JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Flexible Microarray Construction and Fast DNA Hybridization Conducted on a Microfluidic Chip for Greenhouse Plant Fungal Pathogen Detection

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This study employed a microfluidic method in which probe creation does not require pin-spotting and fast hybridization is conducted on the same microarray chip for the detection of three greenhouse pathogens (*Botrytis cinerea*, *Didymella bryoniae*, and *Botrytis squamosa*). In this method, 16 oligonucleotide probe line arrays were created on a glass substrate by a microfluidic printing method. Then, low amounts of the DNA samples (1 fmol of oligonucelotides or 1.4 ng of PCR products) were introduced into the microchannels that were orthogonal to these probe lines. The hybridizations of 16 samples (21-mer complementary oligonuleotides and ~260 bp PCR products) were fulfilled at the channel−probe line intersections and in a short time (minutes). The optimization of probe immobilization and sample hybridization are described in detail. The method successfully detected and discriminated between two 260 bp PCR products with a one-base-pair difference from closely related greenhouse plant fungal pathogens (*B. cinerea* and *B. squamosa*).

**KEYWORDS: Microfluidic microarray; DNA hybridization; fungal pathogen; single base-pair difference discrimination**

## INTRODUCTION

Plant diseases from fungal, bacterial, and viral organisms have caused serious economic losses in greenhouse vegetable industry annually (*1, 2*). Effective disease control requires rapid identification of disease microorganisms. Currently, DNA microarray chips have been widely used in various applications, such as expression profiling, genotyping, and species characterization (*3*). For example, Xu et al. and Bordoni et al. have identified genetically modified soybean and maize using oligonucleotide microarrays (*4, 5*). Warsen et al. and Ronning et al. discriminated between fish pathogens or closely related crops, respectively, with microarray techonology (*6, 7*). The conventional DNA microarrays are generally constructed either by on-chip synthesis of oligonucleotide probes or by spotting of presynthesized probes on activated substrates (*3, 8*). For assays, samples containing the labeled target were applied by manually spreading 10–50 $\mu$L of solutions on the microarray area for hybridization, and the process usually requires long incubation times of up to 16 h (*9, 10*).

Because the microfluidic method is capable of reducing the sample volume and accelerating diffusion and reaction kinetics, DNA hybridization has been conducted on oligonucleotide probes spotted within microchannels (*11–14*). Other than spotting, the probe creation has also been achieved using a microfluidic method. For instance, Liu et al. designed a glass chip containing four line arrays of oligonuclecotide probes, and parallel hybridizations of human genomic DNA targets were completed by using a second chip consisting of four sample microfluidic channels (*15*). Furthermore, Lee et al. created a 3 × 3 array on a gold substrate and conducted microfluidic hybridizations to detect RNA fragments derived from a transgenic plant, *Arabidopsis thaliana* (*16*). Situma et al. have achieved the detection of two different low-abundant DNA point mutations in *KRAS2* oncogenes with poly(methyl methacrylate) and polycarbonate microfluidic chips (*17*). Moreover, Benn et al. studied the mass transfer efficiency and hybridization kinetics inside microfluidic channels using 60-mer oligonucleotide samples on an 8 × 8 array (*18*). In all of these approaches, the number of probes to be printed is limited. However, in applications such as pathogen detection or mutation studies, a small set of probes is sufficient, and so a low-density DNA microarray can be constructed, as compared to the high-density microarrays needed for large-scale gene expression profiling.

To date, the application of the microfluidic microarray method to agricultural problems has been limited. Here, we dubbed the method as microfluidic micorarray assembly (MMA) and a 16 × 16 DNA array was designed to aim at fast assays. In this work, we use the MMA technique successfully to identify three PCR products prepared from the plant fungal pathogens *Botrytis cinerea*, *Botrytis squamosa*, and *Didymella bryoniae*, which cause various greenhouse crop diseases. The first two *Botrytis* amplicons are different from each other, with only a one-base-pair difference in the center. We employed two 21-mer

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10.1021/jf0721242 CCC: $37.00 
Published on Web 11/30/2007

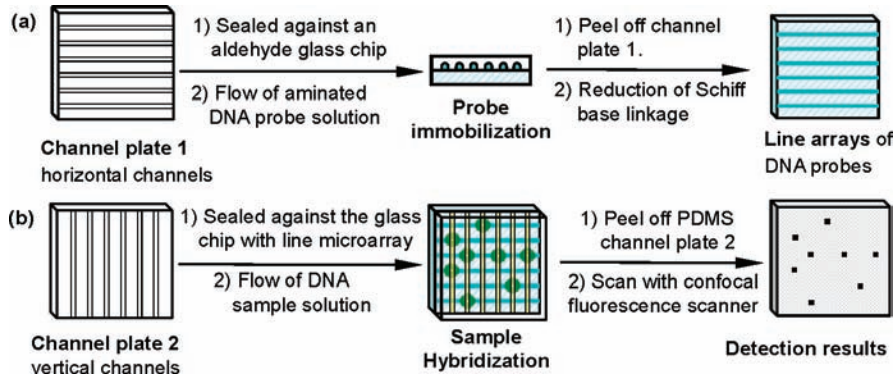



**Figure 1.** Microfluidic microarray method using straight microchannels: (**a**) creation of a DNA probe line array on an aldehyde-modified glass slide via straight microchannels; (**b**) hybridization of DNA samples in straight channels orthogonal to the straight probe lines printed on the glass slide.

**Table 1.** Oligonucleotides and PCR Products Used in This Study

| Acronym | Length | Sequence (5'-3') |
|---|---|---|
| AB | 21-mer | $NH_2$-$(CH_2)_6$ –CGC CAG AGA ATA CCA AAA CTC |
| ALB | 21-mer | $NH_2$-$(CH_2)_{12}$ –CGC CAG AGA ATA CCA AAA CTC |
| AD | 22-mer | $NH_2$-$(CH_2)_6$ –CGC CGA TTG GAC AAA ACT TAA A |
| ADF | 22-mer | $NH_2$-$(CH_2)_6$ –CGC CGA TTG GAC AAA ACT TAA A-Fluorescein |
| B'F | 21-mer | Fluorescein –GAG TTT TGG TAT TCT CTG GCG |
| D'F | 21-mer | Fluorescein –T TTA AGT TTT GTC CAA TCG GCG |
| D'C | 21-mer | Cy5–T TTA AGT TTT GTC CAA TCG GCG |
| B'PF | 264-bp | Fluorescein –…–GAG TTT TGG TAT TCT CTG GCG–…<br>… –CTC AAA ACC ATA AGA GAC CGC–… |
| BN'PF | 264-bp | Fluorescein –…–GAG TTT TGG T**T**T TCT CAG GCG–…<br>… –CTC AAA ACC A**A**A AGA GTC CGC–… |
| D'PF | 259-bp | Fluorescein –…–T TTA AGT TTT GTC CAA TCG GCG–…<br>… –A AAT TCA AAA CAG GTT CGC CGC–… |

oligonucleotide probes and managed to distinguish the PCR products with the microfluidic microarray method. It has been demonstrated that 1.4 ng of PCR products (~260 bp, 1.4 ng/$\mu$L, 1 $\mu$L) was detected at 50 °C and one-base-pair discrimination was achieved in 5 min. The results obtained from the printed glass slide in this work showed an improvement over our previous work (*10*), in which hybridizations were detected only when the probes were spotted on agarose-coated glass slides.

Here, the MMA method consists of two steps of an assembly process (see **Figure 1**). In the first step, channel plate 1 is assembled with the glass chip via reversible bonding. Aminated DNA probes are introduced into the microchannels and are immobilized on the glass chip. A line microarray of probes is thus created. After plate 1 is peeled off, channel plate 2 is then assembled with the same glass chip. The sample solution that flows through the microchannels will intersect the line microarray, and hybridization is accomplished in a few minutes. Both the creation of the probe microarray and the hybridization process in microfluidic channels are capable of reducing the sample volume ($<1$ $\mu$L) and preventing evaporation and cross-contamination. In this work, it was found that the MMA method not only provides a flexible probe creation method but also enhances the detection sensitivity and achieves differentiation of various greenhouse pathogens.

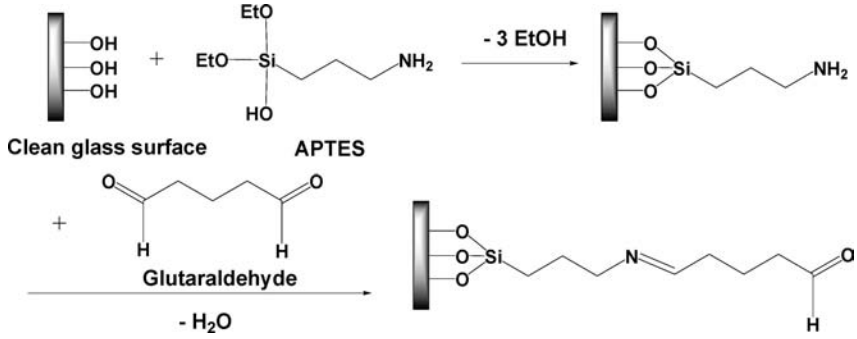
## MATERIALS AND METHODS

**Materials.** 3-Aminopropyltriethoxysilane (APTES), 50% glutaraldehyde, sodium dodecyl sulfate (SDS), and Triton X-100 were obtained from Sigma-Aldrich. Ultrapure water (18 M$\Omega$ cm$^{-1}$) was obtained from an Easypure RF purification system (Dubuque, IA). Negative photoresist (SU-8 50) and its developer were purchased from MicroChem Corp. (Newton, MA). An elastomer base, Sylgard 184 silicone, and its curing agent used to make polydimethysiloxane (PDMS) were obtained from Dow Corning Corp. (Midland, MI). The plain 3 in. $\times$ 2 in. glass microscope slides were purchased from Fisher Scientific Co. (Ottawa, ON, Canada). All other chemicals and solvents were purchased from BDH Tech Inc. (Toronto, ON, Canada) and used without further purification.

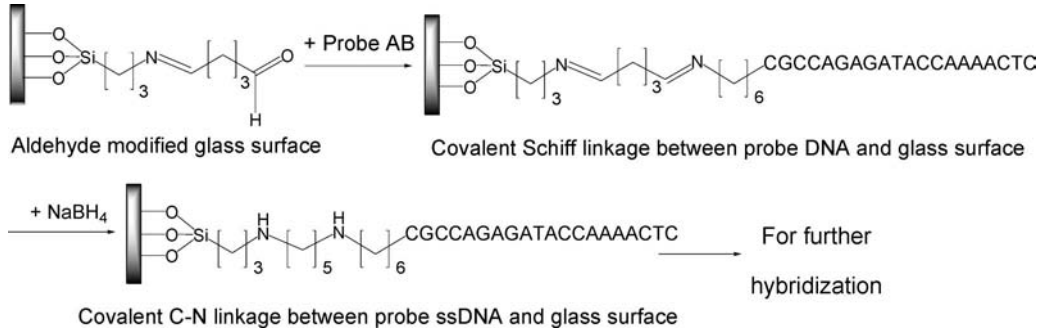
All oligonucleotides were synthesized and modified by Sigma-Genosys (Oakville, ON, Canada). The sequences of probe oligonucleotides were designed to detect two greenhouse plant pathogens, *Botrytis cinerea* (with probes AB and ALB) and *Didymella bryoniae* (with probe AD) (*9, 10*). The probes were modified with an amine group at the 5′-end. In AB and AD molecules, this amine group was spaced from their DNA sequences with a C6 linker, whereas a C12 linker was used in ALB. Dual-labeled probe ADF, which has the same DNA sequence as probe AD, has its 3′-end also labeled with fluorescein. The probe was used as a marker in probe immobilization. Oligonucleotide samples B′F and D′F, which are complementary to the sequences of probes AB and AD, respectively, were labeled with fluorescein at the 5′-end. D′C has the same sequence as D′F but was labeled with Cy5 dye at the 5′-end. The sequences of oligonucelotides used and their acronyms are listed in **Table 1**.

Genomic DNA samples were extracted from cucumber−dextrose broth of *B. cinerea*, *D. bryoniae*, and *B. squamosa* at Agriculture and Agri-Food Canada (*10*). Three PCR products (B′PF, 264 bp; D′PF, 259 bp; and BN′PF, 264 bp) were amplified and labeled with fluorescein from the three species, respectively. The concentration of B′PF and D′PF was ~40 ng/$\mu$L (~250 nM), whereas that of BN′PF was lower at about 6 ng/$\mu$L (~40 nM). The central sequences of the sense strand

**Scheme 1.** Surface Modification To Generate Aldehyde-Functionalized Glass Slides




**Scheme 2.** Covalent Attachment of Aminated Probe DNA to Aldehyde Glass Surface




of B′PF and D′PF are complementary to the sequences of probes AB and AD, respectively, whereas BN′PF has a one-base-pair difference (TTT:ATA instead of TAT:ATA in the center) from B′PF (*20*).

**Surface Modification of Glass Chips.** The glass substrates were chemically modified to produce aldehyde-functionalized surfaces using an established procedure (see **Scheme 1**) (*21*). Briefly, plain glass slides were cleaned with a 10% NaOH solution for 10 min at ~100 °C. After being rinsed with distilled water, the slides were treated with a piranha solution (70:30 v/v sulfuric acid to 30% hydrogen peroxide) for 1 h at ~80 °C. The slides were then rinsed with water and dried under a stream of nitrogen.

The cleaned slides were treated with a mixture of ethanol/$H_2O$/APTES (95:3:2 by volume) for 2 h under stirring, rinsed with 95% ethanol and deionized $H_2O$, dried under nitrogen, and baked at ~120 °C for 1 h. The aminated glass slides were then immersed in 5% glutaraldehyde in a 10× phosphate-buffered saline (PBS) solution overnight and washed with acetone and deionized $H_2O$. After being dried in a nitrogen gas stream, the aldehyde-modified glass slides were stored in a dark place at 4 °C before probe printing.

**Fabrication of PDMS Channel Plates.** A 2 in. × 2 in. PDMS channel plate was fabricated using an established photolithographic method (*22*). The channel pattern was designed using Visual Basic (Microsoft) and was printed on a transparency to create the photomask at a resolution of 3368 dpi. Molding masters were fabricated in a modular clean room (577 series, Clean Air Products, Minneapolis, MN). First, a 4 in. silicon wafer was spin-coated with a layer of SU-8 photoresist by a spin coater (WS-400, Laurell Technologies Corp., North Wales, PA). Then the channel patterns were created on the SU-8 coated wafer with the photomask using a UV exposure system (model LS-150-3, Bachur & Associates, San Jose, CA). The SU-8-coated wafer was developed to produce the molding master. PDMS prepolymer was cast against the molding master and cured at 50 °C for 12 h to yield an elastomeric channel plate. The width of the straight channels was 300 $\mu$m, and the channel height was 20 $\mu$m. The length of the straight section of each channel was 30 mm. Solution reservoirs (1 mm in diameter) at both ends of channels were punched on the PDMS channel plate using a flat-end syringe needle.

**Probe Line Array Creation.** As shown in **Figure 1a**, the PDMS channel plate was sealed against the aldehyde glass slide. Then, 0.8 $\mu$L of probe DNA prepared in the spotting solution (1.0 M NaCl + 0.15 M $NaHCO_3$) was added into the inlet reservoirs using a micropipet. The probe solution was filled through the channels by applying vacuum pumping at the outlets. With incubation at room temperature for 30 min, covalent Schiff linkage was formed between the amine ends of the probe oligonucelotides and the aldehyde groups on the glass surface (**Scheme 2**) (*23*). After the microchannels had been washed with 1 $\mu$L of washing solution (0.15% Triton-X 100, 1.0 M NaCl, and 0.15 M $NaHCO_3$), the PDMS channel plate was then peeled off and the glass slide was chemically reduced with a $NaBH_4$ solution (100 mg of $NaBH_4$ dissolved in 30 mL of 1× PBS and 10 mL of 95% EtOH) for 15 min to reduce the Schiff linkage to the more stable C−N single bond (**Scheme 2**). The glass chip was then rinsed with deionized water for 2 min and dried by nitrogen gas and was ready for hybridization. As an immobilization control, ADF was used, and this was manifested as continuous probe lines shown in **Figure 3**. All procedures were conducted at room temperature.

**Sample Hybridization and Microarray Scanning.** In DNA target hybridization, the glass chip with probe line arrays was covered with a PDMS channel plate. The straight channels were orthogonal to the printed probe lines on the slide (as shown in **Figure 1b**). The DNA samples (oligonucelotides or PCR products) were prepared in the hybridization buffer (4× SSC + 0.2% SDS, unless stated otherwise). The PCR products were denatured at 95 °C for 4 min and then quickly cooled in an ice–water bath just before hybridization. DNA targets (1.0 $\mu$L) were added to the inlet reservoirs using a micropipet. Sample solutions in different reservoirs were then pumped into channels by vacuum suction simultaneously applied at the 16 outlets. The flow of DNA targets and their binding on the immobilized probes in a microchannel are shown in **Scheme 3**. Two methods were used to control the hybridization temperature inside the microchannels. In the continuous-flow mode, a Peltier device (CP1-12715, Thermal Enterprises, Kendall Park, NJ) was placed under the glass slide assembly and the hybridization temperature was adjusted by tuning the voltage applied to the Peltier device. In the stop-flow incubation mode, the assembly was incubated in a humidity box placed in an oven at a specified temperature. Hybridization was achieved between complementary targets in solution and probe lines at the intersections, showing the hybridization patches of 300 $\mu$m × 300 $\mu$m. The microchannels were rinsed immediately with 2 $\mu$L of hybridization buffer following hybridization.

Following the hybridization and washing procedures, the glass slide was scanned on a confocal laser fluorescent scanner (Typhoon 9410, Molecular Dynamics, Amersham Biosystems). The resolution is 25 $\mu$m. The excitation wavelength was 488 or 633 nm for fluorescent-labeled

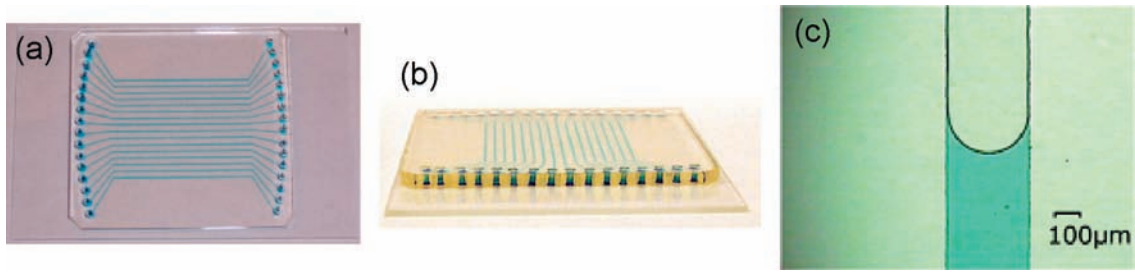



**Figure 2.** Images of the assembly of a 2 in. × 2 in. PDMS channel plate on a 3 in. × 2 in. glass slide: (**a**) top view, 16 channels filled with blue dye solutions; (**b**) side view showing the inlet reservoirs filled with dye solution; (**c**) microscopic view of a straight channel partially filled with the dye solution.

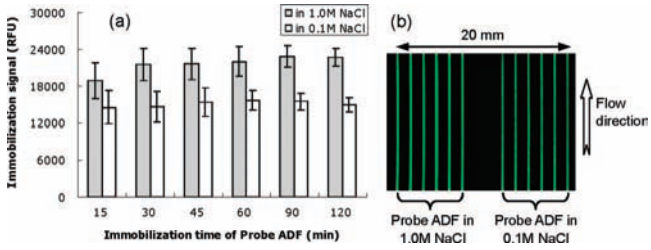
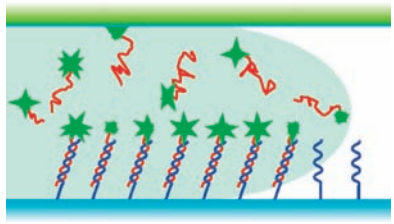



**Figure 3.** Probe immobilization: (**a**) Effect of ionic strength of spotting solutions and immobilization time on the ADF signal. The probe line arrays were made by incubating 0.8 $\mu$L of 25 $\mu$M ADF prepared in 1.0 M NaCl (gray bar) or 0.1 M NaCl (white bar) in microchannels at different durations. The slide was chemically reduced and then washed with distilled water. The fluorescent signals were measured by scanning the slide at 488 nm. (**b**) Fluorescent image of ADF probe lines (vertical green stripes) printed on the glass slide using different immobilization times. The brighter the probe lines, the stronger the fluorescent signals.

**Scheme 3.** Microfluidic Hybridization of Target DNA Strands (in Red) in the Parabolic Liquid Front to the Probe DNA Strands (in Blue) Immobilized on the Glass Chip Surface



or Cy5-labeled samples, respectively. The photomultiplier tube (PMT) voltage was set to 600 V. The scanned image was analyzed by ImageQuant 5.2 software. In the data quantification procedure, square frames (13 pixels × 13 pixels) were overlaid on the square hybridization patches in the image. The average fluorescent signals of the 169 pixels were measured in relative fluorescent units (RFU).

## RESULTS AND DISCUSSION

**Flexible Probe Immobilization Without Spotting.** In conventional microarrays using the robotic spotting method, it usually took 8–16 h for probe immobilization to complete after spotting (*9*). To reduce solvent evaporation during this process, a humidity chamber was used and DMSO might have to be added in the probe solution. The latter may either increase the spot size on the slide or reduce the actual amount of DNA fixed on the solid support (*24*). In the microfluidic printing method, the probe solution, which was confined to the PDMS microchannels, would not dry out even in several hours. Furthermore, shrinking of the channel dimensions to microscale decreased the diffusion time and hence the incubation time needed to complete probe immobilization (*25, 26*). To study the effect of immobilization time on the signal intensity, a dual-labeled oligonucleotide probe (ADF) was flowed through and incubated in the microchannels for different durations. It was found that the fluorescent intensity was higher with an immobilization time of 30 min than with 15 min, as shown in **Figure 3a**. When immobilization time was >30 min, the signal intensity from immobilized probes did not increase by >3%. Thus, 30 min was considered to be enough to achieve effective probe immobilization. The effect of solution ionic strength on probe immobilization was also studied with the same chip. In the spotting buffer containing 1.0 M NaCl, we observed a greater ADF signal intensity, as compared with that obtained in 0.1 M NaCl (**Figure 4a**). Our observation is consistent with the previously reported findings by Peterson et al. that single-stranded DNA is less adsorbed using a buffer of a low salt concentration (*27*). This is because the charged DNA strands are better electrostatically shielded under the high ionic strength condition (*28*), and so it could be easier for aminated oligonucleotides to come into contact and to react with the aldehyde groups on the glass surface.

The effect of the probe concentration on probe immobilization was also studied. As shown in **Figure 4a**, the fluorescent intensity is much higher using 25 $\mu$M than 10 $\mu$M ADF, but the rate of increase is reduced when the probe DNA concentration goes from 25 to 400 $\mu$M. It was found that the signal obtained at 25 $\mu$M DNA probes has reached around 60% of the value obtained from 400 $\mu$M probes. Apparently, the amount of immobilized oligonucleotides kept increasing with the higher probe concentration, and the results did not generate an optimized probe concentration for microfluidic probe printing. Further experiments were carried out by measuring the hybridization signals obtained from these probe lines. Here, complementary oligonucleotides (D′C) labeled with a second fluorescent tag, Cy5, were used to distinguish the hybridization signals (at 633 nm) from the immobilization signals (at 488 nm). It is clear from **Figure 4b** that the hybridization signal has reached its highest value with probe lines prepared with 50 $\mu$M ADF, and the amount of hybridized samples decreased when higher probe concentrations were used. Our observations were consistent with the findings of Le Berre et al. and Peterson et al. that higher probe density on the glass surface could reduce the efficiency of duplex formation and the kinetics of target capture procedure (*24, 27*). The decreased hybridization signals from probe lines at high concentrations might be due to steric constraints resulting from the high probe density on the glass substrate. Accordingly, a 25 $\mu$M probe was chosen as a compromise for both signal sensitivity and reagent savings. The surface coverage of the DNA probes on the surface was also estimated. This was achieved by establishing a calibration graph correlating the fluorescence signals to the concentrations of ADF solutions when they were completely filled inside channels (data not shown). It was found that the fluorescent intensity of the

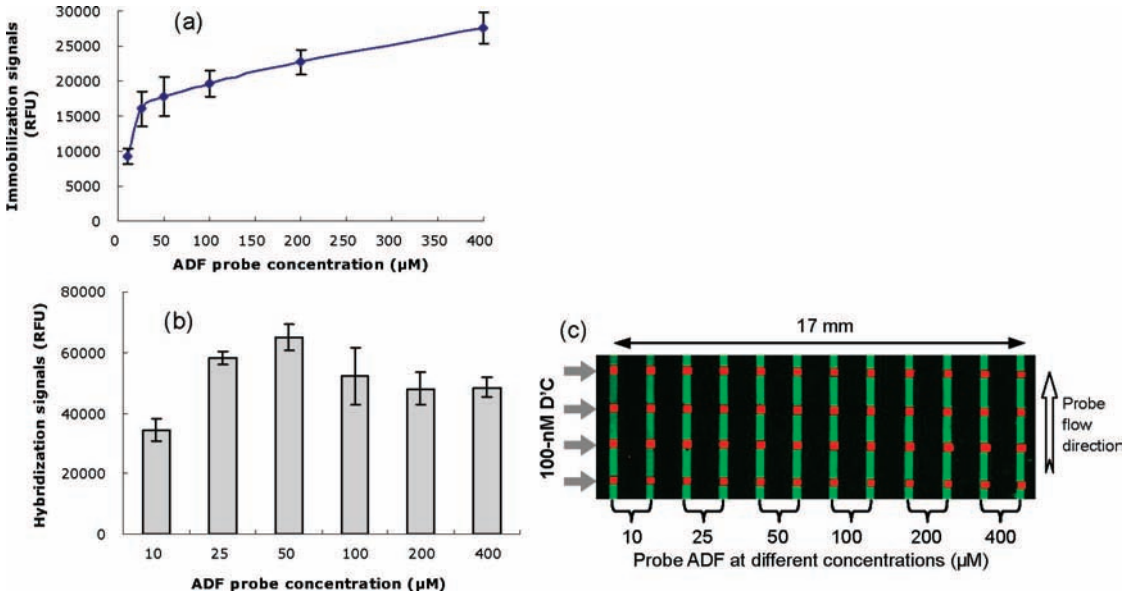



**Figure 4.** Sample hybridization: (**a**) Immobilization signal of various ADF probe solutions (0.8 $\mu$L) at different concentrations (10−400 $\mu$M) that were incubated in microchannels for 2 h. After washing, the slide was scanned at 488 nm. (**b**) Hybridization signals resulted from the above probe lines. Complementary oligonucleotides (D′C) labeled with Cy5 (100 nM, prepared in 1× SSC + 0.2% SDS) were hybridized to the ADF probe lines for 10 min. After washing, the slide was scanned at 633 nm. (**c**) Overlaid dual-channel image of the same glass slide showing both printed probe lines (vertical green lines) and square hybridization patches (red) at intersections. ADF probe immobilization was achieved in duplicate at each concentration.

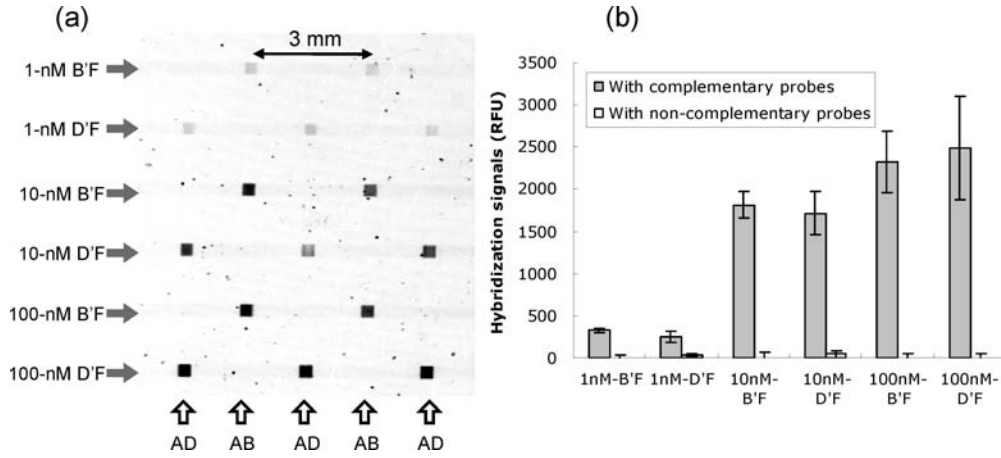



**Figure 5.** Hybridization of oligonucleotide samples to printed probe lines: (**a**) Fluorescent images of the hybridizations of oligonulceotides (B′F and D′F prepared in 1× SSC + 0.2% SDS) with probe line arrays for 10 min at room temperature. (**b**) Histogram showing the fluorescent intensities of hybridization versus nonspecific binding at various sample concentrations. The gray bars represent the signals of samples hybridized with their complementary probe sequences, that is, B′F with AB or D′F with AD; the white bars represent the nonspecific binding. The error bars describe the standard deviations of the signals from five or seven hybridization patches.

probe lines created with 25 $\mu$M ADF was comparable to that of a 0.5 $\mu$M ADF solution, leading to the surface density of $5 \times 10^{11}$ strands/cm$^2$, which was comparable to the value of $\sim 3 \times 10^{12}$ strands/cm$^2$ obtained in a recent study (*27*).

**Fast Hybridization of Multiple DNA Samples.** Two sets of fluorescein-labeled oligonucleotide samples, B′F and D′F, at three different concentrations, were hybridized to preprinted oligonucleotide probe lines of AB and AD at room temperature for 10 min. As shown in **Figure 5a**, fluorescent patches showing successful hybridization occur at the intersections between the vertical printed probe lines and horizontal microchannels filled with three samples of different concentrations (1, 10, and 100 nM). It was found that 1 $\mu$L of 1 nM B′F and D′F could be detected. The signal-to-noise ratios of 1 nM of B′F and D′F were $5.6 \pm 0.4$ and $4.1 \pm 1.1$, respectively. Good specificity of hybridization was obtained, as shown in **Figure 5b**. The nonspecific binding signals remained low even at a high sample concentration of 100 nM. The hybridization kinetics was found to be faster in microchannels than in bulk solutions (*29*). This experiment has demonstrated the detection of two different oligonucleotide samples (1 $\mu$L, at three concentration of 1, 10, and 100 nM) after hybridization at room temperature for 10 min. Moreover, the patches formed in microfluidic hybridizations are homogeneous in shape and intensity, an important feature of a spot microarray that can only be achieved in highly controlled microarray facility.

After experiments with oligonucleotide samples, two PCR products (B′PF, 264 bp; D′PF, 259 bp), which were amplified from plant fungal cultures, were tested. Usually, the hybridization for PCR products requires higher temperature and longer duration as compared to oligonucleotide samples, and so in classical DNA microarray experiments, overnight incubation in a thermostatted chamber is needed (*30*). Accordingly, denatured PCR products were hybridized to probe lines at 50 °C, which

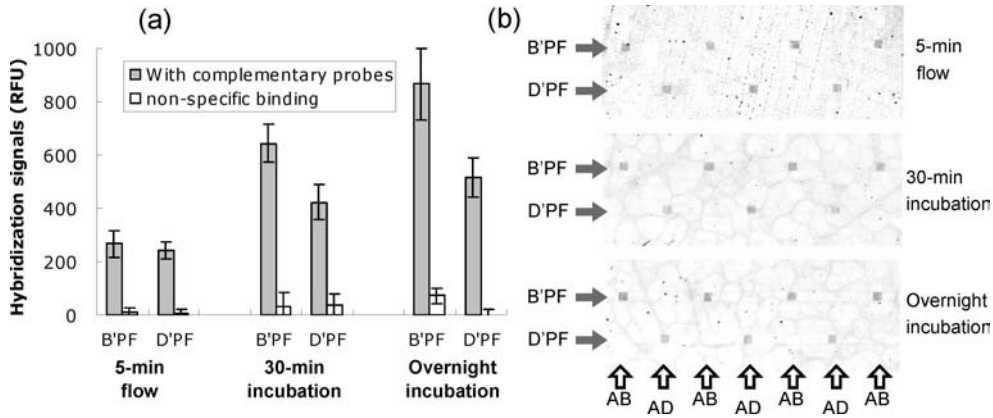
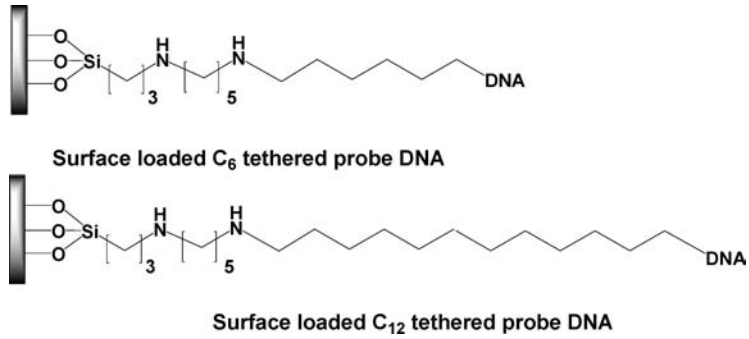



**Figure 6.** Hybridization of PCR products to printed probe lines: (**a**) Fluorescent signals from the hybridization of 2.6 ng of pre-denatured PCR products (B′PF and D′PF) at 50 °C for 5 min of flow, 30 min of incubation, and overnight incubation, respectively. (**b**) Fluorescent images corresponding to the left histogram of hybridizations of two samples to seven probe lines.

**Scheme 4.** Immobilized Oligonucleotide Probe with C6 or C12 Tether As Spaced from the Glass Substrate




was lower than the melting temperature of 66 °C (*31*). Moreover, the results obtained from different hybridization conditions were compared: the first set was carried out by continuous flowing of samples for 5 min, and the second and the third sets were conducted by stop-flow incubation for 30 min or overnight. As shown in **Figure 6a**, it was found that longer hybridization time gave stronger signals, but the 30 min hybridization signal for B′PF reached ∼75% of that obtained from overnight hybridization; it was ∼82% for D′PF. However, the two targets were already distinguished clearly by using 5 min of hybridization at continuous flow of the PCR product samples, which suggested that the hybridization rate was enhanced greatly by the microfluidic flow. **Figure 6b** depicts the fluorescent images of the results obtained for hybridizations at three different flow/incubation conditions. In the case of long-time hybridization, a humidified box had to be used, and the condensation of water vapor on the chemically modified glass slide might create variations on the background signals shown in the fluorescent images. However, in the case of a 5 min flow, a low background was observed because a short hybridization time was used and so no humidified box was used. As compared to our previous work (*10*), in which B′PF and D′PF were detected with pin-spotted DNA microarrays, the new MMA method simplified the probe creation step (no agarose, no spotting), reduced the sample volume (from 50 to 1 $\mu$L), and shortened the hybridization time (from overnight to 5 min). In addition, multisample hybridizations were achieved all on the same chip.

**Effect of Probe Tether Length and One-Base-Pair-Difference Discrimination.** In this work, we also studied the effect of different probe tether lengths on the hybridization efficiency of PCR products with printed probe lines within microfluidic channels. In a study reported by Shchepinov et al., the use of a longer tether in the immobilized probe led to a higher hybridization signal (*32*). In our hands, two aminated probes with two different tethers were used: probe AB (with a C6 tether) and probe ALB (with a C12 tether) (see **Scheme 4**). After immobilization of these two probes (AB and ALB) under the same conditions, the PCR products were hybridized to them at 50 °C for 5 min. As shown in **Figure 7a**,**b**, probe ALB gave a ∼3-fold higher hybridization signal than probe AB when the PCR products (1.4 and 2.6 ng) were applied. This finding is consistent with Shchepinov's result, which is attributed to a less steric hindrance where the longer tether probe is spaced farther away from the glass substrate. However, a longer tether led to a decrease in the hybridization specificity, as more nonspecific binding (signals from D′PF) was observed, shown as small black bars in **Figure 7a**.

The lower specificity obtained when a longer tether probe is used has an impact on our study of the discrimination between two PCR products with a one-base-pair difference. These two PCR products (B′PF, 264 bp; BN′PF 264bp) were related to two closely related subspecies, *B. cinerea* and *B. squamosa*, respectively. They differ in only one base pair at the center of the 264 bp long sequences (see **Table 1**). The duplex formed between denatured BN′PF and probe AB had a one-base-pair mismatch (TTT:ATA instead of TAT:ATA) in the center of the PCR product. The melting temperature of the mismatch duplex was calculated to be 5 °C lower than that of the matched duplex. The hybridization results were shown in **Figure 7c**,**d**. Although probe ALB gave higher hybridization signals for B′PF, the discrimination between B′PF and B′NPF was lower. In terms of differentiation between BN′PF and B′PF, it was found that the longer tethered probes captured more mismatched DNA targets; the shorter tethered probe gave both lower nonspecific binding and a better discrimination between the two PCR products. It can be seen from **Figure 7c** that the discrimination

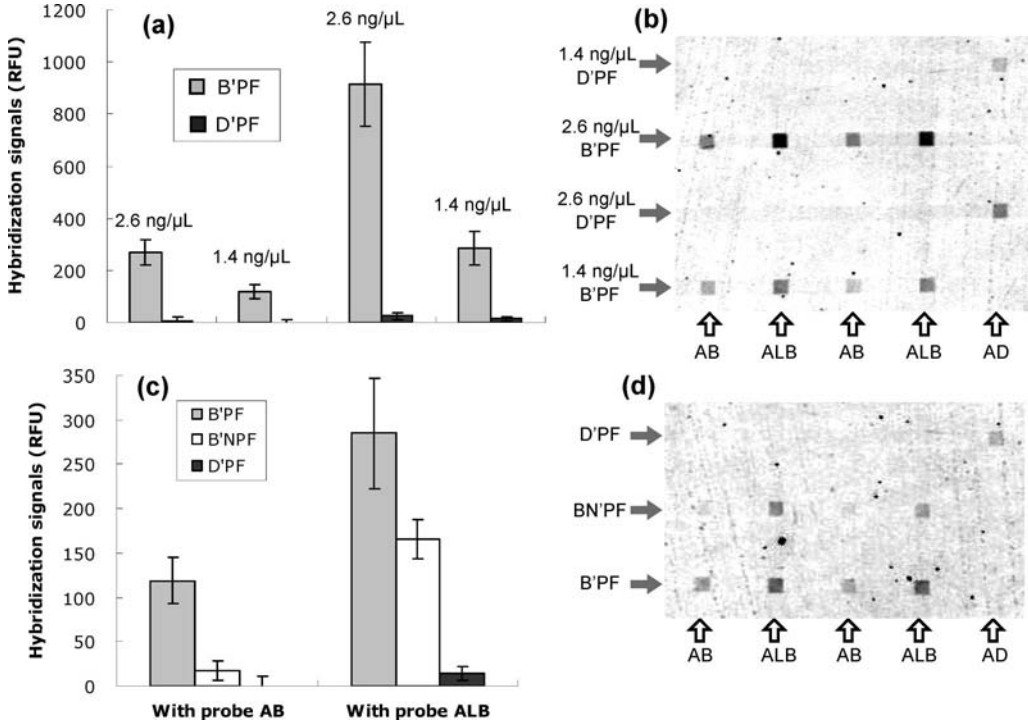



**Figure 7.** Hybridization of PCR products to probes having different tether lengths: (**a**) Fluorescent signals from hybridization of PCR products (1.4 and 2.6 ng) to probes with two different tether lengths. (**b**) Fluorescent image corresponding to the left histogram of hybridizations of samples to five probe lines. (**c**) Effect of probe tether length on discrimination of PCR products with one base pair difference. Fluorescent signals come from the fluorescent patches of three PCR products (B′PF, BN′PF, and D′PF, 1.4 ng for each). The small black bars come from the nonspecific binding signals of D′PF, and they are too low to be seen on probe AB. The error bars describe the standard deviations of the signals from five duplicated tests. (**d**) Fluorescent image corresponding to the left histogram of hybridizations of three samples to five probe lines. In all cases, the DNA targets were hybridized by continuous-flow method for 5 min at 50 °C.

ratio of BN′PF over B′PF is 15% for the probe AB, whereas the ratio becomes worse, to ~60%, for the longer tethered probe ALB. Therefore, by using the short tethered probes, we have demonstrated the capability of the MMA method to discriminate between low amounts of PCR products (1.4 ng) with a one-base-pair differentiation.

**Conclusion.** In this paper, the microfluidic microarray assembly (MMA) method was employed in which flexible probe array creation and fast DNA sample hybridization were conducted in microchannels. The hybridization could be fulfilled in minutes at the intersections between the sample channels and printed probe lines. The process conducted in microfluidic channels was capable of reducing the sample volume (<1 μL) and protecting the liquids from evaporation and cross-contamination. It was demonstrated that 1 fmol of DNA (1 nM, 1 μL) of oligonucleotide samples was detected in 10 min at room temperature. The microfluidic method was also applied for greenhouse plant fungal pathogen detection of two ~260 bp PCR products, *B. cinerea* and *D. bryoniae* (1.4 ng/μL, 1 μL, at 50 °C for 5 min). In addition, discrimination between two 260 bp PCR products with a one-base-pair difference was distinguished with a discrimination ratio of 15%. It is demonstrated that the MMA method provides the advantages of flexible probe creation, low sample volume, good spot homogeneity, and fast hybridization rate, as applied to agricultural problems.

## ABBREVIATIONS USED

MMA, microfluidic microarray assembly; PDMS, polydimethysiloxane; APTES, 3-aminopropyltriethoxysilane; SDS, sodium dodecyl sulfate; SSC, sodium chloride−sodium citrate buffer; PBS, phosphate-buffered saline; AB, C6 aminated oligonucleotide probes for the detection of *Botrytis cinerea*; ALB, C12 aminated oligonucleotide probes for the detection of *Botrytis cinerea*; AD, C6 aminated oligonucleotide probes for the detection of *Didymella bryoniae*; B′F, fluorescein-labeled 21-mer oligonucleotides complementary to the sequences of probe AB; B′C, Cy5-labeled 21-mer oligonucleotides complementary to the sequences of probe AB; D′F, fluorescein-labeled 22-mer oligonucleotides complementary to the sequences of probe AD; B′PF, fluorescein-labeled 264 bp PCR products from genomic DNA of *Botrytis cinerea*, the central 21 bp being complementary to the sequences of probe AB; BN′PF, fluorescein-labeled 264 bp PCR products from genomic DNA of *Botrytis squamosa* having one base pair difference in the center from the sequence of B′PF; D′PF, fluorescein-labeled 259 bp PCR products from genomic DNA of *Didymella bryoniae*, the central 22 bp being complementary to the sequences of probe AD.

## ACKNOWLEDGMENT

We are grateful to Carol Koch of Agriculture and Agri-Food Canada for kindly providing the PCR products of the three greenhouse plant fungal pathogens and Dr. Dipankar Sen for the use of the confocal laser fluorescent scanner.

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**Received for review July 16, 2007. Revised manuscript received September 27, 2007. Accepted October 22, 2007. We thank the Natural Sciences and Engineering Research Council of Canada for an Idea-to-Innovation Fund.**

JF0721242