A MICROFLUIDIC DEVICE FOR 500-SECOND DNA MICROARRAY HYBRIDIZATION THAT REDUCES TARGET CONCENTRATION BIAS

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Abstract: The long hybridization time, the large sample volume required, and the signal bias caused by concentration level variation (higher concentration equilibrates faster) are some major drawbacks of current DNA microarray experiment. This work describes a novel and simple modification of the current microarray format that resolves these drawbacks. The microfluidic device utilizes a simple integration of microchannel and microarray. A serpentine microtrench is designed and fabricated on a PMMA chip using a widely available CO_2 laser scriber. The trench spacing is compatible with the inter-spot distance in standard microarrays spotted on commercial glass slides. The microtrench chip and microarray chip are easily aligned and assembled manually so that the microarray is integrated with a microfluidic channel. Discrete sample plugs are employed in the microchannel for hybridization. Flowing through the microchannel with alternating depths and widths scrambles continuous sample plug into discrete short plugs. These plugs are shuttled back and forth along the channel, sweeping over microarray probes while re-circulation mixing occurs inside the plugs. In this device, targets with different concentration levels show comparable hybridization equilibrium time so that hybridization efficiency bias caused by target concentration is reduced.

Introduction

In conventional DNA microarray experiment, the pairing of complementary targets in reaction mixture to surface bound probes is driven by diffusion process. The process is very slow because of the small diffusion constant of large DNA target molecules. Usually the reaction is performed for 12 to 18 hours or overnight [1, 2]. However, studies have shown that hybridization equilibrium requires longer incubation time [3].

The main reason for the slow reaction is because of the small diffusion constant of large biomolecules. The diffusion constant of DNA molecules in water is expressed as the following equation [4]:

$$Dw = 4.9 \times 10^{-9} \times [bp]^{-0.72} \quad (1)$$

in which the bp is the length of the DNA molecule. For example, for 80-mer target DNA in water, the corresponding diffusion length ($l = \sqrt{2Dt}$, where D is the diffusion constant and t is the diffusion time [5]) is 1.9 mm for 24 hours. Therefore a complete hybridization may take more than 60 hours [3] in a 20 mm x 30 mm array.

In addition to the long reaction time required for hybridization, another major drawback for current DNA microarray experiment is the hybridization signal bias among abundant genes and scarce genes [6].

Thermodynamic equilibrium is critical in heterogeneous DNA hybridization assay. A lower target concentration corresponds to a longer equilibrium time [6]. Genes that are down-regulated require longer times to be measured with the same accuracy as those that are up-regulated [3, 6]. For optimized probes for which cross-hybridization is very low, the time taken to reach equilibrium still depends on target concentration. Therefore, systematic hybridization bias is frequently found when the hybridization reaction is not driven to completion.

We have previously developed a microchannel hybridization method that complete hybridization reaction in ~500 seconds [7]. It has also been found that concentration bias was largle reduced. The current work describes how we utilize droplet mixing effect to expedite the hybridization reaction in a microfluidic device and eliminate the concentration bias.

Materials and Methods

Oligonucleotides: Synthetic 5'-biotin-labeled oligonucleotides are acquired from MWG (Germany). The probe and target oligonucleotide sequences are listed in Table 1. The probe sequence is representative of T-cell acute lymphocytic leukemia gene (TAL1). The microarray slides are home-prepared aminosilane-treated sodalime slides (Kimble) or commercial aminated slides (CORNING UltraGAPS Coated Slides). The thickness of the slides is 1 mm.

Microfluidic chip fabrication: The authors' previous work reported the fabrication of microfluidic chips [7, 8]. Briefly, a commercial CO_2 laser scriber (M-300, Universal Laser Systems, USA) is used to engrave the PMMA substrate to fabricate a microtrench. The microtrench pattern is designed using CorelDraw (Corel)

ISSN: 1727-1983

and then sent to the laser scriber for direct machining onto the PMMA substrate. The trench surface was smoothened to have roughness of ~2.4 nm (RMS) according to our previous works. The trench width and depth can be varied by changing the laser fabrication parameters. Chips with trenches of depths 50 μm to 200 μm are prepared. Normally, a depth of 100 μm is used in hybridization. The microtrench plate has one opening, and a serpentine pattern with 50 straight sections. The overall trench length is 1000 mm. The 50 straight sections encompass 20 mm x 30 mm area. In forming a channel, a PMMA microtrench plate is stacked with a conventional glass microarray. When the two plates are stacked on top of each other, a microarray with microchannels is formed. The microchannel has only a single opening through which to introduce a target solution and to connect to a syringe pump. All reagent solution is introduced and extracted from the opening. The sealed distal terminal of the microchannel is for storing compressed air.

Table 1: The TAL1 probe and the target sequences.

Name	Sequence (5'→ 3')
80 PM	AAGAGGAGACCTTCCCCCCT
	ATGAGATGGAGATTACTGAT
	GGTCCCCCACACCAAAAGTT
	GTGCGGCGTATCTTCACCAA
80 Target	TTGGTGAAGATACGCCGCAC
	AACTTTTGGTGTGGGGGACC
	ATCAGTAATCTCCATCTCAT
	AGGGGGGAAGGTCTCCTCTT

Simple microarray/microchannel integration: The microtrench is manually aligned with the microarray spots as follows. The distance from one corner of the microarray to the two edges of the slide is set using the arrayer. The same distance is used in fabricating the microtrench chip. The distance from the microtrench to the two edges of the microtrench chip is determined during fabrication using the CO_2 laser scriber. Accordingly, when the edges of the microarray and the microtrench chip are placed against two flat surfaces, the microarray is automatically aligned with the microtrench. The mechanical alignment procedure is simple and has a spatial repeatability of 10 μm.

Glass slide modification and microarray spotting: Microarray slides are modified according to the procedure described in our previous work [7]. Oligonucleotide probes are dissolved in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) solution [9] to a final concentration of 30 μM at pH 6.5. Spotting is performed using a home-built robot equipped with solid pin to deposit probe spots on the glass slide to produce a microarray. The spots have a diameter of about 80 μm. The distance between each pair of spots is 300 μm. An example showing the arrangement of probe spots in the microchannel is in Figure 1. Usually 30 repeated probe spots are used. Following spotting, the slides are incubated in a desiccator (~20 % relative humidity) at 25 °C for 18 h. Ultraviolet irradiation (set to deliver 600 mJ of energy) are then used to crosslink the oligonucleotides of the 80-mer oligonucleotide probes onto the slide. After integrated with the microchannel chip, the array is then blocked using 5X SSC, 0.1 % SDS and 0.1 % BSA at 42 °C for 30 min, and rinsed three times in deionized water for 5 min before hybridization.

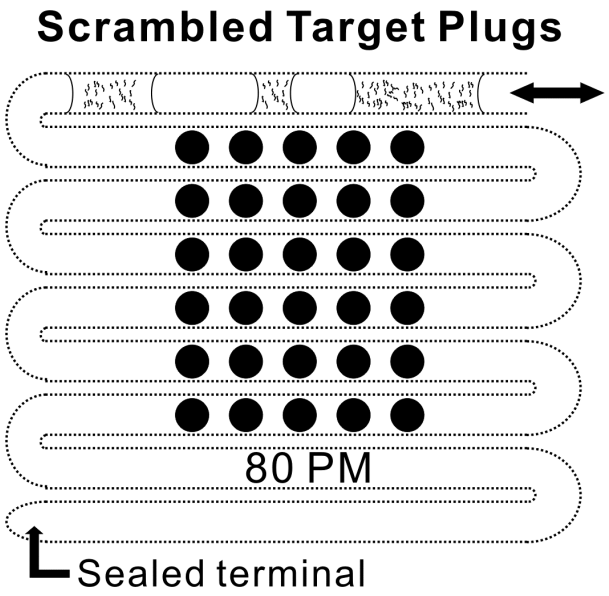

Figure 1: Illustration of scrambled discrete plugs sweeping over probe array. The spot distance is 300 μm and the spot diameter is 80 μm.

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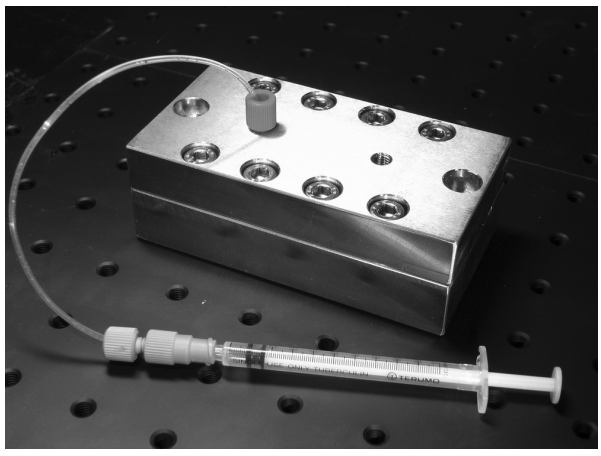


Figure 2: Photo picture of the device. The PMMA microtrench chip and the glass microarray slide are sealed together by steel clamps. 1 ml syringe is used to pump the target sample.

Shuttle hybridization: Target concentration of 50 pM to 90 nM are used throughout the entire study. For 80 mer hybridization, Biotin-labeled target ssDNA is diluted in 50 % formamide, 5X SSC and 0.1 % SDS, to

ISSN: 1727-1983

a final concentration from 50 pM to 90 nM. For shuttle hybridization, 1 µl DNA target solution is introduced into the microchannel opening (Figure 1) using a pipette. A syringe is then connected to the opening, sealing the microchannel (Figure 2). The sample solution is pushed to the distal side of the channel, away from the opening, when the syringe is compressed. The distal terminal of the channel is sealed and the compressed air is stored in the sealed channel. Hence the sample solution bounces back when the compressed syringe is released. In shuttle hybridization, the target solution is mixed with the probes while being pumped back and forth inside the entire channel, sweeping over ALL probes. The cycle time of the shuttling is 2 seconds. The entire microarray/microchannel assembly is placed in a 42 °C water bath to control the temperature. Following hybridization, the target solution is drawn from the channel and discarded. Several buffer solutions are introduced into, and then drawn out, to perform washing. 10 µl of 2X SSC, 0.1 % SDS at 42 °C , 20 µl 0.1X SSC, 0.1 % SDS at 25 °C and 20 µl 0.1X SSC at 25 °C are sequentially used. Each wash takes 5 min. The array is then incubated with 1 µl Cy5-conjugated Streptavidin (SA-Cy5, 0.05 mg/ml) (Zymed, CA) with reagent shuttling for 5 min at 25 °C, followed by washing in 2X SSPE/ 0.1% SDS. The microarray is then detached from the microtrench plate to be scanned to detect fluorescence signals. After hybridization and SA-Cy5 staining, the microtrench only shows fluorescence of background level comparable to that of the microarray background.

Conventional flat glass hybridization: Conventional flat glass hybridization is performed according to standard procedure. Briefly, a Gene Frame (Abgene, UK) is attached to the microarray slide to produce a sealed chamber. 100 µl target DNA is applied into the chamber for hybridization. The temperature of hybridization is 42 °C, and the slide is incubated in a humidified chamber. Following hybridization, the slides are washed in 2X SSC, 0.1 % SDS at 42 °C for 5 min, 0.1X SSC, 0.1 % SDS at 25 °C for 10 min and 0.1X SSC at 25 °C for 5 min. The flat glass array is then incubated with 40 µl SA-Cy5 at 25 °C for 60 min, and then washed with 2X SSPE/ 0.1% SDS.

Fluorescence image quantification: Following hybridization and SA-Cy5 incubation, the microarray slides are scanned at a resolution of 5 µm using a GenePix 4000B array scanner (Axon Instrument, CA). The PMT voltage is set to 700 V. The hybridization signal from multiple copies of probes is averaged and used in analyzing the data.

Results

In order to measure that whether target DNA molecules with different concentration achieve complete hybridization at the same reaction time, we performed hybridization with target concentration ranging from 50 pM to 90 nM. The concentration difference is more than three orders of magnitute. Furthermore, same target sequence was used at different concentration so that hybridization signal difference resulted from sequence variation is ruled out.

For comparison, traditional microarray experiment was performed with hybridization time from 0.2 to 18 hours. The result for hybridization time from 2 to 18 hours is shown in Figure 3. Target concentration from 1 nM to 90 nM was compared. The hybridization signal is presented by signal to background ration (S/B), as denoted under each image. It is clearly seen from these fluorescence images that higher target concentration results in faster signal growth. For example, fluorescence spot is clearly observed at 1 hour (data not shown) for 90 nM target while for 1 nM target the fluorescence spot is not clearly observed until after 4 hour. Moreover, signal intensities for 90 nM target at 2 hours and 4 hours do not differ significantly while for 1 nM target the signal growth during 2 hours to 4 hours is clearly observed. The result shows that for target at low concentration the signal growth is slower than that for high target concentration.

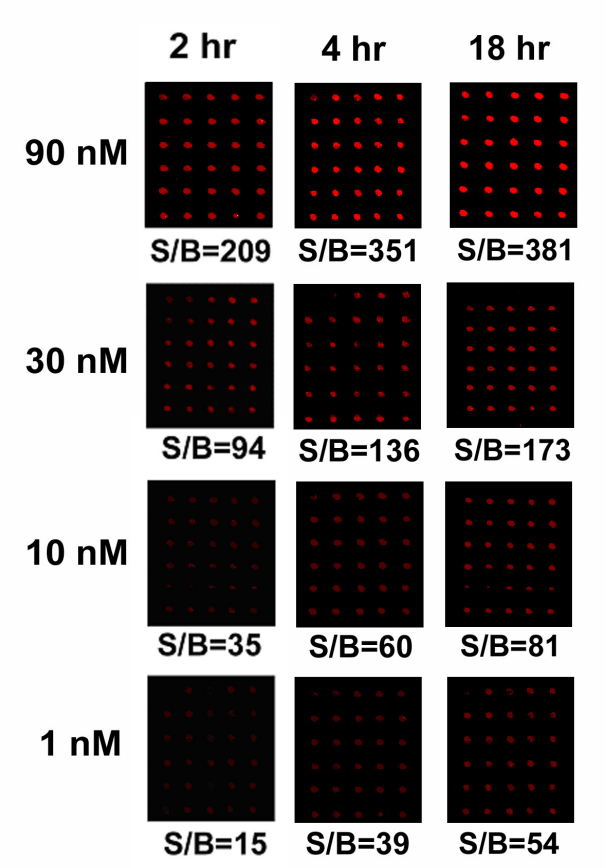


Figure 3: Fluorescence images and corresponding S/B ratio of conventional hybridization at various time (2, 4, 18 hours) with different target concentration.

Contrary to the long hybridization time required to result in observable signal, Figure 4 shows the fluorescence image of microarray hybridized for 500 and 1000 seconds in our microfluidic hybridization device. Compared to fluorescence image for 500 seconds hybridization time, longer reaction time at 1000 seconds result in less than 5 % signal growth. This

ISSN: 1727-1983

indicates that hybridization signal reaches equilibrium at around 500 seconds for all the concentration tested.

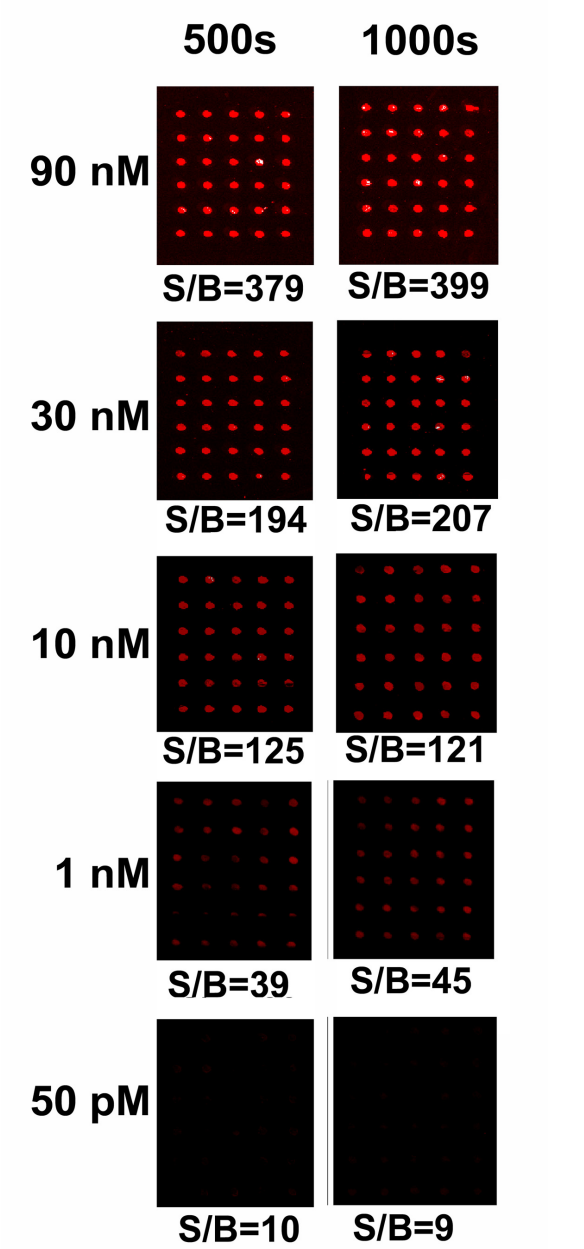
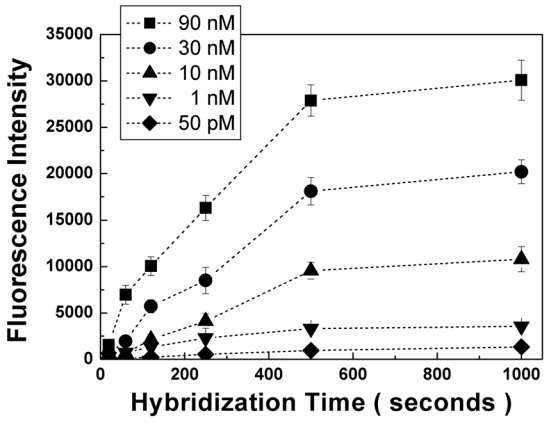


Figure 4: Fluorescence images and corresponding S/B ratio of shuttle hybridization at 500 seconds and 1000 seconds hybridization time with different target concentration.

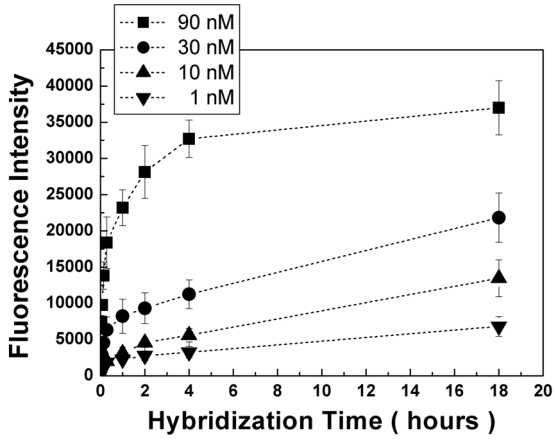
Figure 5 quantitatively compares the signal growth of the conventional hybridization and shuttle hybridization. The Figure shows that signal from high target concentration, e.g. 90 nM, reaches a plateau at about 4 hours. On the contrary, signal from low target concentration continues to grow after 18 hours of incubation. This result clearly indicates that hybridization does not reach equilibrium until 18 hours of incubation and suggests that hybridization signal does not accurately reveal the actual relative gene concentrations in a sample without driving the DNA-pairing reaction to completion. Therefore the DNA microarray platform is prone to yield biased gene level information when the hybridization does not reach equilibrium.

Discussion

In our shuttle hybridization, the principle that expedites the hybridization reaction is the chaotic droplet mixing [7, 10, 11]. Ismagilov et al. [11] showed that for constant channel dimension and plug flow velocity the droplet flow chaotic mixing time is proportional to log(1/D), where D is the diffusion constant. Combing with equation (1), the mixing time for 800 bp DNA is only 1.5 times longer than that of 80-mer DNA. Therefore the use of long target DNA is thus expected to extend the required hybridization time only slightly. Although 80 mer oligonucleotide is used in this study, longer DNA molecules are expected to behave similarly. Our recent experiment result confirmed this speculation (data not shown). In summary, our shuttle hybridization device reduces signal bias and increases quantitative analysis accuracy of DNA microarray data. Furthermore, the modification to traditional microarray format is simple. Common microarray slide and scanner that is currently in use can be used in this shuttle hybridization device.



(a)



(b)

Figure 5: Hybridization signal growth of (a) shuttle hybridization and (b) conventional hybridization.

ISSN: 1727-1983

Conclusions

In this work, we described our efforts to overcome some major drawbacks of current DNA microarray experiment. A novel and simple modification of the current microarray format was proved to be efficient, especially for eliminating the signal bias resulted from different target concentration. The microfluidic device utilizes a simple integration of microchannel and microarray. An economic microfluidic chip fabrication platform that utilizes CO_2 laser scribing on PMMA is used to fabricate the microfluidic chip used in this study. Discrete sample plugs inside the microchannel induces effective chaotic droplet mixing and thus reduces hybridization time. Furthermore, the hybridization method drives the hybridization to completion and eliminates the concentration signal bias. This advantage over traditional hybridization method is valuable for quantitatively analysis of microarray data.

Acknowledgment

We appreciate the financial support from National Science Council, Taiwan (contract number: NSC 94-2113-M-001-048) and Academia Sinica, Taiwan.

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ISSN: 1727-1983