized intensity of each spot was calculated and set as 1 and values from different print-chips were color-coated, respectively.

This process results in DNA chips with a lower DNA load than other direct loading processes (*in situ* synthesis or spotting). Application of the print-chips in combination with currently available detection systems could result in a reduction in the dynamic range of targets that can be accommodated, but this is anticipated to be compensated by the increased sensitivity of fluorescent scanners in the near future. Aspects to be explored further include better chemistry for loading the master-chips, precise control of the 'printing' intensity and polymers other than acrylamide as the printing matrix. It is anticipated that the integration of this method with other existing chip fabrication technologies will have the potential to boost the DNA chip production speed by a factor of 10–100.

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#### REFERENCES

 Southern,E.M. et al. (1999) Microchip review series. Nature Genet., 21 (Suppl.), 5–60.

- Rogers, Y.H., Jiang-Baucom, P., Huang, Z.J., Bogdanov, V., Anderson, S. and Boyce-Jacino, M.T. (1999) Immobilization of oligonucleotides onto a glass support via disulfide bonds: A method for preparation of DNA microarrays. *Anal. Biochem.*, 266, 23–30.
- Beier, M. and Hoheisel, J.D. (1999) Versatile derivatisation of solid support media for covalent bonding on DNA-microchips. *Nucleic Acids Res.*, 27, 1970–1977.
- Kumar,A., Larsson,O., Parodi,D. and Liang,Z. (2000) Silanized nucleic acid as a universal platform for DNA immobilization. *Nucleic Acids Res.*, 28, e71.
- Fodor,S.P.A., Read,J.L., Pirrung,M.C., Stryer,L., Lu,A.T. and Solas,D. (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science*, 251, 767–773.
- Okamoto, T., Suzuki, T. and Yamamoto, N. (2000) Microarray fabrication with covalent attachment of DNA using bubble jet technology. *Nature Biotechnol.*, 18, 438–441.
- Singh-Gasson,S., Green,R.D., Yue,Y., Nelson,C., Blattner,F., Sussman,M.R. and Cerrina,F. (1999) Maskless fabrication of light-directed oligonucleotide microarrays using a digital micromirror array. *Nature Biotechnol.*, 17, 974–978.
- Singhvi,R., Kumar,A., Sacramento,C.A., George,M., Ingber,D.E., Lopez,G.P., Wang,D.I.C. and Stephanopoulos,G.N. (1998) Method of formation of microstamped patterns on plates for adhesion of cells and other biological materials, devices and uses therefore. US patent US5776748.
- Mitra,R.D. and Church,G.M. (1999) In situ localized amplification and contact replication of many individual DNA molecules. *Nucleic Acids Res.*, 27, e34.