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Parallel Production of Oligonucleotide Arrays Using Membranes and Reagent Jet Printing

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ABSTRACT

Here, we present a low-cost method to produce compact arrays using microporous materials and reagent jetting. Oligonucleotides are immobilized on membrane sheets as a series of lines. The membrane sheet is then rolled and bound, and the spiral structure is cut like a "jelly roll" to produce identical arrays. The spiral arrays behave much like larger formats using membranes, and hybridization detection can be accomplished using standard signal-generation mechanisms. The method is particularly useful for producing identical arrays from pre-synthesized oligonucleotides.

INTRODUCTION

There is a growing need for production of nucleic acid arrays with uniform and reproducible binding properties. Applications include allele-specific oligonucleotide (ASO) hybridization (4), reverse dot blot (14), sequencing by hybridization (SBH) (6), enhanced SBH (3), expression analysis (13) and organization of cDNA libraries (5). The use of membranes to carry out hybridization analysis is well documented (1), and the microporous materials currently available have been optimized for such applications. However, the membrane-based arrays produced to date are quite large, on the order of 22 × 22 cm, and analysis requires large volumes of reagents. Many methods have been advanced to produce "microarrays" with a high spatial density and thousands of binding zones (2,7–9, 11,12,15). In many cases, the arrays are constructed on solid supports to produce "chips" that only provide a small number of immobilized oligonucleotides at each array site for the hybridization reaction (16). As a result, the signals generated by such methods require expensive and slow-scanning systems to detect hybridization of the target. In addition, many chip methods are extremely costly to adapt for basic research purposes.

This report describes a method of making microarrays using membrane supports and reagent printing of nucleic acids. Because the method uses the same materials and signal-generation schemes used by larger-format arrays, much of the knowledge base can be applied to the compact spiral device.

MATERIALS AND METHODS

Jetting and Immobilization of Oligonucleotides

Oligonucleotides used in these experiments were obtained from Ransom Hill Bioscience (Ramona, CA, USA) and correspond to the normal and mutant sequence for cystic fibrosis G551D. The sequences for the immobilized probe oligonucleotides are amine-5′-AGTGGAGGTCAACGA-3′ (normal) and amine 5′-AGTGGAGATCAAC-GA-3′ (mutant), which differ by a single base change of G to A. The amino group was included to potentially allow covalent immobilization of the oligonucleotide to a membrane support. The target sequences are the 15-mer complimentary sequences labeled at the 5′ end with biotin. All 4 syntheses were 0.2 µmol, reverse-phase desalted and gave measured values of 19–20 optical density (OD) at 260 nm.

Jetting was carried out using piezoelectric (PZT) actuated delivery (7) and thermal ink-jet printing. Thermal ink printing used an Apple StyleWriter™ II (Apple Computer, Cupertino, CA, USA) wherein the ink cartridge (Cannon BC-02; Cannon Instrument, State College, PA, USA) was disassembled, the ink sponge and ink removed, and 70 µL of DNA solution were introduced into the entry port for the jet head. For thermal printing, the oligonucleotides were diluted 1:20 into 10× phosphate-buffered saline (PBS), pH 7.4 (Sigma Chemical, St. Louis, MO, USA) and 7% isopropanol. For PZT printing, the oligonucleotides were diluted 1:20 into 10× PBS, and 10 µL were loaded into the jet head. Jetting rates for PZT ink-

jet printing were about 0.1 µL/cm and 0.03 µL/cm for thermal ink-jet.

The lines of probe DNA were printed on Predator™ membrane (Pall Gelman Sciences, Port Washington, NY, USA). The membrane is a modified polyethersulfone with a pore size large enough to accommodate colloidal labels. As in the case of chips (18), the amine-labeled probes were immobilized by drying the printed membrane at 60°–80°C for 30 min to 24 h, with substantially the same results throughout the conditions specified. The mechanism of immobilization is not known; however, it is relatively stable, as hybridized target (with gold colloidal label) can be "stripped" from the arrays by an 80°C wash, and sufficient probe remains bound to allow at least one more hybridization.

Generation of Arrays

The sheets of immobilized probe DNA (2-cm wide × 30-cm long) are loosely wrapped around silicon rods (4-mm outer diameter × 4-cm long), then rolled repeatedly between the thumb and forefinger while applying pressure, and the spiral naturally wraps itself into a tightly wound structure. The spiral is then bound with adhesive tape. The spiral bundle, 8-mm outer diameter and 2-cm in length, is placed into a plastic block with a hole drilled slightly larger than 8 mm to support the bundle during cutting. A 1-mm length of the bundle is exposed and cut using a Schick® double-edge razor blade to generate each array. The process is repeated, and with care, about 20 arrays (each 1-mm thick) can be generated from the 2-cm-long bundle. In contrast to a sawing approach, the blade method generates zero waste. Figure 1 outlines this procedure.

Hybridization and Detection

Hybridization was carried out with a 1:100 dilution of the biotinylated target DNA (12 µM) into a hybridization buffer of 1% casein, 5 mM Tris, pH 8.0, 13.8 mM NaCl and 0.27 mM KCl (all chemicals in this section are from Sigma Chemical). First, the array was hydrated and washed with 150 µL of hybridization buffer. Excess buffer was removed with a paper towel, 30 µL of the diluted target were applied to the surface of the array and incubated for 10 min at room temperature (25°C). Next, 30 µL of a 1:1000 dilution of ExtrAvidin® Alkaline Phosphatase Conjugate (Sigma Chemical) in hybridization buffer were applied, and excess liquid was allowed to flow through the array into an absorbent pad. After a 10-min incubation, the array was rinsed with 150 µL of hybridization buffer to remove unbound enzyme, and 30 µL of the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate were added. Color development was stopped after a 1–2-min incubation by rinsing the array with 200 µL of 1/10 Tris-buffered saline, pH 8.0.

For real-time melting, hybridized dot blots were incubated with an ExtrAvidin-FITC conjugate (1:100 dilution in hybridization buffer) and then with an anti-fluorescein isothiocyanate (FITC) gold conjugate (1:1 dilution of conjugate with hybridization buffer), each for 10 min. After signal development, the blots were washed with hybridization buffer at increasing temperatures, and signal intensity was monitored at 30 frames/s. Gold colloid was made by reduction of 0.5 mM AuCl with 1 mM sodium citrate (10). The gold conjugate was made by mixing 3 µL of mouse monoclonal anti-FITC (1 mg/mL) with 1 mL of colloid.

All images were recorded with a Memorex® video camera attached to a stereo microscope and digitized to 8-bit resolution using a VideoSpigot® Frame Grabber (Creative Labs, Milpitas, CA, USA) in a 486 computer. Densitometry of the gray-scale images was carried out with NIH Image software Version 1.55 on a Macintosh® Performa 475 computer (Apple Computer) (software available from **ftp://zippy.nimh.nih. gov/pub/ nih-image**).

RESULTS

To demonstrate the capability of the method, about 200 lines of normal and mutant 15-mer probes were printed on opposite sides of a membrane to produce 400 element arrays. Figure 2 shows a typical result from hybridization at low temperature with either normal or mutant targets (i.e., zones on both sides of the membrane give 400 signals). Printing on both sides was possible because the probe penetration was less than half the membrane thickness of about 140 µm. More stringent washes with warm hybridization buffer eliminated cross-hybridization, so the arrays had 200 spots present on a single side of the membrane (data not shown).

Figure 3A shows the results from an unrolled section of the array membrane after hybridization and signal development as compared to the ink-printed pattern on paper (Figure 3B). The thermal ink-jet provided a very fast method of printing 160-µm-wide DNA lines (200 in ca. 10 s). Some variation in DNA line density and the presence of
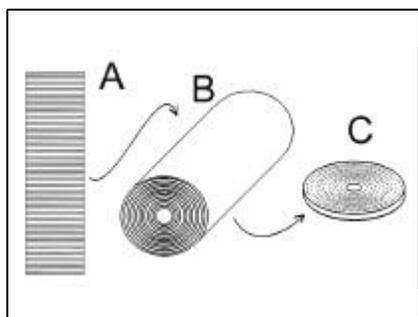
Figure 1. Spiral array construction. (A) Lines of reagent are printed in parallel on a membrane support. (B) The sheet is rolled onto a support rod, reagent lines parallel to the cylindrical axis and bound with an adhesive tape sheath. (C) Individual arrays are generated by cutting slabs from the spiral bundle. Reagents are placed on top of the array and allowed to flow through. Alternatively, the array is soaked in the various solutions.
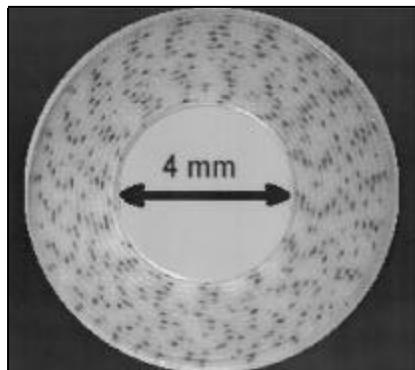
Figure 2. Medium density spiral array. About 200 lines of normal and mutant G551D probes were printed on opposite sides of the membrane using a thermal ink-jet. The hybridization pattern shown is obtained from either a normal or a mutant target at low stringency (result from a normal target is shown). Detection is with alkaline phosphatase and BCIP/NBT.

smaller "satellite" lines was noted, whereas such variations were not observed in the ink-printed lines. Further optimization of the DNA "ink" with respect to density, viscosity and alcohol content would likely improve the jetting quality. Nevertheless, the thermal ink-jet approach appears to be a viable method of dispensing oligonucleotides.

Although in a spiral pattern, the zones can be analyzed using a standard charge-coupled device (CCD) camera with the appropriate magnification. Figure 4 shows the results from a partial array generated with PZT jetting. The jetted lines were about 300-μm wide and extended halfway through the membrane. PZT jetting produced lines that were more uniform in density and shape as compared to the thermal ink-jet. As in larger formats, the various probes require different hybridization temperatures for optimum discrimination. Results show that increasing the stringency reduces cross-hybridization of a single base mismatch, and the cross-hybridization shown in Figure 4, B and C can be eliminated by further increases in hybridization temperature with some loss in signal strength for the exact match interactions.

One advantage of microporous supports for array fabrication is the large amount of signal that is generated by enzymatic or microparticle detection methods. A signal that is clearly visible by eye or a standard video camera (with the appropriate magnification) can be generated using colloidal labels. Because the label is directly coupled to the hybridized target, stringency can be changed in real-time to optimize resolution. Figure 5 shows melting curves obtained from a reverse dot blot on a flat sheet using a gold colloid label. Melting temperatures estimated from the 0.5 levels are: 44.5° and 33°C for normal:normal and normal:mutant hybridizations (Figure 5A); and 36° and 44°C for mutant:normal and mutant:mutant hybridizations (Figure 5B). The corresponding results for solution melting of similar oligonucleotide pairs in 15 mM NaCl are: 47°, 34°, 37° and 43°C, respectively (18). The ability to vary stringency in real-time allows for optimum hybridization conditions when targets have different GC contents (17,18). The real-time melting ob-

served in the dot blot is also possible with the spiral array, and a complete study of pure and various ratios of mixed normal:mutant oligonucleotides is underway for comparison to prior work using wave guides (17).

DISCUSSION

The results show the spiral array behaves much like the larger flat-sheet arrays; no attempt was made to optimize sensitivity or specificity in the present
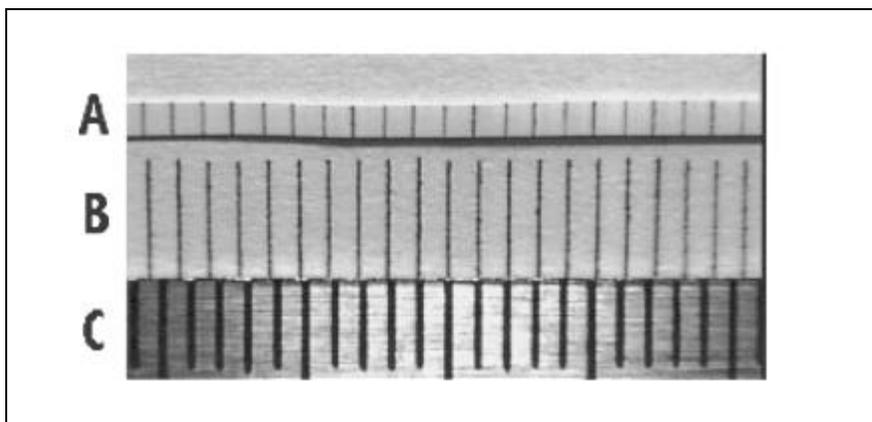

Figure 3. Thermal ink-jet printing. (A) The array developed as in Figure 2 was unrolled to expose the printed reagent lines on the membrane support. (B) The same pattern printed on paper with the standard ink cartridge. (C) A ruler with 1-mm divisions.
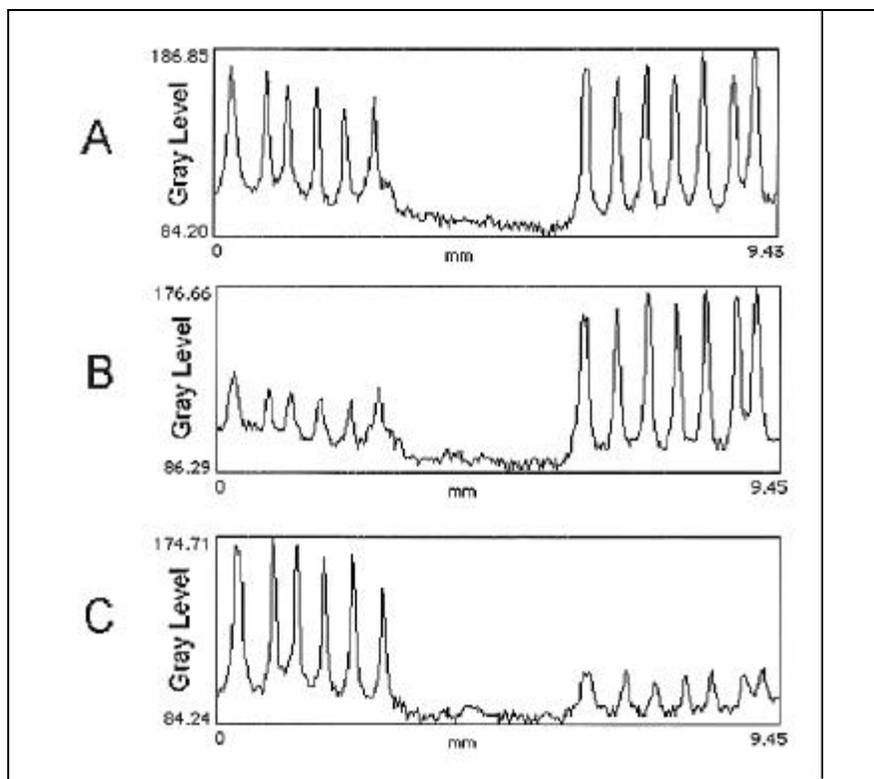

Figure 4. Densitometry analysis of arrays generated by PZT jetting. Arrays were made with 6 mutant and 7 normal probe lines, both applied to the same side of the membrane. The arrays were hybridized at low stringency (25°C) and then washed with warm hybridization buffer before adding the enzyme conjugate. The gray-scale image was scanned by drawing a curved line through the array zones with NIH Image software and plotting the profile of gray level vs. distance. (A) Pattern from normal target at low stringency (pattern from mutant target the same), reading from left, peaks 1–6 are mutant probes, and peaks 7–13 are normal. (B) Pattern from normal target, 47°C wash. (C) Pattern from mutant target, 42°C wash.

study. One strength of the spiral array method is that it makes use of materials, equipment and detection methods that are readily available at relatively low cost. A membrane sheet with 8- × 10-in dimensions is able to hold at least 1270 lines of binding reagent, each 100 μm in width and 100 μm apart. Higher densities may be possible with improvements in jetting technology or use of other dispensing methods. About 200 arrays can be generated from the 8- × 10-in sheet by hand cutting.

A rough estimate of spiral array cost can be made from parameters used in this study. The amine-labeled oligonucleotide cost was $71.25 for 100 μL. Jetting of the 1/20 diluted DNA at a
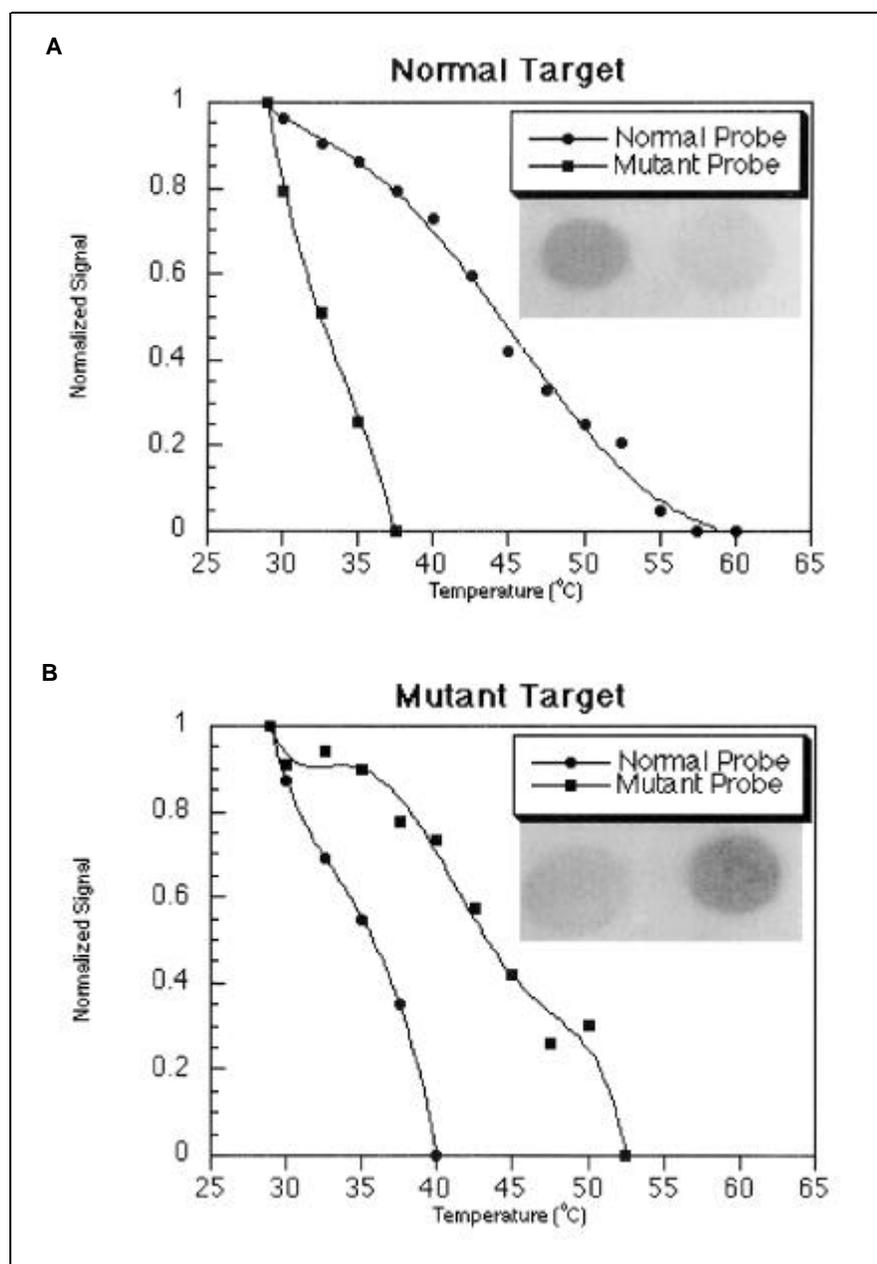
Figure 5. Real-time control of hybridization stringency. Signals on hybridized reverse dot-blot membranes were generated with gold colloid and then subjected to manual washes with buffer of increasing temperature at 2.5°C increments. Melting of target from the zones was recorded as it occurred with a standard CCD camera. Raw gray-level data were normalized (17), and the smooth curves are the result of a polynomial fit. Inset pictures show the 1-mm diameter spots at 30°C, with normal probe on left and mutant probe on right.

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rate of 0.03 µL/cm yields 10 arrays and thus, \$0.00011 per array spot. Compared to chip methods, oligonucleotide reagents for a 65 536 array would cost \$7.00. A factor of 10 or more improvement in cost is anticipated by printing thinner reagent lines, cutting thinner arrays (<1 mm) and/or printing discontinuous lines of reagents. The main technical requirement for implementation is a printing method such as reagent jetting—a reagent "pen" or manual spotting of oligonucleotides to form a line. On a small scale, 10–100 arrays are easily generated by hand and can be useful in the research setting.

CONCLUSION

Current trends in chip development have tended toward greater densities and smaller feature size to account for the required redundancy in signal acquisition. Applications are beginning to emerge that only require a few hundred to a few thousand probes for detection of known mutations. At this scale, the jelly-roll method with pre-synthesized oligonucleotides appears to be an effective means of producing arrays. One anticipated use of the method is the development of diagnostic tests for the detection of human genetic disease. With a limited investment of resources, the spiral array provides an easy method for the generation of arrays in the research setting. Although the method is at a rudimentary stage, we trust a new dimension has been added to the production of oligonucleotide arrays.

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