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Electrokinetically Based Approach for Single-Nucleotide Polymorphism Discrimination Using a Microfluidic Device

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In this work, we describe and implement an electrokinetic approach for single-nucleotide polymorphism (SNP) discrimination using a PDMS/glass-based microfluidic chip. The technique takes advantage of precise control of the coupled thermal (Joule heating), shear (electroosmosis), and electrical (electrophoresis) energies present at an array of probes afforded by the application of external electrical potentials. Temperature controllers and embedded thermal devices are not required. The chips can be easily and inexpensively fabricated using standard microarray printing methods combined with soft-lithography patterned PDMS fluidics, making these systems easily adaptable to applications using higher density arrays. Extensive numerical simulations of the coupled flow and thermal properties and microscale thermometry experiments are described and used to characterize the in-channel conditions. It was found that optimal conditions for SNP detection occur at a lower temperature on-chip than for typical microarray experiments, thereby revealing the importance of the electrical and shear forces to the overall process. To demonstrate the clinical utility of the technique, the detection of single-base pair mutations in the survival motor neuron gene, associated with the childhood disease spinal muscular atrophy, is conducted.

Single-nucleotide polymorphism (SNP) detection[1] has proven itself to be a particularly useful technique for the diagnosis of a variety of genetic diseases and cancers.[2] Many techniques based on electrophoretic separation have been used for SNP detection, such as heteroduplex analysis, single-strand conformation polymorphism, denaturing gradient gel electrophoresis, temperature gradient gel electrophoresis (TGGE), allele-specific PCR amplification, and ligase chain reaction/ligase detection reaction. DNA microarray technologies[3,4] involving large-scale DNA sequence detection based on hybridization between complementary DNA sequences on a solid support can be used for large-scale SNP screening,[5] genotyping,[6] and loss of heterozygosity[7] and also to detect specific SNPs for diagnostic purposes.[8] High-throughput DNA microarray technologies simplify SNP analysis, but most methods require several hours for hybridization (due to diffusion-limited reaction kinetics) and complicated washing steps demanding strict temperature and buffer salt content control. In addition, microarray hybridization and washing manipulation is conducted on centimeter-scale glass substrates thereby requiring that large quantities of DNA target sample be used.

Microfluidic (lab-on-a-chip) technologies provide the advantage of processing small liquid volumes in a rapid and controlled manner, which has proven especially useful for analyses in life sciences.[9−11] To increase the overall reaction rate and simplify sample manipulation, several researchers began to investigate the integration of several microfluidic-based technologies into single microscale platforms.[12−14] Various designs such as paramagnetic bead-based devices,[15] poly(methyl methacrylate) chips using universal zip code arrays,[16] and polycarbonate chips designed to be credit card sized[17] or that offer continuous monitoring of the hybridization reaction[18] have been used for accelerating and

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automating detection of hybridization (see Erickson and Li[12] for a more comprehensive review). Hydrodynamic pumping techniques are adopted in most microfluidic devices that are designed for detection of hybridization despite the unique features offered by high-voltage-driven flow in microfluidic chips. A likely reason relates to the difficulty associated with handling high voltages in the high salt content buffer that is required for DNA hybridization.

A necessity for obtaining maximum discrimination for oligonucleotides between fully matched hybrid and a single-base-mismatched hybrid in a microfluidic chip is accurate control of the thermal conditions at the microarray interface. In the past, this has been largely accomplished through the integration of external heaters and thermal sensors (e.g., RTDs). PCR amplification was one of the first on-chip applications that required this type of control.[19−21] For example Kopp et al. used a copper heating block for continuous-flow PCR in a microfluidic channel,[22] while others such as Lao et al.[23] have used high thermal conductivity silicon-based devices to achieve precise temperature control and rapid cooling. For SNP detection, Buch et al.[24] reported a TGGE method based on the temperature gradient on a polymer microfluidic chip. Thermal denaturation of surface-immobilized DNA, or DNA present in free solution, was investigated using microfluidic platforms and molecular beacon-based SNP detection.[25,26] Platforms for high-throughput on-chip temperature gradient assays were established by Mao et al.[27] for discriminating fully complementary DNA strands from those containing a single T−G or C−A mismatch. This work was done using SYBR Green I intercalating dye and was reported for parallel and combinatorial measurements.[28] Using temperature control units, the incorporated external heaters and thermal sensors on the microfluidic chips made it possible to control and rapidly change the temperature and to produce spatial temperature gradients.

Joule heating is an inherent consequence of electrokinetic transport resulting from ohmic heat generation as electric current flows through a buffer. In capillary electrophoresis (CE) systems and other microfluidic separation devices, Joule heating usually results in band broadening and deterioration of separation efficiency. The better heat dissipation characteristics associated with glass chips were originally touted as one of the large advantages of moving from capillary- to chip-based CE. The recent trend toward polymeric microfluidic devices, however, largely negates this advantage due to the relatively low thermal conductivity of the materials involved. Recently, Erickson et al.[29] conducted a combined experimental and numerical study of the mechanisms of dissipation and control of Joule heating in polymeric and hybrid polymer/glass chips. The work indicated that while chips that are formed entirely from polymeric materials are slow to respond and heat, hybrid chips do provide a more rapid response and better thermal control. De Mello et al.[30] recently demonstrated controlled ohmic heating of ionic liquids in a co-running channel that was external to the main fluidic system. To date, however, precise control of Joule heating has not been exploited for use in on-chip analytical applications, despite its ubiquitous presence in electrokinetic microfluidics.

The new work herein describes and demonstrates an on-chip electrokinetically based microfluidic technique for SNP discrimination. The traditional advantages in terms of flow and sample handling afforded by electrokinetic transport[31] are extended here to include thermal control through on-line manipulation of the Joule heating conditions. Precise control of the thermal and electrical conditions at the probe sites are shown to enable discrimination of single-base-mismatch SNP sample targets on the chip by exploiting differences in denaturation energetics. To characterize the Joule heating conditions for optimal discrimination, a combined numerical and experimental heat-transfer study has been conducted. To demonstrate the clinical utility of the technique, the detection of genetic markers associated with the childhood disease spinal muscular atrophy (SMA) is investigated. SMA is characterized by the loss of α-motor neurons leading to symmetrical wasting of the voluntary muscles.[32] Asymmetric PCR products containing the SMA gene sequence were directly analyzed on the microfluidic chip.

EXPERIMENTAL SECTION

Reagents. All buffers were made using chemicals from Sigma-Aldrich Canada (Oakville, ON, Canada) and were prepared with double-distilled water. A solution of 0.5× SSC with 100 mM phosphate buffer (pH 7.0) was made by diluting and mixing 20× SSC stock solution (pH 7.0) and 200 mM phosphate buffer (pH 7.0). The buffer solution for on-line experiments and off-line hybridization was made by dissolving 0.1% (w/v) poly(vinylpyrrolidone) (PVP) (average molecular mass 36 000 Da) into the 0.5× SSC and 100 mM phosphate buffer. This buffer was used for on-line hybridization as well as for DNA sample dilution. Before use, the buffer was filtered with a 0.25-μm Acrodisc syringe filter (Pall Corp., Ann Arbor, MI 48103) and degassed for 20 min. The 10% Polybrene (hexadimethrine bromide) solution for precoating the hybridization chip channel walls in the temperature measurement was made by adding an appropriate amount of solid Polybrene to distilled water. Laser grade rhodamine B dye (Acros Organics, Pittsburgh, PA) was dissolved in deionized water at a concentration of 1 mM and stored at −20 °C. This solution was diluted to 50 μM in the hybridization buffer (0.5× SSC + 100 mM phosphate

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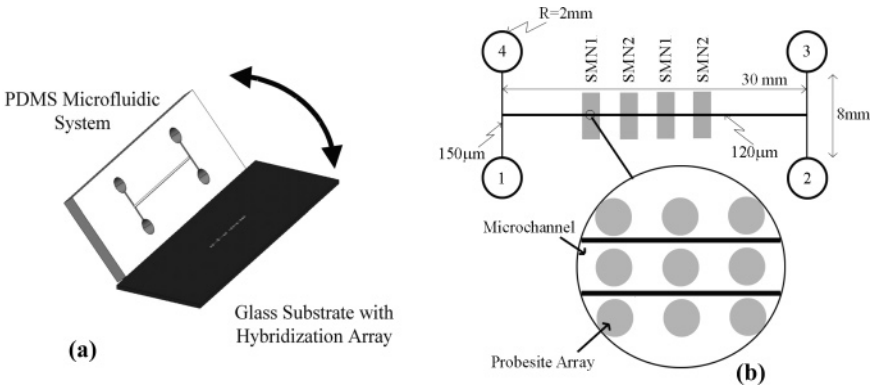

Figure 1. (a) 3D overview of the chip and assembly procedure showing PDMS fluidics and immobilized hybridization array. (b) H-type channel structure probe site layout for electrokinetically based single-nucleotide polymorphism discrimination. Four regions were robotically spotted onto the chip each with eight individual probe sites shown as SMN1 and SMN2 in the figure. For the purposes of this work, the same probes were used for each spot in the individual regions for DNA hybridization chip. Input ports labeled as (1) sample port, (2) waste port, (3) buffer port, and (4) auxiliary port.

Table 1. Oligonucleotide Sequences Used as Immobilized Probes and Labeled Targets in SNP Analysis Experiments[a]

oligonucleotide	sequence
SMN1 (immobilized probe)	5′-NH_2−C_{12}-A TTT TGT CT***G*** AAA CCC TGT-3′
SMN1A (Cy3-labeled target, complementary to SMN1)	5′-Cy3-ACA GGG TTT ***C***AG ACA AAA T-3′
SMN2 (immobilized probe)	5′-NH_2−C_{12}-A TTT TGT CT***A*** AAA CCC TGT-3′
SMN2A (Cy3-labeled target, complementary to SMN2)	5′-Cy3-ACA GGG TTT ***T***AG ACA AAA T-3′
uidA (reference immobilized probe)	5′-NH_2−C_{12}-AGT CTT ACT TCC ATG ATT TCT TTA ACT ATG-3′

[a] SNP sites are shown in boldface italic type.

buffer + 0.1% PVP) for all in-channel temperature measurements. The rhodamine B solution was filtered with a 0.25-μm Acrodisc syringe filter (Pall Corp.) prior to use.

Oligonucleotides and PCR Products. All oligonucleotide probes, targets, and PCR primers were obtained from Integrated DNA Technologies (Coralville, IA). Oligonucleotide probes were 19 nt in length (Table 1). SMN1 or SMN2 probes were covalently immobilized to the glass surface with (3-glycidoxypropyl)trimethoxyslane (GOPS) modification using robotic printing technology for DNA microarrays. SMN1A and SMN2A were the Cy3-labeled complementary oligonucleotides of SMN1 and SMN2, respectively. The uidA sequence is a reference probe, which was used for study of nonspecific adsorption.

Following the PCR protocol described by Watterson et al.,[33] 5′-(Cy3)-CCTTTTATTTTCCTTACAGGGTTT-3′ (SMN202-F) and 5′-TGATTGTTTTACATTAACCTTTCAA-3′ (SMN202-R) were used as forward and downstream primers. Amplifications were done asymmetrically using a primer ratio of 10:1 for SMN202-F/SMN202-R. The annealing temperature was set to 49 °C. After 35 cycles of PCR amplification, PCR products were checked using 1% agarose gel electrophoresis and ethidium bromide staining. Cy3-labeled 202-bp PCR product was amplified from the template containing SMN1 gene sequence. The PCR products were stored in a freezer held at −20 °C. Before analysis on a chip, the PCR sample was thawed and diluted with the hybridization buffer in a 1:2 ratio.

Microfabrication and Chip Assembly. The microfluidic chips developed for this application are based on those described previously by Erickson et al.[34] and are composed of a PDMS upper substrate and a glass lower substrate as shown in Figure 1. The PDMS contained the microchannel structure and sample/waste/auxiliary ports, and the oligonucleotide probes were immobilized on the glass substrate. A schematic of the channel layout and microarray integration technique is shown in Figure 1b. PDMS fluidic chips were manufactured using a standard single-layer soft-lithography technique described by Duffy et al.[35] and reported in our previous work[34] and thus will not be described in detail here. Chemicals used in the GOPS surface modification were obtained from Sigma-Aldrich Canada. Oligonucleotide immobilization on glass slides (detailed description can be found in ref 34) was done using the protocol developed by Maskos and Southern.[36] Briefly, a glass slide (Fisher Finest, 75 mm × 25 mm, 1.0 mm thick) was cleaned and sequentially treated with a mixture of hydrogen peroxide and ammonia and a mixture of hydrogen peroxide and hydrochloric acid. Then the slide was silanized with GOPS and washed with methanol, dichloromethane, and anhydrous ethyl ether sequentially. Before use, the slide was kept in a vacuum desiccator. A robotic printer (SpotArray 72, Perkin-Elmer) was used for printing 24 × 8 arrays on the GOPS-modified slides. SMN1 and SMN2 probes with a 5′-amino modifier and C_{12} linker in 3× SSC buffer (shown in Table 1) were used for printing by deposition of 20 μM solutions. Four zones of 24 × 8 arrays were printed on the middle of a glass slide for the on-line hybridization experiments. Two of these arrays consisted of SMN1 spots, while the remaining two were of SMN2 spots, as shown in Figure 1. In

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Table 2. Voltage Program in the SNP Detection on the Microfluidic Hybridization Chip[a]

step	function	duration (s)	voltage (V) at port 1	at port 2	at port 3	at port 4
1	sample loading and dispensing	120	0	float	300	20
2	continued sample transport and hybridization	60	115	float	500	0
3	washing and temperature control with Joule heating	60	460	float	2000	0
4	final flushing for chip regeneration	60	575	float	2500	0

[a] The sample/waste/auxiliary port positions are shown in Figure 1.

each 24 × 8 array, there were 192 identical spots of 100-μm diameter and 160-μm center-to-center spacing distance. After overnight incubation in a humidity chamber, the slides were thoroughly washed with 1× SSC buffer for 10 min and rinsed with water. Then the slides were denatured in 95 °C water for further removal of oligonucleotides adsorbed on the slides. To form the enclosed chip, the PDMS substrate was first plasma treated. It was then aligned and placed in direct contact with the glass substrate. In this way, eight spots from each array (each with a 100-μm diameter) were aligned in the middle of the 120-μm-wide channel on the chip.

Instrumentation and On-Line Chip Operation and Detection Protocols. Target transport visualization and on-line hybridization detection was conducted using a test bench that had been built in-house.[34] Cy3-labeled oligonucleotide transport was visualized using a Leica DM-LB fluorescence microscope with: 10×, 0.3 NA, long working distance objective, the appropriate filter set (excitation band-pass 546 nm/12 nm; emission 600 nm/40 nm band-pass), and a 100-W broadband mercury lamp. A 12-bit Retiga-1300 cooled digital CCD camera was used for imaging and light intensity digitization. Hybridization scanning was conducted using this optical system with a 32×, 0.6 NA objective, and a Leica DMSTC computer-controlled X−Y translation stage. The scanning program extracted the hybridization channel intensity profile by capturing and storing an image every 50 μm along the length of the channel. The average intensity of the pixels in five different 9 μm by 9 μm squares at the middle of the DNA microarray spots was then taken as the local point-intensity value. The result was that intensity along the channel length could be measured with a resolution of 10 μm.

Prior to initiating experiments, the four ports were filled with the hybridization buffer, 0.5× SSC, and 100 mM phosphate buffer (pH 7.0) with 0.1% PVP. The chip was then placed into an electrical harness, and platinum electrodes, wired to the switching board, were inserted into the ports. A fluorescence intensity scan of the channel was then conducted to obtain the background intensity of the hybridization channel. The sample reservoir buffer (typically port 1 in Figure 1) was then replaced with the sample containing the Cy3-labeled DNA targets at a concentration of 0.5 μM in hybridization buffer. The voltage program for the entire procedure for on-chip analysis of SNP, including sample-dispensing, hybridization and washing, and the final flushing steps, is shown in Table 2. The voltages were set using a high-voltage power supply (Spellman, Hauppauge, NY) and a four-lead electrical switching board, each with an independently adjustable output voltage. First, the sample plug was electrokinetically dispensed and sent to the hybridization array from the sample port. After the sample plug was dispensed into the hybridization channel, a programmed voltage protocol was used for moving the sample through the hybridization channel, which exposed the sample to four probe sites and allowed for hybridization. Following this, a higher voltage was applied at port 4 for washing and controlling the temperature in the channel. During this step, the optical scanning of the hybridization channel was done in the direction of flow of the target material. A corrected hybridization intensity profile was obtained by subtracting the prescan image from the image obtained after hybridization was complete. The final step was to remove all of the hybridized targets for the next round determination. Further evaluation of the effect of the electronic denaturing method involved attempts at regeneration by flushing with formamide for 10 min (to denature the hybrids) followed by a 10-min buffer flush without the final flushing step.

A uidA sequence (immobilized probe) was used as a reference probe to evaluate the nonspecific adsorption in the microfluidic system. It was found that there was no significant nonspecific adsorption in the system. Consequently, uidA spots were omitted in further experimentation. Only SMN1 and SMN2 probes were printed on the glass slides prepared for chip assembly.

Off-Line Microarray Experiments. Off-line standard microarray experiments were also conducted in order to have a benchmark against which to compare the on-line chip results. In these experiments, solutions of 0.5× SSC and 100 mM phosphate buffer with 0.1% PVP was used as the hybridization buffer as well as the washing buffer. Cy3-labeled SMN1A target (see Table 1) was diluted to 0.1 μM in the hybridization buffer and applied to the printed glass slide with immobilized SMN1 and SMN2 probe spot matrixes similar to those shown in Figure 1b. Then the slide was incubated in a humidity chamber for 10 min. Following hybridization, the slide was kept in a plastic, buffer-filled hybridization case and placed into a thermostated water bath for washing (0.1 °C precision). A 10-min washing time was used, and the effect of different temperatures was examined in 5 °C increments. Fluorescence intensity was measured using a dual-channel Bio-Rad chip reader (Hercules, CA), and the output was in the form of 16-bit TIFF files. The correlation of the average intensity signal of all 192 spots in the 24 × 8 matrix with change of temperature was examined.

Microscale Thermometry Experiments. Rhodamine B was used for temperature measurement in the channel of the PDMS/glass chip, following previously established methods.[29,37] Because we used a hybridization buffer different from that of the previously reported experiments, it was considered prudent to recalibrate the rhodamine B in the buffer that was used in the new work. To achieve this, a method was devised using a microfabricated

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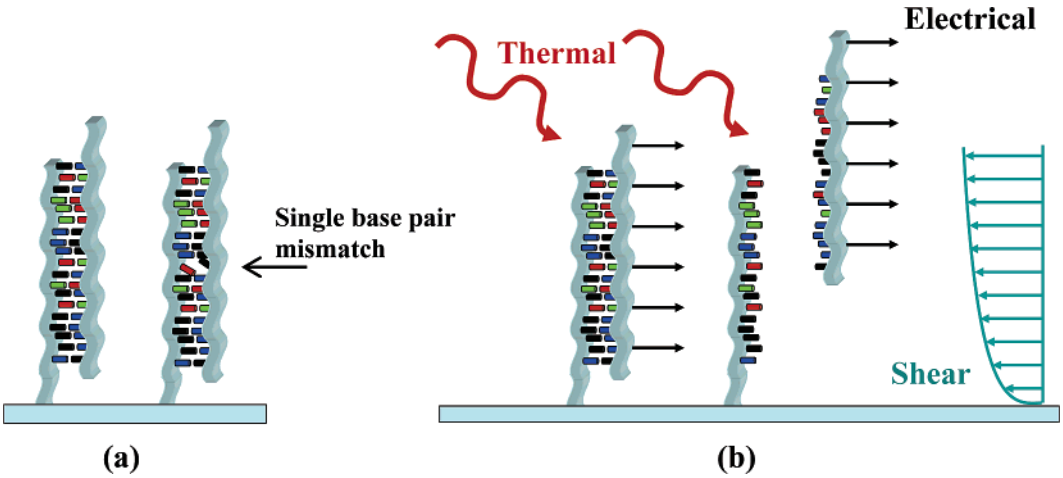

Figure 2. Electrokinetically based SNP discrimination technique. (a) At standard conditions, both the fully matched and single-base pair mismatch are hybridized. (b) Applying an electrokinetic driving potential allows for precise manipulation of the thermal (through Joule heating), shear (through electroosmotic flow), and electrical (through electrophoresis) forces enabling the SNP to be discriminated.

thermal reservoir, PID temperature controller (CN132, Omega, Stamford, CT), and a Kapton insulated flexible heater (KHLV-102/5, Omegalux, Stamford, CT). The reservoir was constructed by punching a hole of 4.8-mm diameter in 5-mm-thick PDMS. This reservoir was then filled with 50 μM rhodamine B solution in the hybridization buffer, and a thermocouple probe was immersed in the liquid. The reservoir was covered with a film of PDMS (~1 mm thick) to prevent liquid evaporation at elevated temperature. The fluid was heated to 80 °C, and the film heater was turned off. As the liquid cooled, intensity images were captured every 5 s using the fluorescence microscope system described previously, and the corresponding temperature was recorded. This was repeated a number of times to ensure repeatability of the calibration method.

Initial in-channel temperature measurements were unsuccessful because it was found that the dye adsorbed onto the PDMS surfaces. This resulted in nonuniform dye intensity along the channel and generally led to unreliable results. To reduce the adsorption of rhodamine B on the PDMS, a coating procedure was applied prior to the fluorescence intensity measurement. Polybrene solution (10% w/v, in water) was continuously pumped through the channel for 5 min. The positively charged polymer prevented rhodamine B from adsorbing onto the PDMS/glass surface.[38] This was followed by 5-min flushes each using deionized water and hybridization buffer, respectively. The microfluidic chip was then permitted to sit for 15 min prior to a temperature measurement experiment. After the chip preparation and conditioning, in-channel temperature was measured as a function of the applied voltage. Raw intensity values were adjusted using room-temperature intensities and compared with the previously obtained calibration curve. This was repeated for different applied voltages such that the full temperature profile across the voltage range used in the hybridization experiment was collected. The intensities were compared with the initial scan data to ensure that no significant dye adsorption had taken place. The Polybrene surface treatment is well known to change the surface ζ-potential from its native negative value to positive, thereby reversing the direction of flow and changing the thermal convection conditions from those experienced during chip operation. Here, however, both the hybridization array and experimental temperature measurement locations were far enough away from the channel intersection so that convective effects were small (i.e., the velocity in the direction of observed gradients in temperature was negligible) and thus did not significantly influence the internal temperature distribution.

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RESULTS AND DISCUSSION

Electrokinetically Based Technique for SNP Detection. Implementation of hybridization methods using standard microarray technology requires precise temperature and shear control in both the hybridization and washing steps for effective discrimination between one-base pair-mismatch and fully matched DNA sequences. If a strict experimental protocol is observed, single-base pair-mismatched DNA could be washed away leaving only fully matched spots on the microarray. Unfortunately, this process was both time and labor consuming. In previous work, we introduced an electrokinetically controlled DNA hybridization chip[34] that was shown to enable rapid and quantitative target analysis. Here we significantly extend that work by demonstrating how that technique can be applied to automatic SNP detection. A schematic of the discrimination technique is shown in Figure 2. As can be seen, under standard conditions or at low applied voltage (Figure 2a), both the fully matched hybrid and that with the single-base pair mismatch remain hybridized, making discrimination difficult. Applying an electrokinetic driving potential allows for manipulation of the thermal (through Joule heating), shear (through electroosmotic flow), and electrical (through electrophoresis) forces above the microarray. While control of the latter two of these effects is reasonably straightforward, precise thermal control through Joule heating is more difficult and requires an understanding of the heat-transfer mechanisms involved. In our previous work,[29] it was demonstrated that, while higher temperatures were typically obtainable in polymeric systems, thermal runoff limited the degree to which the temperature could be controlled. When a thermal sink (in the form of a glass lower substrate) was employed, lower temperatures were

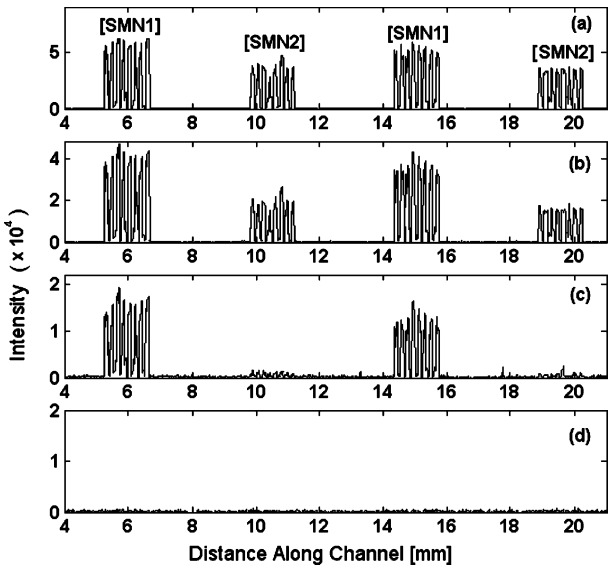

Figure 3. Hybridization intensity of SMN1A target at matched (SMN1) and single-base pair-mismatched probe sites (SMN2) at washing voltages of (a) 116 V/cm in the hybridization channel, (b) 348, (c) 463, and (d) 577 V/cm. The signal intensity for the mismatched probe sites decreases more rapidly than then matched probe sites with increasing voltage until (c) where the mismatch is indistinguishable from the background noise but a strong matched signal is still apparent. As such, by simply using the proper washing voltage, simple electrokinetics can be used to fully discriminate SNPs.

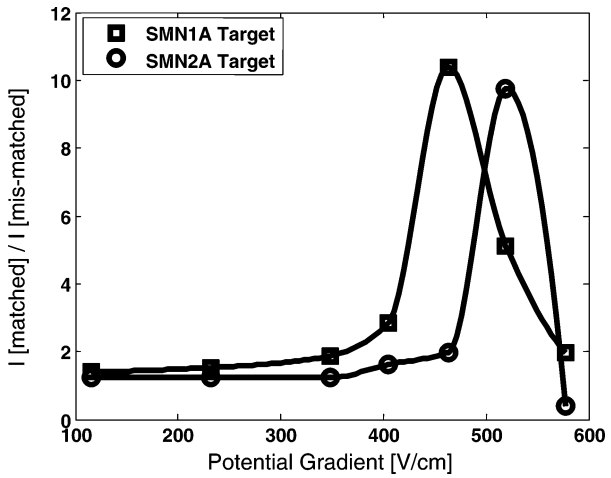

Figure 4. Optimal discrimination for both SMN1A and SMN2A targets. Data points show ratio of average intensity over the matched probe site to the average intensity of the mismatched probe site. Peak represents point where ratio of match intensity to mismatch intensity is greatest.

achieved. However, a higher degree of control and uniformity could be obtained.

To determine the optimal conditions for SNP discrimination, a series of incrementally increasing voltages were applied during the discrimination step, ranging from 100 to 600 V/cm within the hybridization array (computation of the potential gradients within the hybridization channel is discussed in the proceeding section). Figure 3 shows the hybridization signal from SMN1A targets on both SMN1 and SMN2 probes at illustrative points during these experiments. At a lower voltage, SMN1A registered signals on both zones (Figure 3a). When the higher voltages were applied, the intensity on the SMN2 probes decreased dramatically, while the intensity decrease on the SMN1 probes was much less substantial (Figure 3b). Optimal discrimination was obtained with an applied potential gradient of 460 V/cm, shown in Figure 3c, where the signal on SMN2 probe sites was completely eliminated. Significantly increasing the voltage above this point (Figure 3d) resulted in denaturation at all probe sites. Use of larger potentials has therefore been exploited for chip regeneration. Figure 4 illustrates the effect in greater detail by comparing the intensity of the matched probe sites with that of the mismatched probe sites for both SMN1A and SMN2A targets. While the intensity ratios remained constant at lower voltages, a clear optimum in the discrimination existed for each different selectivity, at 460 and 525 V/cm, respectively (though there exists a significant overlap region where both SNPs can be well discriminated). The difference in the optimal potential gradient between these two cases can be attributed to the change in the thermodynamic stability associated with the SMN1A/SMN2 hybrid. This is similar to traditional hybridization experiments where the optimal thermal conditions for discrimination vary (as above) dependent on probe length, type, and mismatch location. The advantage here is that the response time of the system is much quicker and several optimal conditions could scanned through rapidly (as only the microchannels needs to reach equilibrium instead of heating the entire microarray chip).

In our previous study,[34] the oligonucleotide target was directly diluted in the hybridization buffer and introduced into the chip. Electrophoretic mobility was used to overcome electroosmotic flow in the reversed direction and thus constituted only a negative factor serving to reduce the apparent mobility of the oligonucleotides in the channel and increased the time required for sample dispensing and washing. To reduce the analysis time, here the electroosmotic flow was suppressed by dynamically coating[39,40] PVP onto the PDMS/glass channel walls by adding some low-mass polymer to the hybridization buffer. Experiments conducted over a range of PVP concentrations revealed that 0.1% (w/v) PVP was sufficient to suppress almost all of the electroosmotic (EO) flow without significantly reducing the electrophoritic mobility due to sieving effects. As a result of EO flow suppression, a 3-fold decrease in the time required to transport the sample over the hybridization array was observed. Additionally the PVP coating provided a much more uniform electrokinetic transport environment, reducing the chip-to-chip variability in surface properties. Accordingly, 0.1% PVP in the hybridization buffer was used for all experiments. Experimental results also showed that the dynamic PVP coating was stable at relatively high temperatures. An additional benefit of dynamic PVP coating was the reduction in the adsorption of DNA on the glass/PDMS surfaces. PVP was used as one main component in modified Denhardt's solution to reduce DNA adsorption onto the glass surfaces.[38] Off-line experimentation with conventional microarray slides verified that the adsorption of the DNA on the glass surfaces could be eliminated by adding PVP in the hybridization buffer.

Microfluidic Chip Thermal Characterization. There are three phenomena that may contribute to the electrokinetically induced denaturation: Joule heating, electroosmotic shear, and

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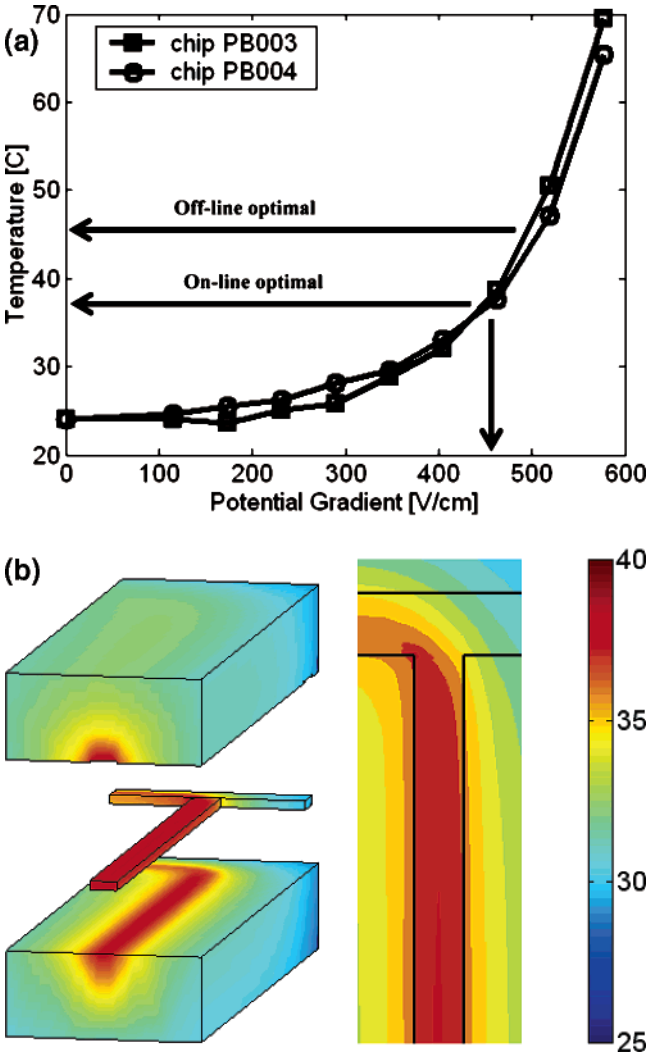

Figure 5. (a) Hybridization channel temperature increase with applied potential gradient. Temperature was measured using the Rhodamine B thermometry technique outlined in the text. Potential gradients compute via 3D numerical simulation of the temperature and flow and electrical field. Arrows show temperatures for on-line (in chip) and off-line (standard microarray experiments) optimal SNP detection. b: Computed 3D temperature profile during hybridization discrimination stage at channel intersection for an applied voltage of 2000 V (460 V/cm).

the electrical force on hybrids induced by electrophoresis of the ssDNA targets. To characterize the relative importance of the first of these effects in comparison to the others, a detailed thermal characterization of the microfluidic system used herein was undertaken. Figure 5a shows the measured in-channel temperature with increasing applied potential field using the Rhodamine B thermometry technique described in the Experimental Section. Results from two different chips with identical geometry are also shown in order to illustrate the chip-to-chip repeatability of the technique. The data reported were taken 30 s after the application of the external potential. The new results about current stability and direct dynamic temperature control, as well as some previously reported results,[29] suggest this time is more than sufficient to reach a steady state. The rapid increase in channel temperature at higher voltages is as a result of the increasing buffer conductivity with temperature, which induces a higher current load on the system than would be obtained from a proportional increase in

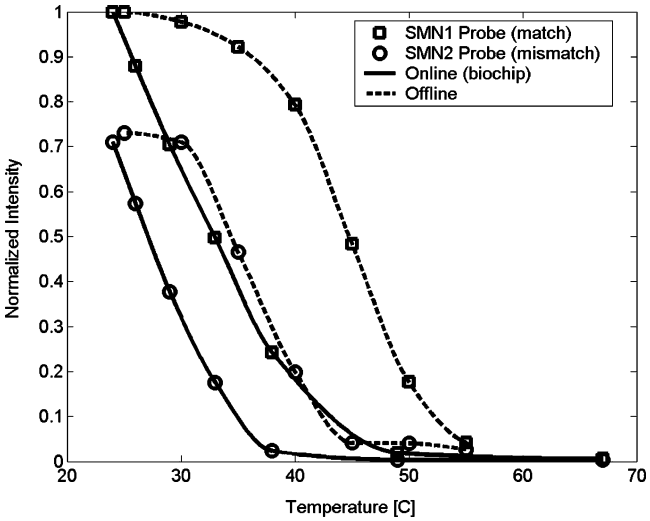

Figure 6. Comparison of the normalized intensities between on-line electronically controlled chip and the off-line microarray. The 0.5 μM SMN1A in the hybridization buffer was used as the target. Solid lines, on-line results of SMN1A target on SMN1 and SMN2 probes, the temperature in the channel was measured by Rhodamine B thermal characterization at different potential gradients; dashed lines, off-line results of SMN1A target on the SMN1 and SMN2 probes, at different washing temperature.

applied potential. Comparing Figure 5a with the results shown in Figure 4 reveals that the optimal on-line SNP discrimination occurs between 36 and 38 °C.

Computation of the potential gradients within the hybridization channel was complicated by the dependence of buffer conductivity on temperature; thus, full 3D numerical simulations of the temperature field[29] were required. Figure 5b illustrates the numerically predicted temperature profile in the hybridization channel under a computed voltage gradient of 460 V/cm. As can be seen, the temperature profile in the hybridization channel is higher than that in the dispensing arms as a result of the increase of the potential field gradient within the smallest channel width section of the chip. To ensure that other sections of the chip do not overheat prior to reaching the desired discrimination temperature in the hybridization array, it was necessary (for this case) that the hybridization channel have a smaller channel width than the dispensing arm.

Comparison with Off-Line Hybridization Array Experiments. Off-line hybridization melting curves for SMN1 probes obtained using a glass microarray are shown in Figure 6 and are compared with intensity data obtained from on-line temperature control. The similarity of the intensity−potential gradient relationship to the off-line melting curves for fully matched and one-base-mismatched hybrids on the glass suggests that Joule heating is the dominant effect leading to the SNP discrimination. This means that SNP discrimination can be achieved by optimization of the applied potential gradient on the microfluidic chips, similar to the procedure of optimization of the hybridization/washing temperature when using a DNA microarray. The melt curve showed that best discrimination between the single-base-mismatched and complementary targets in off-line experiments occurred at 40−45 °C. Compared to the off-line results under the same buffer conditions and the same kind of glass slide, the electrokinetically controlled on-line results showed a lower optimum temperature.

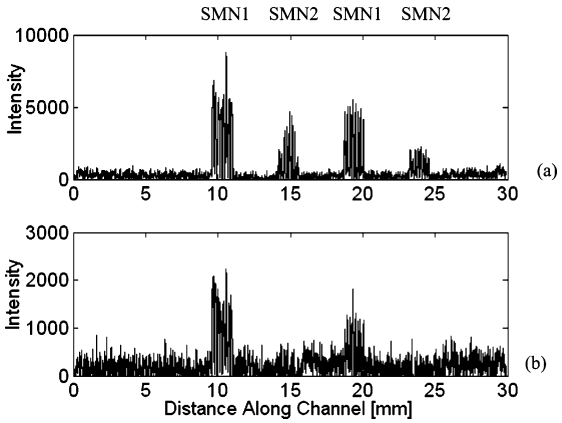

Figure 7. Hybridization intensity of asymmetric PCR product containing SMN1A sequence at matched (SMN1) and single-base pair-mismatched probe sites (SMN2) at washing voltages of (a) 116 V/cm in the hybridization channel and (b) 463 V/cm.

This suggests that, besides the Joule heating effect, the coupled electroosmotic shear and electrical forces do play a role in inducing denaturation. The reduction in the melt temperature difference between the matched and mismatched hybrids, as illustrated in Figure 6, suggests a reduction in the specificity of the reaction for the on-line case. As this figure does not include the effects of shear and electrical on the reaction specificity, it is difficult to make a direct comparison between the two.

The function, duration, and voltage conditions for each stage in the on-line analysis protocol are detailed in Table 2. As can be seen, the total analysis time for the on-line hybridization, including the time of final flushing for chip regeneration, was ~5 min. The reduction in analysis time (as compared with several hours for traditional microarray experiments) coupled with the ease of sample handling and SNP discrimination represented a significant improvement in the overall process.

PCR Products for SNP Detection. To demonstrate the clinical relevance of the hybridization chip, asymmetric PCR products were used for microfluidic SNP analysis. The protocol for detection of the PCR products was the same as that used for oligonucleotide targets, except for the dilution methods. Considering the operational convenience and the small sample volume required, 1:2 or 1:4 mixing of the PCR product with the running buffer was done before sample introduction without further treatment of the PCR solution. Despite the low sensitivity of the epifluorescence microscope system that relied on excitation using a mercury lamp, the SNP fluorescence intensity signal could still be determined on the hybridization chip with reasonable signal-to-noise ratio. The total amplicon concentration in the asymmetric PCR products was ~200 nM (including dsDNA and ssDNA, approximately in 1:1 ratio). Figure 7 shows the hybridization results following high-temperature washing, using the Joule heating protocol described previously. The eight hybridization spots present within the SMN1 zone can be seen clearly against the background noise, while the hybridization signals that were previously seen at the SMN2 zones were absent. The components in the PCR cocktail, including the excess dye-labeled primer (in asymmetric amplification, 10:1 ratio of forward primer and reversed primer was used) and any primer−dimer, did not interfere with the hybridization/washing process on the chip. The chips could be regenerated by using an appropriately high voltage.

CONCLUSIONS

Electrokinetic sample delivery, washing, and Joule heating temperature control provided a fast, simple, and robust single-nucleotide polymorphism detection method on a microfluidic PDMS/glass chip. Genetic sequences prepared by asymmetric PCR amplification could be detected on the chip. The electrokinetically driven flow was used to provide sample delivery and washing. The Joule heating resulting from electric current produced in the channel was exploited for temperature control. The concurrent effect of electroosmotic shear, electrophoretic forces, and thermal energy within the microfluidic channel allowed for effective discrimination between complementary and SNP targets. Pumping, flow regulation units, external heaters, and thermal sensors were all unnecessary in this system. Because of the short analytical time required, simplicity of equipment, and ease of manipulation, this method has great potential for use in clinic diagnostic laboratories for applications in rapid genetic diagnostics.

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