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Rapid serial analysis of multiple oligonucleotide samples on a microchip using optically-gated injection

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Abstract

Optically-gated injection of fluorescently-labeled DNA has been accomplished for the first time. Rapid, serial analysis of oligonucleotide ladders has been shown on a microchip using this injection technique. Separations of five- and six-component samples have been completed in 60 s or less with a capability to carry out serial injections of these samples every 15 s. The technique has been shown to have better than five base resolution for small oligonucleotides and excellent reproducibility in migration times (≤0.75% RSD). Currently, the limit of detection for the system is 0.23 μ*M*. Additionally, multiple unique samples of DNA have been consecutively analyzed in a single separation lane using optical gating. Six consecutive injections of three different samples have been achieved with no sample carryover and a total analysis time of ~10 min. These results show the potential of optical gating as an alternative injection technique for high-throughput DNA applications, such as genotyping and monitoring dynamic processes.

Keywords: Chip technology; Injection methods; Optical gating; Oligonucleotides; DNA

1. Introduction

There has been tremendous growth in the amount of genetic information collected in the last decade. The genomes of 60 species have been sequenced to date and this number is rapidly increasing [1]. The knowledge gained from sequencing the human genome promises to revolutionize the way medicine is practiced through the use of genetic diagnostic assays. There are currently 1500 diseases for which a mutated gene has been identified and can be used for diagnosis [1]. The number of available genetic assays is expected to continue to rapidly expand with the assays becoming routine diagnostic tools in clinical settings. These developments have driven the search for new analytical techniques which can perform genetic analysis more rapidly and at a lower cost than conventional methods. Electrophoresis on microfabricated chips is one of the techniques that has been extensively studied for this purpose and shows great promise in becoming a realistic, clinical tool [2–6].

DNA analysis on a microfabricated chip was first shown in 1994 [7,8]. Since then, there have been an astounding number of publications demonstrating the use of these devices for DNA sizing, sequencing and genotyping (see reviews in Refs. [2–6,9,10]). Microchips provide rapid, accurate DNA analysis with low reagent consumption and the ability to integrate sample preparation, separation and detection components onto a single device. A primary goal of much of the work in this field has been to achieve

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high throughput analysis of unique samples. This has been accomplished by decreasing separation times and either repeating several serial injections in a single separation lane [7,11–16] or multiplexing many parallel separation lanes onto a single chip [17–20]. Combinations of both approaches have been shown as well, where serial separations of samples are performed in individual lanes of a parallel array [16,18,20]. The requirement for automated analysis of multiple samples is critical to the utility of a genetic diagnostic device.

DNA analysis can be accomplished on microfabricated chips ~50–100 times faster than on conventional slab gels and 5–10 times faster than in capillaries [5,6,9]. This increase in speed is attributed in part to the use of shorter separation distances on chips. As separation distance decreases, the ability to form well-defined, narrow injection plugs becomes increasingly important in order to maintain good peak efficiency [2,6]. In most applications, this has been accomplished using the cross-T method. This technique and its variations are well-characterized and have demonstrated the ability to create reproducible, picoliter-sized injection plugs [2,6,21].

Another injection technique, originally developed to generate very narrow injection plugs in fast separations with capillary electrophoresis, is optical gating [22,23]. In this method, two laser beams are focused onto the capillary; a gating beam, of high intensity, is used for injection and a probe beam is used for laser-induced fluorescence (LIF) detection. The gating beam photobleaches fluorescent sample as it is continuously electrophoresed through the capillary. A shutter is used to momentarily block the gating beam, allowing a small plug of sample to move past the injection window without being photobleached. The fluorescently-labeled plug is separated into its components while moving down the capillary and is subsequently detected at the probe beam. Optically-gated sample introduction not only has the ability to form narrow injection plugs, as mentioned above, but is also capable of performing rapid, serial injections with ease, making it ideal for high throughput applications and studying dynamic processes. In capillaries, it has been applied to multidimensional separations [24,25], kinetic studies [26] and dynamic chemical monitoring [27,28]. Recently, optical gating has been accomplished on a microchip using fluorescently-labeled amino acids, and has demonstrated comparable results to the cross-T method in its injection volume and reproducibility [15]. Although the cross-T injection mode has many advantages, optically-gated injection could find an important niche with its ability to do rapid, serial analysis while maintaining a constant potential across the separation lane. In addition, there is the potential to conserve chip space that results from the need for only two reservoirs per separation lane, instead of three or four [15].

In this paper, optical gating is applied to separations of DNA samples. Short, fluorescently-labeled oligonucleotides are rapidly injected and separated in series on a microchip. Resolution, efficiency and peak area are examined under various conditions for these separations. Additionally, three unique samples are analyzed in series in the same separation lane, demonstrating the potential of this technique for high-throughput applications, such as genotyping. The samples are deposited into different sample wells on the chip and are consecutively electrophoresed into the separation lane. Optically-gated injection, followed by separation and LIF detection, is performed on each sample. The ability to analyze multiple, unique samples in an automated fashion is critical for the potential of optical gating on a microchip to be used as a practical diagnostic technique and advancement toward this goal is presented here.

2. Experimental

2.1. Microfabrication

The microfabricated chips were constructed, as previously described [15], using traditional photolithography techniques at the EMPRL Nanofabrication Facility (The Pennsylvania State University, University Park, PA, USA). Borofloat glass plates (Technical Glass Products, Mentor, OH, USA) were used to fabricate these chips. A simple cross-T design was used and the final chip had 230-μm wide, 74-μm deep, channels. Pipette tips were epoxied (ITW Performance Polymers, Riviera Beach, FL, USA) over holes drilled into the top plate of the chip

to form larger reservoirs and minimize sample and buffer evaporation.

2.2. Sieving matrix and sample preparation

In these experiments 3% linear polyacrylamide (LPA) was used as the sieving medium. It was prepared by dissolving acrylamide powder (ICN Biomedicals, Aurora, OH, USA) in TBE buffer (10 m*M* Tris, 10 m*M* borate, 1 m*M* EDTA) and polymerizing with 10% ammonium persulfate (Aldrich, Milwaukee, WI, USA) and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Sigma, St. Louis, MO, USA). Single-stranded poly-T oligonucleotides of various lengths were obtained from either the Penn State Nucleic Acids Facility or IDT (Coralville, IA, USA). The oligonucleotides were labeled at the 5′ end with 6-carboxyfluorescein (FAM; excitation wavelength (λ_{ex}) = 496 nm, emission wavelength (λ_{em}) = 516 nm) or fluorescein (λ_{ex} = 492 nm, λ_{em} = 520 nm) and were diluted to the desired concentration in the LPA solution.

2.3. Instrumentation

Optical gating of the fluorescent DNA sample was performed with the 488-nm line from an argon ion laser (Innova 70, Coherent, Santa Clara, CA, USA), as described previously [15]. The beam was split and focused onto the microfabricated chip in two places. Previous to the split the laser power was ~300 mW. The higher intensity gating beam was passed through a shutter before reaching the chip to facilitate the optical gating process. The gating beam diameter at the chip surface was ~1 mm. Confocal, LIF detection was utilized at the probe beam with a 60× 0.70 NA microscope objective (Olympus, Melville, NY, USA). The resultant signal was collected with the same objective, passed through a dichroic mirror (Chroma Technology, Brattleboro, VT, USA), spatially (100-μm pinhole, Edmund Scientific, Barrington, NJ, USA) and optically filtered (515-nm cut-on, Oriel, Stratford, CT, USA and 520-nm bandpass, Omega Optical, Brattleboro, VT, USA), and collected with a photomultiplier tube (Hammamatsu, Bridgewater, NJ, USA). The signal was then amplified (Keithley Instruments, Cleveland, OH, USA) and collected at 100 Hz using a Labview program (National Instruments, TX, USA) written in the laboratory. Data analysis and baseline subtraction were performed with PeakFit v4.11 (SPSS, Chicago, IL, USA).

2.4. Procedures

A vacuum pump was used to fill the chips with the DNA/LPA sample. Labeled DNA was placed in the sample reservoir and held at ground. The detection reservoir was filled with LPA and held at positive potential from 500 V to 1.3 kV with a high-voltage power supply (Bertan, Hicksville, NY, USA). Electric fields are noted in the figure legends. The gating and probe beams were aligned onto the long arm of the chip ~1.7 cm apart, defining the separation distance.

In the experiments where multiple samples were analyzed in a single separation lane, the chip was filled with LPA and three of the reservoirs were filled with different fluorescent samples. Migration of sample into the separation channel was accomplished by toggling the ground between each of the three reservoirs with a manual switch, floating the remaining two reservoirs. Each arm of the T was loaded for 15 s prior to the start of the experiment. An injection was performed from one sample reservoir, then 40 s later the ground was switched to the next reservoir. Approximately 1 min later an injection was performed by optical gating.

3. Results and discussion

3.1. Characterization of oligonucleotide separations with optical gating

Optical gating is utilized to make several serial injections of fluorescently-labeled oligonucleotides on a microchip. Fig. 1 depicts repeated injections of a sample containing two FAM-labeled oligonucleotides: 30- and 40-mer poly-Ts; and four fluorescein-labeled oligonucleotides: 10-, 20-, 60- and 80-mer poly-Ts. Two separations are shown at each of the five injection times between 100 and 500 ms. Each separation is completed 40 s after injection, indicated by the arrows. This is comparable with other reported separation times for oligonucleotides on

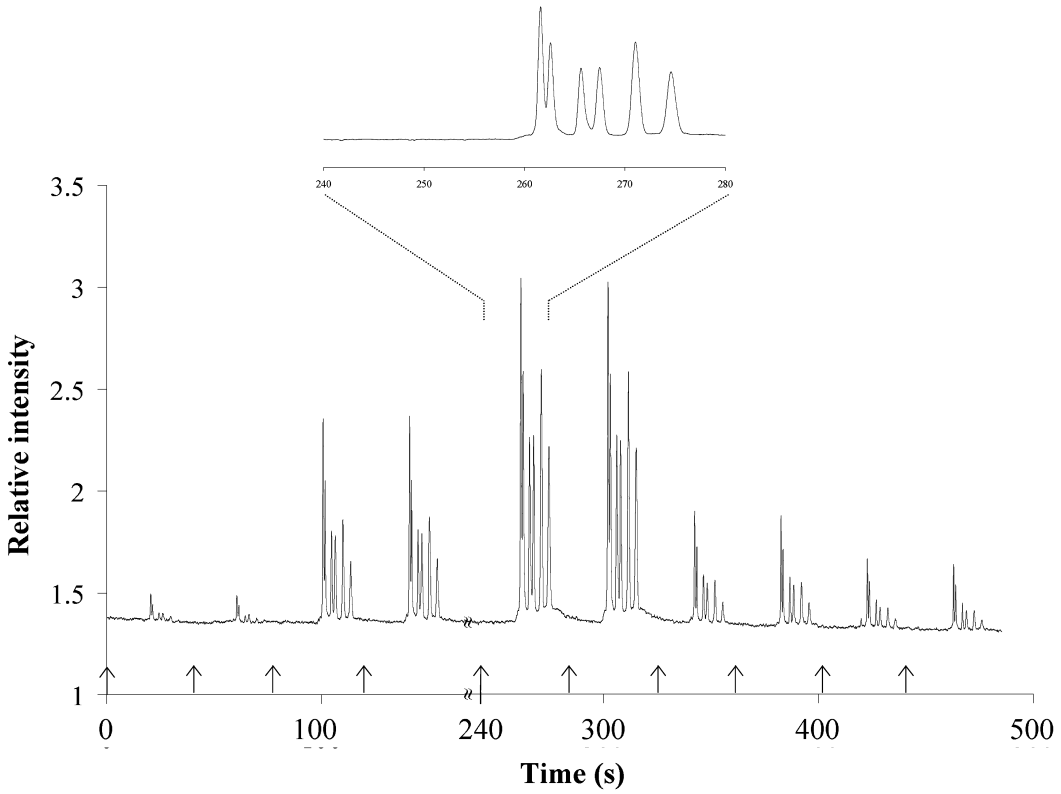

Fig. 1. Serial separations of six fluorescently-labeled oligonucleotides at various injection times. Fluorescein-labeled 10-, 20- (0.5 μ*M*), 60- (1.25 μ*M*) and 80-mer (2 μ*M*) fragments and FAM-labeled 30- and 40-mer (1.25 μ*M*) fragments were injected twice at each of the following injection times: 100, 300, 500, 200 and 150 ms. The arrows indicate the point of injection. The first 500-ms injection is enlarged as shown in the inset. Two 200-ms injections were removed from the plot to eliminate redundancy as shown in the plot. Separation conditions: 324 V/cm, 1.72-cm separation distance.

chips, making it a competitive technique for DNA analysis [7,8,29]. There is a 60-s delay between each injection; however, this could clearly be reduced to increase throughput. Since throughput is ultimately defined by the time from detection of the first peak to detection of the last peak, the delay time could be decreased to ~15 s in this case. In the following discussion, several parameters will be examined to characterize the separations shown in Fig. 1.

As shown in the inset of Fig. 1, there is clear resolution between each of the fragments with the electric field, *E*, used in this experiment. The effect of *E* on resolution is examined in Fig. 2. It shows that resolution between the 10- and 20-mer fragments increases with increasing *E*, from 162 to 324 V/cm. Electric fields over 324 V/cm are avoided because of suspected Joule heating in the chip, resulting in poor baselines. Another important factor that contributes to resolution is the concentration of LPA. In all of the experiments shown here 3% LPA

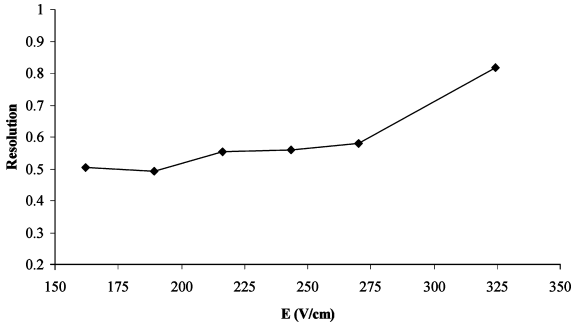

Fig. 2. Resolution between the 10- and 20-mer fragments versus electric field, *E*. All injections were 500 ms. Other experimental conditions were the same as in Fig. 1.

is used. Lower concentrations of 2 and 2.5% LPA were examined, but exhibited greatly reduced resolution for these small oligonucleotides (data not shown). At concentrations above 3%, it becomes difficult to fill the microchip and clean it for repeated use with the current set-up.

Increasing injection time reduces both resolution and efficiency in the separations shown in Fig. 1. There is a 26% decrease in resolution as injection time increases from 150 to 500 ms; therefore, it is advantageous to work at the shortest injection time possible when resolution is of primary importance in the experiment. Additionally, efficiency, *N*, decreases from $\sim 6\times10^5$ plates/m to 4×10^5 plates/m as injection time increases from 150 to 500 ms, as shown in Fig. 3. At higher injection times, more fluorescent sample is allowed to pass through the gating region before photobleaching resumes. This results in larger sample plugs and less efficient peaks. Furthermore, the peaks for the smaller fragments in the sample generally have higher efficiencies than the larger fragments. One reason for this could be that the smaller fragments move through the separation region faster than the larger peaks, and are therefore subject to less band-broadening.

Perhaps the most obvious feature of the data shown in Fig. 1 is that peak height increases with injection time. As expected, this holds true for peak area as well. Fig. 4 shows a plot of peak area/concentration versus injection time for these data. There is a clear, linear trend in increasing peak area as injection time increase from 150 to 500 ms. It should be pointed out that the smaller fragments of

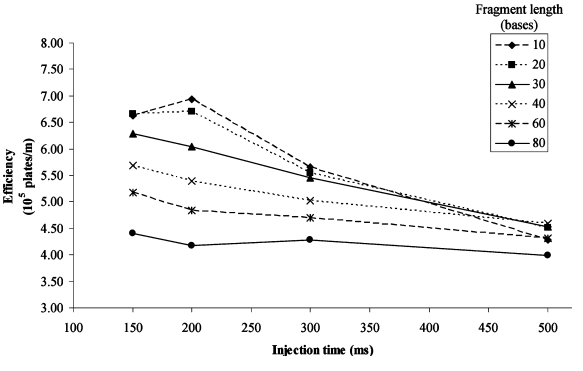

Fig. 3. Efficiency, plates/m, versus injection time for the data shown in Fig. 1.

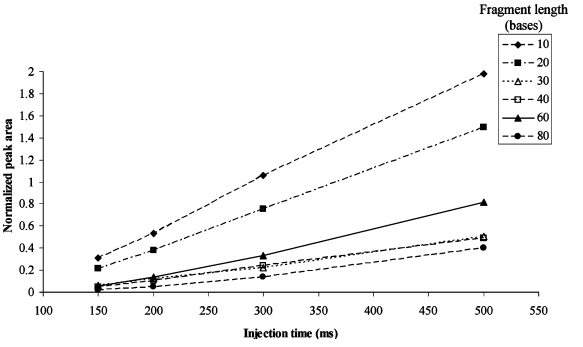

Fig. 4. Normalized peak area versus injection time for the data shown in Fig. 1. Peak area was normalized by dividing the original value by the concentration of the fragment.

the ladder have greater peak areas than the larger fragments due to preferential electrokinetic loading of high mobility analytes over low mobility analytes. This biased injection was anticipated when preparing the sample. Consequently, the concentrations of the various fragments are adjusted so the smaller fragments are at lower concentrations than the larger fragments. The peak areas used in Fig. 4 are normalized by dividing the original values by the concentration of the fragment. Despite the existence of this preferential loading, all of the fragments are at concentrations of 2 μM or less. The lowest concentration used in these experiments is 0.5 μM, but it is estimated that the concentration limit of detection (LOD) is currently 0.23 μM. The LOD is ultimately defined by the residual background fluorescence resulting from incomplete photobleaching of the sample. Further study is required to reduce this limit of detection.

Two of the peaks, the 30- and 40-mer, clearly do not follow the same trend as the others in terms of peak area. There are several possible reasons for this, including that they were labeled with FAM instead of fluorescein. Additionally, they were obtained from the Penn State University Nucleic Acids Facility (NAF), which presumably uses different labeling and purification procedures than IDT. The peak areas for the 30- and 40-mer are smaller than expected, which may be a result of lower labeling efficiency for the procedure used at NAF compared with that used at IDT, resulting in less labeled DNA in the sample than is actually reported. Furthermore, the migration

times of the fragments in Fig. 1 are not linear with respect to fragment length. To correct this irregularity, an experiment was performed using samples only from IDT.

Fluorescein-labeled 10-, 20-, 30-, 60- and 80-mer poly-Ts, obtained from IDT were analyzed with the same system (data not shown). All of the fragments were baseline resolved. The migration times exhibited excellent linearity with increasing base number (correlation coefficient = 0.9999; $n = 13$) and were evenly spaced. The relative standard deviations for these data range from 0.22 to 0.75%, which illustrates the exceptional reproducibility of optical gating as an injection method.

To further examine the limit of resolution of this technique, separations of fluorescein-labeled 10-, 15- and 20-mer fragments were performed. Fig. 5 shows clear resolution between each of these fragments, demonstrating that this method has the ability to achieve better than 5-base resolution. The excellent reproducibility in migration time, linearity of migration time with increasing fragment size and high resolving power all indicate that this technique is ideal for the sizing of small oligonucleotides, adding to its value as a genotyping tool.

In addition to demonstrating optical gating as a viable and reproducible technique for DNA analysis, Fig. 1 shows the potential for this method to be used in monitoring dynamic processes, such as enzyme digests and polymerase chain reactions (PCRs). The speed and ease with which optical gating can make serial injections from a single sample reservoir makes it a potential alternative to the cross-T method. Cross-T has also been used to make repeated injections of oligonucleotides from one sample reservoir at a rate of ~1 sample/min [7]. As mentioned previously, optical gating has the advantage of maintaining a constant potential across only two reservoirs throughout the experiment, facilitating the use of a simple chip design and electronics set-up.

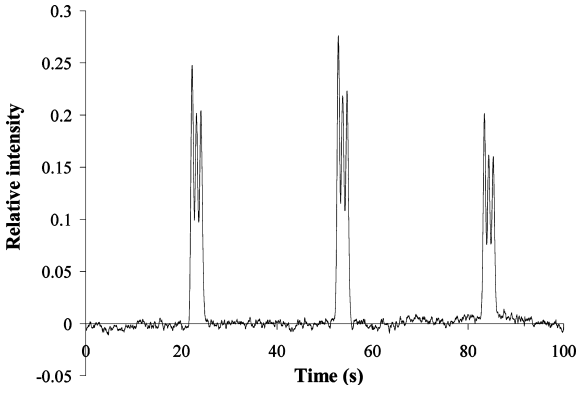

Fig. 5. Three serial separations of fluorescein-labeled 10-, 15- and 20-mer (1 μM) fragments. The first two injections were at 400 ms and the third injection was at 300 ms. Separation conditions: 360 V/cm, 1.65-cm separation distance. The data were baseline corrected using PeakFit.

3.2. Optical gating of multiple samples on a single chip

The application of optical gating on a microchip to high-throughput analysis techniques, such as genotyping, requires the ability to sequentially analyze unique samples without user intervention. A move toward this important advancement has been accomplished by using optical gating to analyze three distinct samples, in series, in a single separation channel. The chip layout for these experiments is shown in Fig. 6A. Three sample reservoirs, 1, 2 and 3, contain different combinations of fluorescein-labeled oligonucleotides. In this case, reservoir 1 contains a mixture of 10-, 20-, 30-, 60- and 80-mers, reservoir 2 contains 10- and 30-mers, and reservoir 3 contains only a 10-mer. The samples are sequentially electrophoresed into the channel, optically gated and detected. In Fig. 6B, the order of analysis is sample 1, sample 2, sample 3. Without making any adjustments to the microchip, a second run is started and the order of analysis is reversed, as shown in Fig. 6C. About 1 min of run time is allowed between each analysis to flush the separation channel of one sample and fill it with the next. It is expected that this time could be reduced by optimizing the chip design and the method by which potentials are applied to each of the reservoirs. Nevertheless, if the experiments in Fig. 6B and C were run consecutively, the total analysis time for six separations would be less than 10 min. This is comparable to what has been achieved using the cross-T technique to inject and separate four DNA digests in a single separation lane [16]. However, four additional sample reservoirs were added to the cross-T design for a total of eight

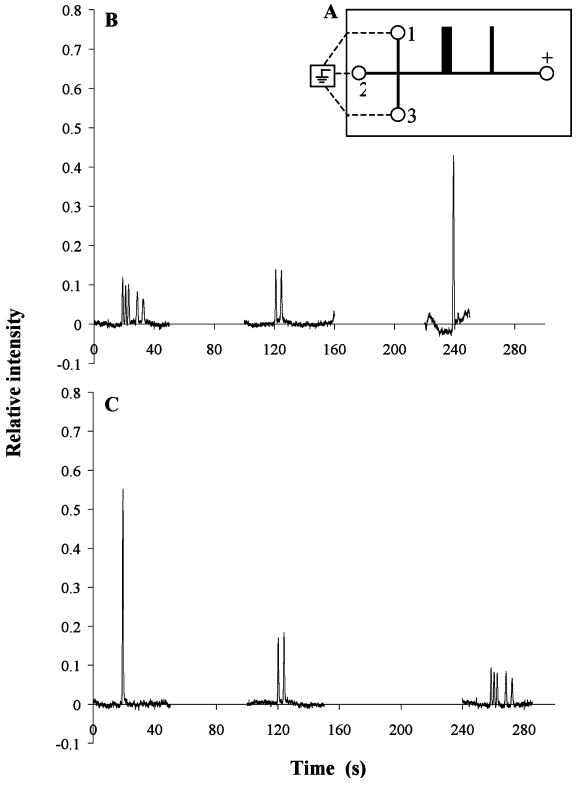

Fig. 6. (A) Schematic of the chip set-up for injection of multiple samples into a single separation lane. Different fluorescein-labeled samples were placed into reservoirs 1, 2 and 3. The ground was toggled between each of the reservoirs using a manual switch. The gating beam and probe beam are represented by the thick and thin lines, respectively. (B) Serial separations of three unique samples. Reservoir 1 contained 10-, 20- (0.5 μM), 30-, 60- (1 μM) and 80-mer (2 μM) fragments. Reservoir 2 contained 10- (0.5 μM) and 30-mer (1 μM) fragments and reservoir 3 contained just a 10-mer (1.5 μM) fragment. The order of injection was reservoirs 1, 2, 3. Each injection was 800 ms. The ground was switched to the next reservoir 40 s after injection of the first sample and injection of the second sample occurred 1–1.7 min later. The data between the switch from the first reservoir and the injection of the next sample were removed for clarity. Separation conditions: 360 V/cm, 1.65-cm separation distance. Data were baseline corrected using PeakFit. (C) Three serial injections performed directly after those shown in B. The order of injection was reversed from the previous run to reservoirs 3, 2, 1. All other experimental conditions were the same.

reservoirs. Optical gating requires a simpler chip design where the total number of reservoirs is $n + 1$, where n is the number of samples to be analyzed. Additionally, the separations shown in Fig. 6 are achieved with a single power supply at constant potential.

A primary concern when analyzing several samples in one separation lane is that there could be sample carryover between analyses. However, the results in Fig. 6B and C show no observable sample carryover in any of the six separations, despite the fact that the separation channel is not flushed with run buffer between samples; rather, the samples can be injected consecutively saving time and increasing the overall throughput of this technique. These results also show that the technique retains all of the qualities discussed in the previous section when applied to serial analysis of unique samples, including high resolution and efficiency, and reproducibility in migration times. These results demonstrate the probable efficacy of optical gating on a microchip for high throughput DNA analysis.

4. Conclusions

The results presented in this paper demonstrate that optical gating has the ability to perform rapid, serial separations from a single reservoir, suggesting its use for monitoring dynamic processes. It can also perform rapid separations of multiple samples in a single separation lane, on a time-scale equivalent to what has been shown with the cross-T. This can be achieved with a simple chip design and no sample carryover between samples. Work is currently in progress to combine the serial method demonstrated here with the more traditional approach for attaining high throughput of multiplexing several parallel lanes on a chip. This will further increase the number of samples that could be analyzed on a single chip.

Clearly, optical gating and cross-T injection both have advantages when used on microchips. The benefit of optical gating as an injection technique may become more apparent as microchip dimensions become smaller and smaller and injection width becomes more important to maintain adequate peak efficiencies. At present, optical gating has demonstrated excellent reproducibility, high resolution and the potential for high-throughput, indicating that it could be a very useful technique for analysis of small DNA fragments.

Acknowledgements

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References

[1] L. Peltonen, V.A. McKusick, Science 291 (2001) 1224.
[2] V. Dolnik, S.R. Liu, S. Jovanovich, Electrophoresis 21 (2000) 41.
[3] C.L. Colyer, T. Tang, N. Chiem, D.J. Harrison, Electrophoresis 18 (1997) 1733.
[4] D. Figeys, D. Pinto, Anal. Chem. 72 (2000) 330A.
[5] J. Khandurina, A. Guttman, J. Chromatogr. A 943 (2002) 159.
[6] C.H. Mastrangelo, M.A. Burns, D.T. Burke, Proc. IEEE 86 (1998) 1769.
[7] C.S. Effenhauser, A. Paulus, A. Manz, H.M. Widmer, Anal. Chem. 66 (1994) 2949.
[8] A.T. Woolley, R.A. Mathies, Proc. Natl. Acad. Sci. USA 91 (1994) 11348.
[9] E. Carrilho, Electrophoresis 21 (2000) 55.
[10] A.G. Ewing, P.F. Gavin, P.B. Hietpas, K.M. Bullard, Nat. Med. 3 (1997) 97.
[11] X. Huang, Q. Pu, Z. Fang, Analyst 126 (2001) 281.
[12] S. Attiya, A.B. Jemere, T. Tang, G. Fitzpatrick, K. Seiler, N. Chiem, D.J. Harrison, Electrophoresis 22 (2001) 318.
[13] Q. Fang, G. Xu, Z. Fang, Anal. Chem. 74 (2002) 1223.
[14] Y.H. Lin, G.B. Lee, C.W. Li, G.R. Huang, S.H. Chen, J. Chromatogr. A 937 (2001) 115.
[15] J.A. Lapos, A.G. Ewing, Anal. Chem. 72 (2000) 4598.
[16] P.C. Simpson, A.T. Woolley, R.A. Mathies, Biomed. Microdevices 1 (1998) 7.
[17] A.T. Woolley, G.F. Sensabaugh, R.A. Mathies, Anal. Chem. 69 (1997) 2181.
[18] P.C. Simpson, D. Roach, A.T. Woolley, T. Thorsen, R. Johnston, G.F. Sensabaugh, R.A. Mathies, Proc. Natl. Acad. Sci. USA 95 (1998) 2256.
[19] Z. Huang, N. Munro, A.F.R. Huhmer, J.P. Landers, Anal. Chem. 71 (1999) 5309.
[20] E.M. Smith, H.W. Xu, A.G. Ewing, Electrophoresis 22 (2001) 363.
[21] C.S. Effenhauser, G.J.M. Bruin, A. Paulus, Electrophoresis 18 (1997) 2203.
[22] C.A. Monnig, J.W. Jorgenson, Anal. Chem. 63 (1991) 802.
[23] C.A. Monnig, D.M. Dohmeier, J.W. Jorgenson, Anal. Chem. 63 (1991) 807.
[24] A.W. Moore, J.W. Jorgenson, Anal. Chem. 67 (1995) 3448.
[25] A.W. Moore, J.W. Jorgenson, Anal. Chem. 67 (1995) 3456.
[26] A.W. Moore, J.W. Jorgenson, Anal. Chem. 67 (1995) 3464.
[27] L. Tao, J.E. Thompson, R.T. Kennedy, Anal. Chem. 70 (1998) 4015.
[28] J.E. Thompson, T.W. Vickroy, R.T. Kennedy, Anal. Chem. 71 (1999) 2379.
[29] D. Schmalzing, L. Koutny, A. Adourian, P. Belgrader, P. Matsudaira, D. Ehrlich, Proc. Natl. Acad. Sci. USA 94 (1997) 10273.