

## Replication of DNA Microarrays from Zip Code Masters

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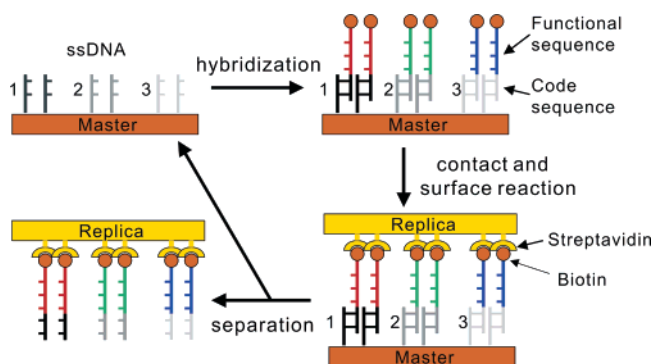
**Abstract:** This report describes a mechanical method for efficient and accurate replication of DNA microarrays from a zip code master. The zip code master is a DNA array that defines the location of oligonucleotides consisting of two parts: a code sequence, which is complementary to one or more of the zip codes, and the functional sequence, which is terminated with biotin. Following hybridization of the zip code to the code sequence, a replica surface functionalized with streptavidin is brought into conformal contact with the surface of the master. When the two surfaces are separated, the functional and code sequences are transferred to the replica, and the zip code remains on the surface of the master. Using this approach it is possible to prepare replica arrays having any configuration from a single, universal master array. Here we demonstrate that this approach can be used to replicate master arrays having up to three different sequences, that feature sizes as small as 100  $\mu\text{m}$  can be replicated, and that master arrays can be used to prepare multiple replicas.

## Introduction

Here we report an efficient and accurate method for the replication of DNA microarrays. The general strategy is illustrated in Scheme 1. First, a zip code master is prepared by spotting different single-stranded oligonucleotides onto an appropriate surface. Each spot represents a different zip code that will direct the placement of a second oligonucleotide.<sup>1,2</sup> Second, the zip code master is exposed to a solution containing biotin-functionalized oligonucleotides that consist of two parts: a code sequence and a functional sequence. Because each code sequence is designed to be complementary to just one specific zip code on the master, the biotin-functionalized oligonucleotides will be directed to their appropriate zip code locations on the master. Third, a replica surface-modified with streptavidin is brought into conformal contact with the zip code master. This results in binding of the replica surface to the biotinylated DNA. Fourth, the replica is separated from the master by mechanical force. This results in transfer of the biotinylated oligonucleotide from the master to the replica. The replica is now ready to be used as a DNA array, and the zip code master can be rehybridized to generate additional replicas.

Previously, we showed that oligonucleotides spotted onto a glass surface could hybridize to their biotin-functionalized complements, and then the complement could be transferred to a streptavidin-modified replica surface.<sup>3</sup> A similar replication

Scheme 1



approach was recently reported by Stellacci and co-workers, who showed that dehybridization was facilitated by heating,<sup>4</sup> and that DNA lines as thin as 50 nm could be replicated.<sup>5</sup> Gaub and co-workers used a related principle to construct force sensors that could distinguish between strong and weak intermolecular interactions, but they were not concerned with pattern replication.<sup>6,7</sup> In contrast to these earlier studies, the zip code approach provides a means for using a single master DNA array to prepare oligonucleotide replicates having any functional sequence positioned anywhere on the array. Importantly, this new approach will also make it possible to use a master DNA array to produce replicates of any other material (for example,

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**Table 1.** Sequences of Zip Codes, Probes, and Targets Used in This Study<sup>a</sup>

name	sequence <sup>a</sup>
zip code 1	5' AAC ATG CAA GGG CAA ATG 3'
zip code 2	5' GCT GAG GTC GAT GCT GAG 3'
zip code 3	5' GGT CCG ATT ACC GGT CCG 3'
1'A	5' GGT GAT ATG GCT TGA TGT ACC ATT TGC CCT TGC ATG TT 3'
2'B	5' GGT GAT ATC GCT TGA TGT ACC TCA GCA TCG ACC TCA GC 3'
3'C	5' TGA TTT TCA GCA GGC CTT ATC GGA CCG GTA ATC GGA CC 3'
A'	5' GTA CAT CAA GCC ATA TCA CC 3'
B'	5' GTA CAT CAA GCG ATA TCA CC 3'
C'	5' ATA AGG CCT GCT GAA AAT CA 3'

<sup>a</sup> If a functional group, such as an amine or biotin, was attached to a DNA sequence, it was always attached at the 5' position. Fluorescein was attached at the 3' end for 1'A, 2'B, and 3'C or to the 5' end for A', B', and C'. All oligonucleotides incorporated a spacer. A C12 spacer was used for amine-functionalized DNA. A TEG spacer was used for biotin-modified DNA. The spacer used for fluorescein is known as Spacer 18. More detailed information about it, and the other spacers, can be obtained at the web site of Integrated DNA Technologies (<http://www.idtdna.com/Home/Home.aspx>). All oligonucleotides were purified by HPLC.

proteins, carbohydrates, or inorganic nanoparticles) that can be labeled with a short oligonucleotide code. Thus, the important aspect of the present work is that it represents a major expansion of the scope of our original report.

DNA microarrays have been increasingly used in high-throughput analysis for a wide range of applications, including monitoring gene expression,<sup>8</sup> drug screening based on drug–DNA interactions,<sup>9</sup> and fundamental studies of genetic diseases and cancers.<sup>10,11</sup> In situ synthesis and ex situ spotting are the two families of methods that have been used commercially to fabricate DNA microarrays.<sup>12,13</sup> The best known in situ method integrates photolithography and solid-state synthesis.<sup>12</sup> Each synthesis cycle consists of protection, photodeprotection, and addition of a nucleotide to directly grow oligonucleotides on a substrate. The growth of oligonucleotides is spatially defined by photolithographic masks, and the number of synthesis cycles required is proportional to the length of the oligonucleotides. This method has the advantages of small spot size (~8 μm spot) and design flexibility,<sup>14</sup> but the inefficiency of solid-state reactions limits the maximum oligonucleotide length to about 60 basepairs (bps)<sup>13,14</sup> and leads to increased cost. The second general method for fabricating microarrays is ex situ spotting of presynthesized oligonucleotides.<sup>15</sup> Spotting to a DNA chip surface can be implemented by either contact printing using rigid pins<sup>16</sup> or by projection through microfabricated nozzles.<sup>17</sup> Spotting does not impose length restrictions on the patterned oligonucleotides.<sup>18</sup> However, the expense and time required to prepare an array is proportional to the dimensionality of the array and the size of the individual array elements, which are large (75–500 μm) compared to those prepared by in situ methods.<sup>13</sup> Moreover, as for any sequential process involving multiple repetitive steps, both in situ synthesis and ex situ

spotting are subject to an accumulation of errors.<sup>13</sup> Other ex situ methods for delivering pre-synthesized oligonucleotides include patterning using microfluidic channels,<sup>19</sup> microcontact printing,<sup>20</sup> and dip-pen nanolithography;<sup>21,22</sup> however, all these methods involve manual loading of the oligonucleotides and therefore, at least for now, are not well-suited for creating large-scale, complex microarrays.

## Experimental Section

**Chemicals and Materials.** CodeLink slides (Amersham Bioscience, Piscataway, NJ), coated with a three-dimensional polymeric scaffold functionalized with *N*-hydroxysuccinimide (NHS), were used to fabricate masters. The poly(dimethylsiloxane) (PDMS) replicas were prepared from liquid precursors (Sylgard Silicone Elastomer-184 from Dow Corning, Midland, MI). 3-Mercaptopropyltrimethoxysilane (97% from Alfa Aesar, Ward Hill, MA) and streptavidin-maleimide (from Sigma-Aldrich, St. Louis, MO) were used as received. All chemicals used to prepare buffers were purchased from Sigma-Aldrich: sodium phosphate monobasic (Sigma S0751), sodium phosphate dibasic (Sigma S0876), Trizma Base (Sigma T6791), Trizma HCl (Sigma T6666), ethanolamine (Sigma E9508), sodium dodecyl sulfate (SDS) (Sigma L4522), and 20x SSC (Sigma S6639).

All the oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). The sequences of the oligonucleotides are provided in Table 1. Fluorescence micrographs were captured using an inverted microscope (Eclipse TE300, Nikon) equipped with a CCD camera (Cascade, Photometrics, Tucson, AZ). The filter set (XC102: 475 nm excitation filter, 505 nm dichroic mirror, and 510 nm long-pass emission filter) was purchased from Omega Optical, Inc. (Brattleboro, VT).

**Fabrication of Master Arrays.** The master slides were fabricated using CodeLink slides according to the instructions provided by the vendor (Amersham Bioscience, Piscataway, NJ). Twenty-five micromolar solutions of 5'-amine-modified oligonucleotides in 50 mM pH 8.5 phosphate buffer were spotted onto a CodeLink slide using a pipet (Pipettor 40000-264, VWR) or a microarrayer (Omnigrid Microarrayer, San Carlos, CA). After spotting, the CodeLink slide was placed inside a sealed chamber above a saturated NaCl solution and incubated at 22 ± 2 °C for 15–20 h. Next, the slide was placed in a solution containing 50 mM ethanolamine and 0.1 M TRIS buffer (pH 9.0) at 50 °C for 30 min to block residual reactive NHS groups. After rinsing with purified water twice, the slide was placed in a buffer containing 4x SSC and

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0.1% SDS, which was pre-warmed at 50 °C for 30 min. After rinsing with water again, the slide was dried under a stream of N<sub>2</sub>.

Only about 50% of the masters prepared using the microarrayer could be replicated, but the masters spotted manually worked 100% of time. Apparently the contact spotting configuration used by the microarrayer causes some damage to the surface of CodeLink slides. Further investigation is underway to clarify this issue. Note, however, that when an array can be replicated, the replica is always 100% faithful to the master and can always be hybridized to the complement of the functional sequence. No false positive signals were ever observed.

**Fabrication of Streptavidin-Functionalized PDMS.** Following our previously reported procedures,<sup>3</sup> thiol groups were first introduced onto a PDMS surface by silanization with 3-mercaptopropyltrimethoxysilane (MPS), and then streptavidin was immobilized onto MPS-modified PDMS through the reaction between maleimide and thiol groups.

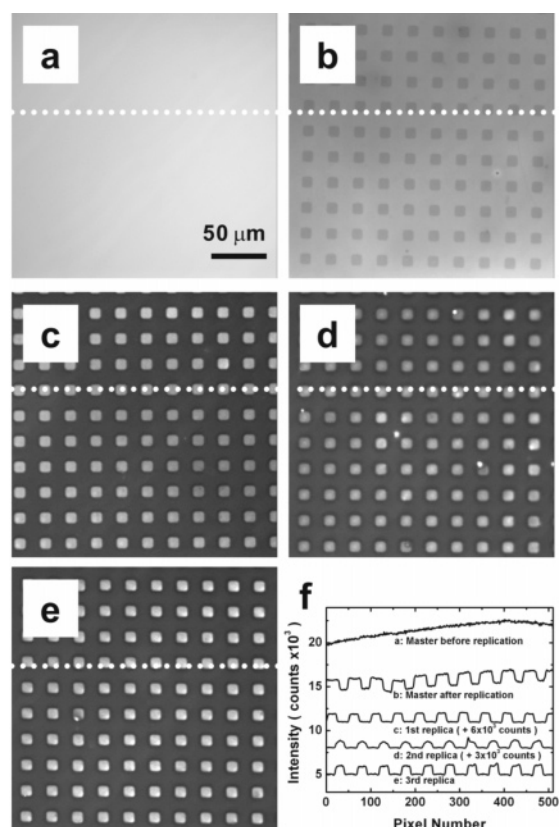
**Replication of DNA Microarrays.** The master was exposed to a solution containing 10 μM oligonucleotide for at least 4 h, and then replication was achieved by contacting the hybridized master with a streptavidin-functionalized PDMS surface. In a typical replication process, 10 μL of pH 7.2 buffer was used to wet the master surface, and then the streptavidin-functionalized PDMS was placed on top of the master with a pressure of 1.4 N/cm<sup>2</sup> at 22 ± 2 °C. Although a pH 7.2 buffer solution was used to wet the master surface in all the experiments presented here, we later found that water (no buffer) worked just as well. After 10 min of contact, the PDMS replica was manually peeled off the master, rinsed, and blown dry. This experimental approach is based on methodology reported by Gaub and co-workers.<sup>6,7</sup>

## Results and Discussion

### Replication of a Zip Code Master Having One Zip Code.

Figure 1 shows that multiple replicas can be prepared from a single zip code master. The master was prepared by applying a solution of amine-modified oligonucleotide (zip code 1, Table 1) onto a CodeLink slide (see Experimental Section for details). Next, the master array was exposed to oligonucleotide 1'A (Table 1). The first 18 bases from the 3' end of oligonucleotide 1'A are the exact complement of zip code 1, and 1'A is labeled with fluorescein at the 3' end and biotin at 5' end. Following hybridization, the master was thoroughly rinsed, and the fluorescence micrograph shown in Figure 1a was obtained. Uniform fluorescence emission from the master surface confirms homogeneous hybridization of oligonucleotide 1'A to the zip code master.

Panels b and c of Figure 1 are fluorescence micrographs of the master and replica, respectively, following replication. Fluorescence intensity is clearly transferred from the surface of the master to the replica after contact. The checkerboard pattern results from drainage canals (20 μm on center, 10 μm wide, and 3 μm deep) present on the replica surface that direct buffer solution away from the contact area during replication. Control experiments showed that these canals were essential for successful DNA transfer. Specifically, if both the master and the PDMS were dry, then no transfer of DNA was observed. Additionally, no transfer was observed in the absence of drainage canals regardless whether buffer was present. We suspect that, in the absence of drainage canals, solvent trapped between the two surfaces prevents molecular contact between the biotin-functionalized oligonucleotides on the master and streptavidin present on the replica. Figure 1f provides a quantitative representation of the data shown in panels b and c of Figure 1. After replication, the contrast between the light and dark areas on the master (~1300 counts, Figure 1b) is very



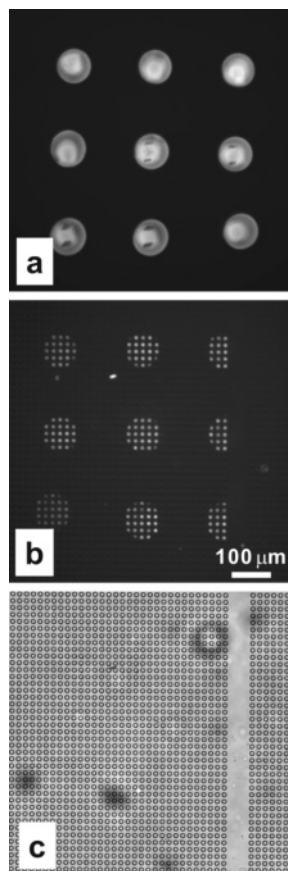
**Figure 1.** Fluorescence micrographs demonstrating transfer of fluorescein-labeled DNA from a master slide to a PDMS replica surface. (a) The master slide modified with zip code 1 (Table 1) and hybridized to fluorescein- and biotin-labeled oligonucleotides 1'A whose code sequence is complementary to zip code 1. (b) The master slide after transfer. (c) The PDMS replica after transfer. (d) The second replica obtained after rehybridization of the master with oligonucleotide 1'A. (e) The third replica obtained after rehybridization of the master with oligonucleotide 1'A. (f) Fluorescence intensity profiles obtained along the dashed lines shown in frames a–e. For clarity, profiles c and d are offset by 6000 and 3000 counts, respectively. The image integration time was 30 s for all frames. The gray scale is 5000–25 000 counts for a and b, and 4500–6500 counts for c, d, and e.

close to that of the replica (~1100 counts, Figure 1c), indicating that only a small fraction of the DNA is lost during transfer.

Panels d and e of Figure 1 show the second and the third replicas obtained from the same master after rehybridization with oligonucleotide 1'A labeled with fluorescein and biotin. The contrast between the light and dark areas for the three consecutive replicas is 1100, 900, and 1200 counts, respectively, indicating good reproducibility and that there is no progressive loss of DNA from the master after formation of three replicas.

The data presented thus far indicate that replication is a consequence of molecular contact and binding between the biotin groups present on the CodeLink slide and streptavidin on the PDMS replica surface. Because in the current experiment the binding force between biotin and streptavidin is stronger than between DNA base pairs,<sup>6,7</sup> the DNA duplexes separate, and the biotin-functionalized oligonucleotides transfer to the replica surface. For very long DNA duplexes, however, it is important to separate the two surfaces slowly to avoid breaking the biotin/streptavidin bond. That is, the force required to separate a DNA duplex is independent of its length if the separation rate is appropriately controlled.<sup>6,7</sup>

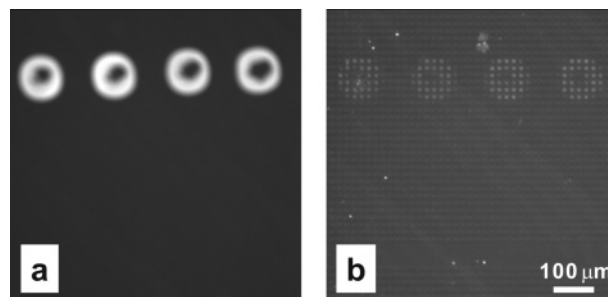
For single-oligonucleotide replicas, the spot size is defined by the spacing of the canals on the replica surface. For example,



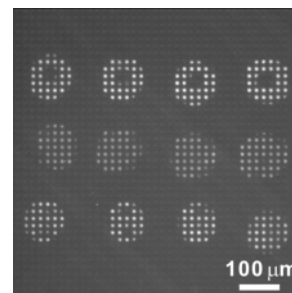
**Figure 2.** Micrographs demonstrating replication of a  $3 \times 3$  master array having just one DNA zip code. (a) A fluorescence micrograph obtained from a master array spotted with zip code 1 (Table 1) and subsequently hybridized to fluorescein- and biotin-labeled oligonucleotide 1'A whose code sequence is the complement of zip code 1. (b) A fluorescence micrograph obtained from the PDMS surface after replication of the master. (c) An optical micrograph of the replica surface showing the drainage canals. The integration time for both a and b was 30 s. The gray scale is 2000–20000 counts for frame a, and 2000–8000 counts for frame b. In frame b, all three spots in the right column are cut off because they happen to intersect a major drainage canal as shown in the optical image, frame c.

each replica spot shown in panels c–e of Figure 1 is  $10 \mu\text{m} \times 10 \mu\text{m}$ , which is comparable to the smallest feature sizes obtained by in situ synthesis ( $\sim 8 \mu\text{m}$ ),<sup>14</sup> and much smaller than those obtained by ex situ spotting ( $\sim 75 \mu\text{m}$ ).<sup>13</sup> However, for replicas patterned with multiple DNA oligonucleotides, the important size parameter is defined by the dimensions of the master, not the replica. Stellacci and co-workers previously demonstrated masters and replicas having feature dimensions as small as  $50 \text{ nm}$ .<sup>5</sup>

To demonstrate replication from a master array instead of from a homogeneous surface, a microarrayer was used to print a  $3 \times 3$  array of nine  $\sim 100 \mu\text{m}$ -diameter spots of zip code 1 (Table 1), and then the master array was copied onto a PDMS replica surface using the procedure discussed earlier for Figure 1. Figure 2a is a fluorescence micrograph obtained from the master after hybridization with fluorescein-labeled and biotin-functionalized DNA sequence 1'A (Table 1). The presence of fluorescence, which is absent prior to hybridization, confirms hybridization of the code sequence. The fluorescence micrograph shown in Figure 2b was obtained from the PDMS replica surface after conformal contact of the two substrates. The  $3 \times 3$  array observed on the replica (Figure 2b) exactly



**Figure 3.** Fluorescence micrographs demonstrating accurate replication of a master having multiple zip codes. (a) A  $4 \times 3$  master array having three zip codes (row 1, zip code 1; row 2, zip code 2; and row 3, zip code 3; Table 1) after hybridization with fluorescein- and biotin-labeled oligonucleotides 1'A whose code sequence is only complementary to zip code 1. (b) A PDMS replica of the master showing only one row of transferred oligonucleotides. The integration time for both a and b was 30 s. The gray scale is 5000–13 000 counts for a, and 5000–8000 counts for b.



**Figure 4.** Fluorescence micrograph demonstrating replication of multiple functional oligonucleotides. First, a  $4 \times 3$  master array having three zip codes (row 1, zip code 1; row 2, zip code 2; and row 3, zip code 3; Table 1) was prepared and hybridized by exposure to a solution containing a mixture of three nonfluorescent, biotin-functionalized oligonucleotides: 1'A, 2'B, and 3'C (Table 1) whose code sequences are complementary to zip code 1, zip code 2, and zip code 3, respectively. After replication, the resulting PDMS surface was exposed to a mixture of fluorescein-labeled targets A', B', and C' that are complementary to the functional sequences of 1'A, 2'B, and 3'C, respectively. The integration time was 30 s, and the gray scale is 5000–8000 counts.

mirrors the master array (Figure 2a), except for the presence of the drainage canals. An optical image of the replica surface (Figure 2c) shows the drainage design of the replica. We have successfully replicated master arrays having up to 100 elements using this procedure: however, they are not shown here because of the limited field of view of the CCD camera used in these experiments.

#### Replication from a Master Having Multiple Zip Codes.

It is important to demonstrate that replication is successful for masters having multiple zip codes. To demonstrate this function, a  $4 \times 3$  master array containing three different zip codes was prepared using a microarrayer. Each row is composed of four spots having a nominal diameter and edge-to-edge distance of  $\sim 100 \mu\text{m}$ . With reference to Table 1, the first, second, and third rows correspond to zip codes 1, 2, and 3, respectively.

Hybridization was carried out for at least 4 h with  $10 \mu\text{M}$  fluorescein-labeled and biotin-functionalized oligonucleotide 1'A (Table 1), which has a code sequence that only matches zip code 1, and afterward fluorescence was observed only from the four spots in the first row (Figure 3a). This result clearly shows that the zip code master correctly directs the proper code sequence to the appropriate location on the master.<sup>1,2</sup> Following replication (Figure 3b), fluorescence is only observed from the top row of spots, corresponding to zip code 1. This confirms

that only the correct functional sequence is transferred to the replica surface.

**Preparation and Functionality of Replica Microarrays Having Multiple Sequences.** Here, we set out to demonstrate that a master array having multiple zip codes could direct placement of multiple codes, that multiple code/functional sequences could be transferred to the replica, and that the replica functional sequences are active. The experiment demonstrating these three points was carried out as follows. First, a  $4 \times 3$  master array having three zip codes was prepared as described for Figure 3. A solution containing a mixture of three nonfluorescent, biotin-functionalized oligonucleotides (1'A, 2'B, and 3'C, Table 1;  $10 \mu\text{M}$  each) was introduced onto the master surface for at least 4 h. The code sequence of each of the three oligonucleotides is complementary to exactly one of the zip codes present on the master surface. Thus, oligonucleotides 1'A, 2'A, and 3'A are directed to zip codes 1, 2, and 3, respectively. Following replication, the replica array was exposed to a solution containing a mixture of three fluorescein-labeled targets (A', B', and C', Table 1;  $10 \mu\text{M}$  each) for at least 4 h. Each target was chosen to match the functional sequence of one of the three oligonucleotides present on the replica surface. The fluorescence image obtained from the replica clearly shows a  $4 \times 3$  array (Figure 4). This experiment demonstrates that a replica array

having multiple sequences can be prepared and used for hybridization-based applications.

## Conclusion

In this work we demonstrated an efficient and accurate method for replication of DNA microarrays from a zip code master. For arrays containing multiple DNA sequences, the replica spots can be as small as  $100 \mu\text{m}$ . Three consecutive replications from the same master were successfully achieved with no significant decrease of oligonucleotide density on the replica surface. Replication from a  $4 \times 3$  master array having three zip codes proved to be accurate, and there was no observable cross-reactivity. Future experiments will focus on larger-scale arrays, smaller spot sizes, and replication of more complex biological materials (proteins and viruses) and inorganic nanomaterials.

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