

Parallel Fabrication of RNA Microarrays by Mechanical Transfer from a DNA Master

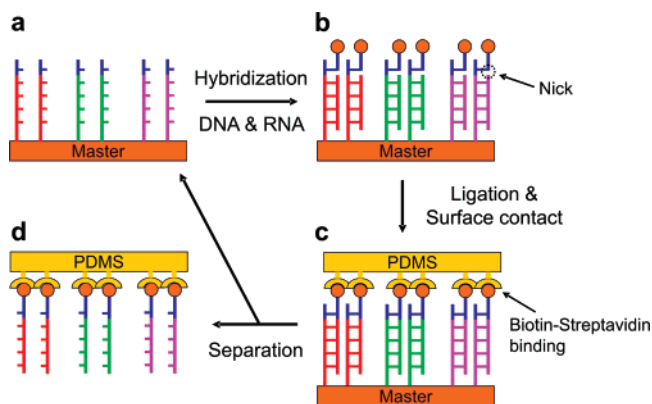
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A new method for fabrication of RNA microarrays is described. The approach involves cohybridization of a short, biotinylated DNA oligonucleotide and an RNA probe sequence to DNA templates spotted onto a master array. Next, the short DNA sequence and the RNA probe are linked using a T4 DNA ligase. Finally, a poly(dimethylsiloxane) (PDMS) monolith modified on the surface with streptavidin is brought into conformal contact with the master array. This results in binding of the biotinylated DNA/RNA oligonucleotides to the PDMS surface. When the two substrates are mechanically separated, the DNA/RNA oligonucleotides transfer to the PDMS replica, and the DNA oligonucleotides remaining on the master array are ready to template another RNA replica array. This sequence can be repeated for at least 18 cycles using a single master array. RNA arrays consisting of up to three different oligonucleotide sequences and consisting of up to 2500 individual $\sim 70 \mu\text{m}$ spots have been prepared.

In this paper we describe a simple means for parallel conversion of DNA master arrays into RNA replicate arrays. The approach is based on a surface enzymatic reaction followed by mechanical transfer. The details of the approach are shown in Scheme 1. First, amine-modified ssDNA templates are immobilized on an epoxy-modified glass slide. The distal ends of all the DNA templates are configured to be identical. Second, this master slide is exposed to single-sequence biotinylated anchor ssDNA, which hybridizes to the distal ends of the templates (the blue sequence in Scheme 1), and to the unmodified probe ssRNA, which is complementary to each unique ssDNA sequence comprising the master array. Third, the nick between the anchor ssDNA and the probe ssRNA is ligated using a T4 DNA ligase.^{1–3} Next, a streptavidin-coated poly(dimethylsiloxane) (PDMS) monolith is brought into conformal contact with the master. This results in binding of the biotin anchor (now linked to the RNA probe) to the streptavidin-modified PDMS surface. When the PDMS monolith is mechanically separated from the master, the RNA array is

Scheme 1



transferred to the PDMS surface while the DNA templates remain on the master surface. This series of steps can be repeated many times without loss of fidelity, resulting in multiple RNA replicate arrays from a single master.

Previously, we showed that an approach similar to that illustrated in Scheme 1 could be used to replicate DNA microarrays.^{4,5} In another case, the replicate oligonucleotides were formed in situ using a surface polymerase reaction.⁶ A related method, which relies on dehybridization by heating rather than by mechanical transfer, has been reported by Stellacci and co-workers.^{7–9} Remarkably, they showed that DNA lines as thin as 50 nm could be replicated.⁸ Kim et al. have recently used this method to replicate DNA arrays fabricated on nylon membranes.¹⁰

RNA microarrays are a powerful tool for the analysis of nucleic acids and proteins. For example, ultrasensitive detection of DNA oligonucleotides was reported using RNA microarrays in conjunction with the enzyme RNase H.^{11,12} The use of RNA aptamer microarrays also allowed simultaneous detection of multiple

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Table 1. Sequences of the Nucleic Acids Used in This Study^a

name	sequence
template DNA I (D _T I)	5'-TTT TTT TTT TTT TTT TTT TTG CAA GCC CCA CCT AGA CCG CAG AG-3AmM-3'
template DNA II (D _T II)	5'-TTT TTT TTT TTT TTT TTT TTT AGC ATT AGG TAC GTC ATT ACA GT-3AmM-3'
template DNA III (D _T III)	5'-TTT TTT TTT TTT TTT TTT TTC GTA AGT TCA GCA CAG TAT GAC CC-3AmM-3'
probe RNA I (R _P I)	5'-CUC UGC GGU CUA GGU GGG GCU UGC-3'
probe RNA II (R _P II)	5'-ACU GUA AUG ACG UAC CUA AUG CUA-3'
probe RNA III (R _P III)	5'-GGG UCA UAC UGU GCU GAA CUU ACG-3'
probe RNA I with dye (R _P I with dye)	5'-DY547-CUC UGC GGU CUA GGU GGG GCU UGC-3'
probe RNA II with dye (R _P II with dye)	5'-DY547-ACU GUA AUG ACG UAC CUA AUG CUA-3'
anchor DNA (D _A)	5'-5Phos-AAA AAA AAA AAA AAA AAA AA-3BioTEG-3'
anchor DNA without PO ₄	5'-AAA AAA AAA AAA AAA AAA AA-3BioTEG-3'
target DNA I	5'-GCA AGC CCC ACC TAG ACC GCA GAG-3Cy3Sp-3'
target DNA II	5'-TAG CAT TAG GTA CGT CAT TAC AGT-3Cy5Sp-3'

^a The abbreviations in the table correspond to the following modifications: 3AmM, an amino modifier on the 3' end of the DNA; DY547, a Cy3 alternate dye attached to the 5' end of the RNA; 5Phos, phosphorylation of the 5' end of the DNA; 3BioTEG, a biotin modifier with a tetraethyleneglycol (TEG) spacer on the 3' end of the DNA; 3Cy3Sp, a Cy3 dye attached to the 3' end of the DNA; 3Cy5Sp, a Cy5 dye attached to the 3' end of the DNA. These are the same notations used by the DNA supplier (Integrated DNA Technologies, Coralville, IA) and the RNA supplier (Dharmacon Inc., Lafayette, CO).

proteins¹³ and demonstrated the potential for diagnosis of cancers.^{14,15} RNA microarrays are normally fabricated by tethering modified RNA oligonucleotides on functionalized surfaces, for example, biotinylated RNA oligonucleotides on streptavidin-functionalized glass slides^{13,16,17} or thiol-modified RNA oligonucleotides on maleimide-terminated gold surfaces.^{11,12} Recently, Corn and co-workers showed that it was possible to prepare RNA microarrays using surface ligation chemistry.^{3,18,19} This approach made it possible to attach unmodified ssRNA to a DNA array, which converts the DNA array to an RNA array. They also showed that the DNA array could be used for at least three ligation–hydrolysis cycles.³

In this paper, we expand the scope of our mechanical-transfer approach for replicating DNA arrays by showing that a related series of steps can be used to prepare many RNA replica arrays from a single DNA master using unmodified RNA oligonucleotides. The key step is a surface ligase reaction, first reported by Lee et al.,³ required to link the anchor DNA to the RNA oligonucleotides. This step is carried out using a T4 DNA ligase. We show that the series of steps illustrated in Scheme 1, which proceed under mild conditions, can be executed at least 18 times using a single DNA master array without loss of fidelity of the replicate RNA array. RNA microarrays consisting of 2500 spots and consisting of up to three different RNA sequences were prepared, and no evidence of cross-hybridization was detected.

EXPERIMENTAL SECTION

Chemicals. Streptavidin–maleimide conjugates (Sigma S9415), 3-mercaptopropyltrimethoxysilane (MPS) (Fluka 63800), and other chemicals for buffers or blocking solutions were obtained from

Sigma-Aldrich: 20× saline–sodium citrate (SSC) buffer (Sigma S6639), 10% sodium dodecyl sulfate (SDS) solution (Sigma L4522), sodium phosphate monobasic (Sigma S0751), sodium phosphate dibasic (Sigma S0876), Triton X-100 (Sigma T8787), Trizma base (Sigma T6791), Trizma HCl (Sigma T6666), ethanolamine (Sigma E9508), 2-mercaptoethanol (Sigma M6250), and *N*-ethylmaleimide (Sigma E3876). The poly(dimethylsiloxane) precursor solution (Sylgard 184) was ordered from Dow Corning Inc. (Midland, MI). T4 DNA ligase (M0202S) provided with 10× reaction buffer (500 mM TRIS–HCl, 100 mM MgCl₂, 100 mM DTT, and 10 mM ATP; pH 7.5 at 25 °C) was used as received from New England BioLabs Inc. (Ipswich, MA). Nuclease-free water was obtained from Fermentas Inc. (Hanover, MD). DNA and RNA oligonucleotides were obtained from Integrated DNA Technologies Inc. (Coralville, IA) and Dharmacon Corp. (Lafayette, CO), respectively. The sequences and modifications are provided in Table 1.

Instrumentation. A fluorescence microscope (Nikon TE2000, Nikon Co., Tokyo, Japan) equipped with appropriate filter sets (filter no. 31002 for DY547 and Cy3 and 41008 for Cy5, Chroma Technology Corp., Rockingham, VT), a mercury lamp (X-Cite 120, Nikon Co), and a CCD camera (Cascade, Photometrics Ltd., Tucson, AZ) was used to acquire optical and fluorescence micrographs. Micrographs were processed using V++ Precision Digital Imaging software (Digital Optics, Auckland, New Zealand). High-density arrays were scanned using a microarray scanner (GenePix 4000B, Molecular Devices Corp., Sunnyvale, CA).

Fabrication of Master DNA Arrays. The master DNA arrays were fabricated using epoxy-modified glass slides (Nexterion Slide E, SCHOTT North America Inc., Elmsford, NY) as previously described.^{4,5} Briefly, template DNA solutions (25 μM in 50 mM sodium phosphate buffer, pH 8.5) were spotted onto the glass slides using either a micropipette or a home-built robotic microarrayer. Next, the spotted slide was incubated in a chamber in which the humidity was in equilibrium with a saturated NaCl solution at 20–25 °C. After incubation, the slide was washed as follows (at 20–25 °C): 1 × 5 min in 0.1% Triton X-100 solution, 2 × 2 min in 1 mM HCl solution, 1 × 10 min in 100 mM KCl solution, and 1 × 1 min in Milli-Q water (18 MΩ·cm, Millipore, Bedford,

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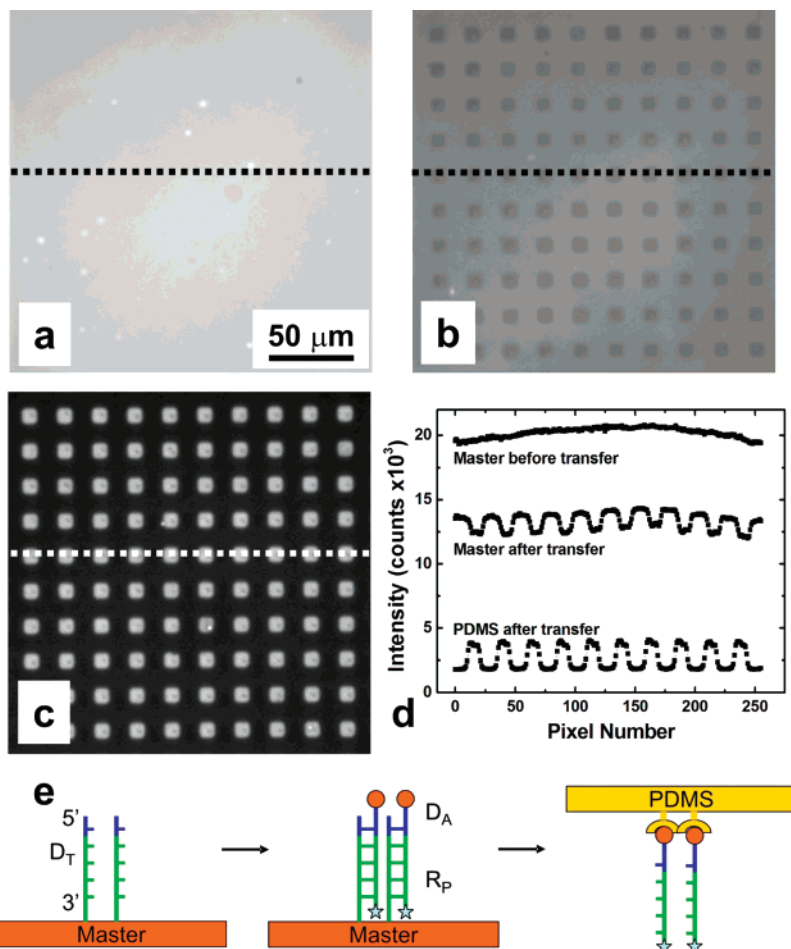


Figure 1. Demonstration of the replication process illustrated in Scheme 1. (a) Fluorescence micrograph obtained from the master after cohybridization and ligation of a short, biotinylated DNA (D_A , Table 1) oligonucleotide and a labeled RNA probe (dye-labeled R_P I, Table 1) onto a DNA template (D_T I, Table 1). (b) Fluorescence micrograph obtained from the master after transfer of the ligated DNA/RNA oligonucleotide to the replica surface. (c) Fluorescence micrograph obtained from the PDMS replica surface after transfer of the ligated DNA/RNA oligonucleotide. (d) Fluorescence intensity profiles obtained along the dashed lines shown in parts a–c. (e) Scheme showing the experimental approach used to obtain the data in parts a–d. The star symbols represent the fluorescent dye. The integration time was 100 ms. The gray scales were 5000–25 000 counts for parts a and b and 1500–5000 counts for part c.

MA). The slide was then placed in a blocking solution (50 mM ethanolamine and 0.1% SDS in 0.1 M TRIS buffer, pH 9.0) for 15 min at 50 °C. After washing with Milli-Q water for 1 min, the slide was blown dry with a N_2 stream and stored under dark and dry conditions.

Fabrication of RNA Replica Arrays. The RNA arrays were fabricated by simultaneously exposing the DNA master array to the anchor DNA oligonucleotide, the RNA probe, and the T4 DNA ligase for 1 h at 25 °C. Specifically, the ligase reaction mixture contained the anchor DNA strands (0.5 μ M), the probe RNA strands (0.5 μ M), and T4 DNA ligase (20 units/ μ L) in a ligase reaction buffer (1 \times : 50 mM TRIS–HCl, 10 mM $MgCl_2$, 10 mM DTT, and 1 mM ATP; pH 7.5 at 25 °C). Incubation chambers (CoverWell, Grace Bio-Labs Inc., OR) were used to ensure uniform spreading of the reaction mixture on the surface. Following ligation, the master slide was rinsed with buffer solutions (at 20–25 °C): 2 \times SSC buffer containing 0.2% SDS and 2 \times SSC buffer. The master slide was washed again as follows (at 20–25 °C): 10 min in 2 \times SSC buffer containing 0.2% SDS, 10 min in 2 \times SSC buffer, and 10 min in 0.2 \times SSC buffer. Next, 4 \times SSC buffer (10 μ L) was dropped on the master to wet the surface, and then

a streptavidin-functionalized PDMS monolith was brought into contact with the surface. A pressure of 1.4 N/cm² was applied at 20–25 °C for 10 min. Note that preparation of the streptavidin-functionalized PDMS monolith was described previously.^{4–6} Finally, the PDMS monolith was peeled off the master surface at a constant separation speed (400 μ m/s) using a linear motion actuator (CMA-25CC, Newport Corp., Irvine, CA), and then both surfaces were washed in buffer and blown dry.

RESULTS AND DISCUSSION

Surface Ligation and Transfer of Ligated RNA Strands.

The fluorescence micrographs shown in Figure 1 demonstrate the viability of the replication procedure shown in Scheme 1. The specific approach for this experiment is illustrated in Figure 1e. Template DNA (D_T I; Table 1) immobilized on the master slide was exposed to a ligase reaction mixture composed of biotinylated anchor DNA (D_A ; Table 1), fluorescently labeled probe RNA (dye-labeled R_P I; Table 1), and a T4 DNA ligase. This resulted in hybridization and ligation of the anchor DNA and probe RNA on the master surface. The ligated RNA/DNA conjugates were then transferred to a PDMS surface.^{4–6}

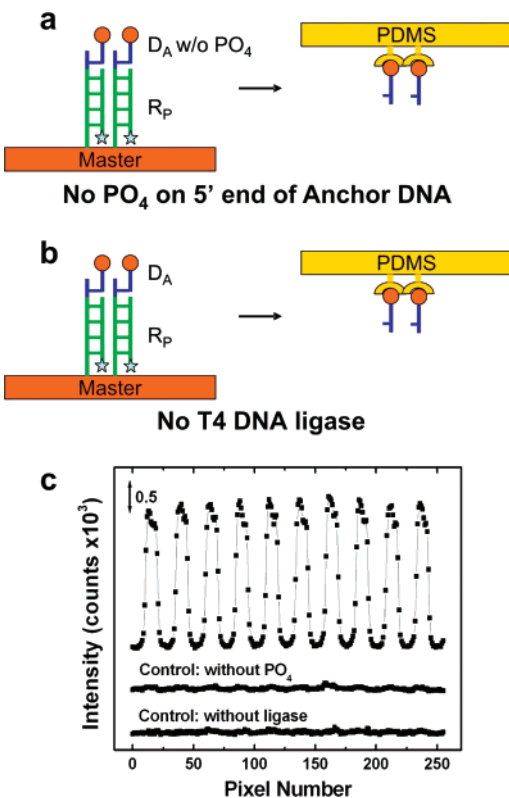


Figure 2. Control experiments confirming replication. (a) Illustration of a control experiment in which a master surface was treated identically to that shown in Figure 1e but in the absence of a 5'-phosphoryl group on the anchor DNA (anchor DNA without PO_4 , Table 1). (b) Illustration of a control experiment in which a master surface was treated identically to that shown in Figure 1e but in the absence of treatment with the T4 DNA ligase. (c) Comparison of fluorescence intensity profiles obtained for the two control experiments (parts a and b) with the profile along the dashed white line shown in Figure 1c (top fluorescence profile). The integration time was 100 ms.

Figure 1a is a fluorescence micrograph obtained from the glass master after exposure to the ligase reaction mixture and subsequent washing. The fluorescence intensity in this micrograph indicates that the labeled probe RNA hybridized with the template DNA. There was no detectable level of fluorescence when the master DNA template was treated identically to the surface shown in Figure 1a but using probe RNA II (dye-labeled R_P II, Table 1) which is not complementary to template DNA I. Parts b and c of Figure 1 are fluorescence micrographs obtained from the glass master and PDMS surface, respectively, after transfer of the ligated RNA strands. Drainage canals ($20 \mu\text{m}$ on center, $10 \mu\text{m}$ wide, and $3 \mu\text{m}$ deep) were microfabricated on the PDMS surface as reported previously^{4–6} to direct buffer solution away from the glass/PDMS interface during conformal contact (Supporting Information, Figure S1). The drainage canals restrict contact between the glass and PDMS surfaces to multiple square areas ($10 \times 10 \mu\text{m}^2$), which results in the grid pattern present on both surfaces. The light areas on the PDMS surface in Figure 1c correspond to transfer of fluorescently labeled probe RNA from the darker regions apparent in Figure 1b. Figure 1d shows fluorescence intensity profiles obtained along the dotted lines in Figure 1a–c. Importantly, the average intensity difference between the bright and dark regions on the master surface (1540 ± 140

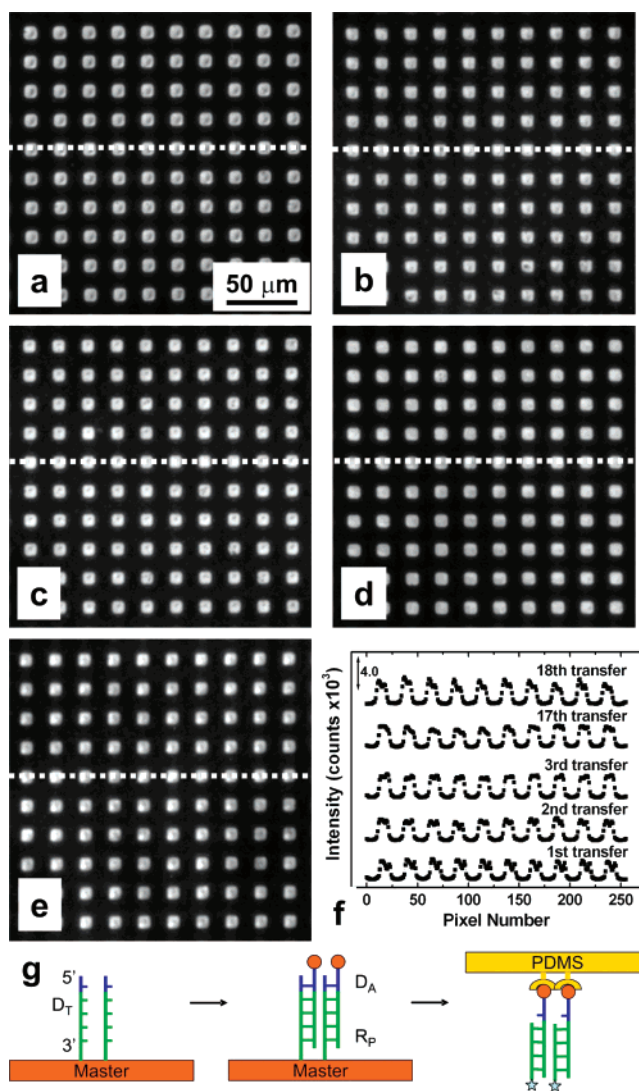


Figure 3. Results showing that at least 18 replicas can be prepared from a single DNA master. (a) Fluorescence micrograph obtained from a PDMS replica surface after hybridization of D_A and R_P I (Table 1) onto the master DNA template, ligation, transfer of the DNA/RNA conjugate to PDMS, and subsequent hybridization of a fluorescently labeled target (target DNA I, Table 1) complementary to the RNA sequence (but not to the sequence of anchor DNA). (b–e) Same as part a, but after 2, 3, 17, and 18 iterations. (f) Fluorescence intensity profiles obtained along the dotted white lines shown in parts a–e. (g) Scheme illustrating the experimental approach used to obtain the data in parts a–f. The star symbols represent the fluorescent dye. The integration time was 100 ms. The gray scale was 1800–5000 counts for parts a–e.

counts, Figure 1b) is close to the intensity difference measured from the PDMS surface (2010 ± 60 counts, Figure 1c), suggesting no significant net loss of ligated RNA during transfer.

It is important to demonstrate that the fluorescence shown in Figure 1c results from transfer of probe RNA strands ligated to the biotinylated anchor DNA rather than from nonspecific adsorption of unligated RNA on the PDMS surface. Accordingly, we carried out the two key control experiments illustrated in Figure 2, parts a and b. Specifically, fluorescence micrographs were obtained from PDMS surfaces treated identically to that shown in Figure 1c but in the absence of the 5'-phosphoryl group of the anchor DNA (anchor DNA without PO_4 , Figure 2a and Table 1)

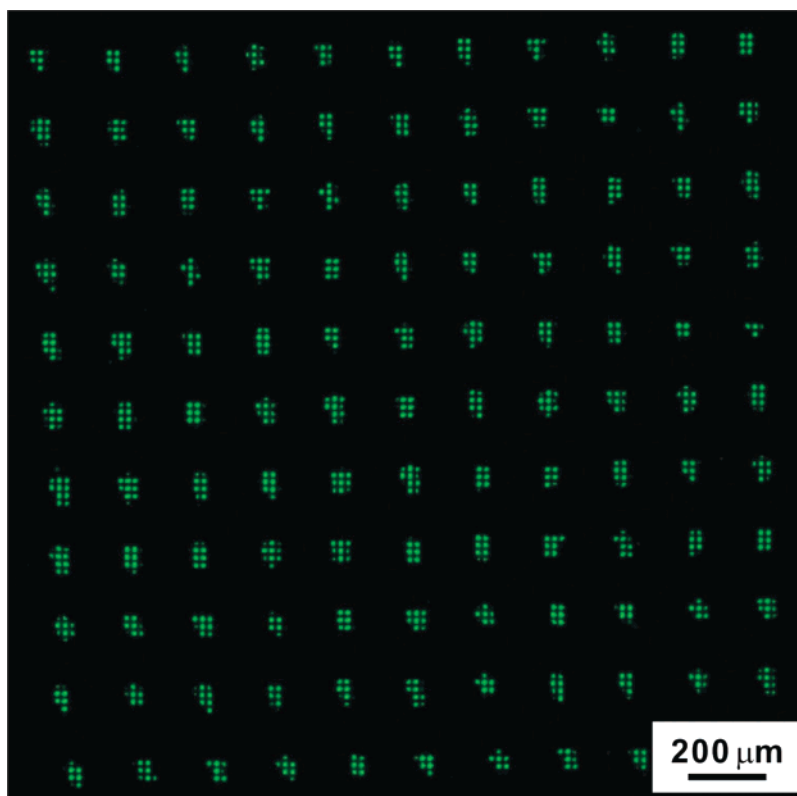


Figure 4. Fluorescence micrograph obtained from an RNA microarray (PDMS surface), which was fabricated using a master DNA array (D_T I) having 2500 microscale spots (nominally $\sim 70 \mu\text{m}$ in diameter). For clarity, only 121 of the 2500 spots are shown. The data were obtained after hybridization of D_A and R_P I onto the master DNA template I, ligation, transfer of the DNA/RNA conjugate to PDMS, and subsequent hybridization of a fluorescently labeled target (target DNA I, Table 1) complementary to the RNA sequence (but not to the sequence of anchor DNA).

and in the absence of the T4 DNA ligase (Figure 2b), respectively. In the absence of 5'-phosphoryl group of the anchor DNA or the T4 DNA ligase, the ligation of the anchor DNA to the probe RNA is not expected to proceed. Indeed, fluorescence micrographs obtained from the PDMS surfaces after these two control experiments were carried out indicated no detectable fluorescence from the PDMS surface after contact with the master. In Figure 2c, fluorescence intensity profiles from these two control experiments are compared to the profile of the replica surface shown in Figure 1c. On the basis of these results, we conclude that both the 5'-phosphoryl group of the anchor DNA and the T4 DNA ligase are required to transfer RNA to the PDMS surface and that there is no detectable level of nonspecific adsorption of RNA on the PDMS.

Multiple Transfers of Ligated RNA Strands from a Single Master. Multiple transfers of ligated RNA strands from a single master to different replica surfaces were demonstrated using the approach shown in Figure 3g. In contrast to the experiments used to obtain the results shown in Figures 1 and 2, the surface ligase reaction here was carried out using unlabeled probe RNA (R_P I, Table 1), and consequently the resulting master surface is not fluorescent. However, after transfer of the nonfluorescent and ligated RNA strands, the PDMS surface was exposed to fluorescently labeled target DNA (target DNA I, Table 1) complementary to the probe RNA sequence but not to the anchor DNA. Figure 3a is a fluorescence micrograph obtained from a PDMS surface after the ligation, transfer, and hybridization steps. The presence of the bright spots indicates that the unlabeled RNA sequence

transferred to the replica surface and that the RNA is functional; that is, it is able to bind fluorescently labeled complementary DNA.

Control experiments analogous to those described earlier indicate no detectable level of fluorescence on the PDMS surfaces in the absence of the 5'-phosphoryl group of the anchor DNA or the absence of the T4 DNA ligase during the ligation step (Supporting Information, Figure S2).

This fabrication cycle, consisting of ligation, transfer, and hybridization with labeled, complementary DNA, was repeated a total 18 times using the same master. Micrographs corresponding to the first, second, third, seventeenth, and eighteenth cycles are presented in Figure 3a–e, respectively. As shown in the fluorescence line scans in Figure 3f, the contrast in fluorescence between the light and dark areas on the surfaces of these replicas were 1860 ± 130 , 2070 ± 180 , 2310 ± 90 , 2120 ± 180 , and 2460 ± 530 counts, respectively. These data indicate that there is no significant or progressive degradation of the master up to the eighteenth round of replication. Note that the master slide was stable, and produced replicas indistinguishable from those shown in Figure 3, for more than 1 month when stored in dark and dry conditions.

Fabrication of an RNA Microarray. In this section we show that it is possible to prepare large-scale RNA microarrays consisting of a single probe RNA sequence (R_P I, Table 1) using the hybridization, ligation, and transfer steps discussed in the context of Figure 3. This was accomplished as follows. First, a master DNA array having 2500 spots (each $\sim 70 \mu\text{m}$ in diameter) of template DNA I (D_T I, Table 1) was fabricated using a robotic

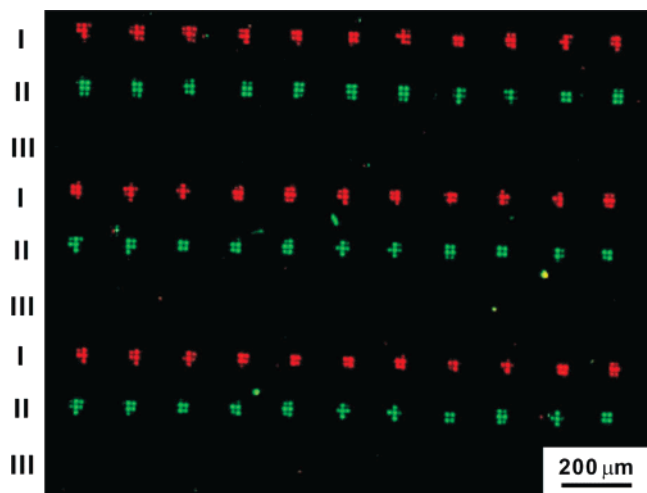


Figure 5. Fluorescence micrograph obtained from an RNA replica array prepared from a master having three different template sequences (D_T I, D_T II, and D_T III; Table 1) in consecutive rows. After ligation and transfer, the PDMS surface was exposed to a mixture of fluorescently labeled DNA targets (target DNA I and target II, Table 1) complementary to the sequence of each RNA probe (R_P I and R_P II, Table 1). The numerals to the left of the micrograph indicate which template sequence is present in that row. As an internal control experiment, the array was not exposed to the DNA complement of R_P III and, hence, that row appears dark in the micrograph.

microarrayer. The T4 DNA ligase reaction was performed on the master surface using unlabeled probe RNA (R_P I, Table 1) as shown in Figure 3g. Finally, the ligated RNA strands were transferred to a PDMS surface. Figure 4 is a fluorescence micrograph obtained by scanning a part of the PDMS replica surface using a microarray scanner after hybridization of target DNA I (Table 1) and washing. The micrograph shows that all 121 RNA spots on the PDMS surface are active toward hybridization of labeled target DNA I. The pixelated appearance of the individual spots is a consequence of the drainage canals present on the PDMS surface.

Fabrication of an RNA Microarray Comprised of Multiple Probe Sequences. RNA microarrays having multiple probe sequences were fabricated using the approach illustrated in Figure 3g. A master DNA array consisting of three different template sequences (D_T I, D_T II, and D_T III; Table 1) and a total of 1500 spots was fabricated using a robotic microarrayer. The three different DNA templates were spotted in consecutive rows as shown in Figure 5. After hybridization and ligation of a mixture of unlabeled RNA probes (R_P I, R_P II, and R_P III; Table 1) on the master, the probe RNA strands were transferred to a PDMS replica surface. Finally, the PDMS surface was exposed to a mixture of fluorescently labeled DNA targets (target DNA I and target DNA II; Table 1) which are complementary to probe RNA I (R_P I) and probe RNA II (R_P II), respectively. Note that only two

different target sequences were introduced onto the PDMS surface. Figure 5 is a fluorescence micrograph obtained by scanning a section of the PDMS surface. It shows that the correct, labeled DNA complements hybridized to the appropriate probe RNA sequences. That is, Cy3-labeled DNA I hybridized with R_P I, Cy5-labeled DNA II hybridized with R_P II, and neither of the labeled DNA targets hybridized with R_P III. No cross-hybridization was observed. As indicated earlier, the pixelation of the spots is a consequence of the presence of the drainage canals.

SUMMARY AND CONCLUSIONS

In this report, we described a method for fabrication of RNA microarrays utilizing a surface ligase reaction and mechanical transfer. Eighteen replicas were prepared from a single master array with no detectable degradation of activity of the resulting replica RNA array or the master DNA array. Finally, a master DNA array consisting of three different sequences was prepared and faithfully replicated as a functional RNA microarray with no observable cross-hybridization. This approach provides a robust means for fabricating RNA microarrays in parallel and with no requirement for RNA modification (for example, with biotin). Finally, this report expands the scope of this general approach for microarray fabrication from DNA to RNA. At present we are examining the possibility of using the same general strategy for preparing replicas of protein arrays.

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SUPPORTING INFORMATION AVAILABLE

Information about the control experiments described in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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