High-throughput Analysis with 96-Capillary Array Electrophoresis and Integrated Sample Preparation for DNA Sequencing Based on Laser Induced Fluorescence Detection

by

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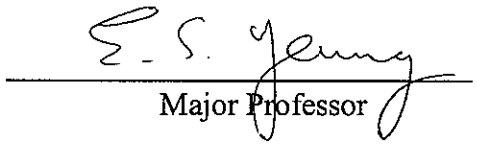

Major Professor

For the Major Program

To

My parents, who encouraged me,

My teachers, who enabled me, and

My wife, who completed me.

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ABSTRACT

The purpose of this research was to improve the fluorescence detection for the multiplexed capillary array electrophoresis, extend its use beyond the genomic analysis, and to develop an integrated micro-sample preparation system for high-throughput DNA sequencing.

We first demonstrated multiplexed capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) separations in a 96-capillary array system with laser-induced fluorescence detection. Migration times of four kinds of fluoresceins and six polyaromatic hydrocarbons (PAHs) are normalized to one of the capillaries using two internal standards. The relative standard deviations (RSD) after normalization are 0.6-1.4% for the fluoresceins and 0.1-1.5% for the PAHs. Quantitative calibration of the separations based on peak areas is also performed, again with substantial improvement over the raw data. This opens up the possibility of performing massively parallel separations for high-throughput chemical analysis for process monitoring, combinatorial synthesis, and clinical diagnosis.

We further improved the fluorescence detection by step laser scanning. A computer-controlled galvanometer scanner is adapted for scanning a focused laser beam across a 96-capillary array for laser-induced fluorescence detection. The signal at a single photo-multiplier tube is temporally sorted to distinguish among the capillaries. The limit of detection for fluorescein is 3×10^{-11} M (S/N = 3) for 5-mW of total laser power scanned at 4 Hz. The observed cross-talk among capillaries is 0.2%. Advantages include the efficient utilization of light due to the high duty-cycle of step scan, good detection performance due to

the reduction of stray light, ruggedness due to the small mass of the galvanometer mirror, low cost due to the simplicity of components, and flexibility due to the independent paths for excitation and emission.

The laser scanning system was slightly modified to achieve two dimensional laser scanning. 488 nm and 514 nm laser lines from a single Ar^+ laser were alternatively scanned for two-color excitation. Again, only one photomultiplier tube was used to record the fluorescent signal from all capillaries. Based on the differences in absorption spectra for the dyes, the peak-height ratios from 488 nm and 514 nm excitation electropherograms were used for peak identification for multiplexed CZE and CGE. Successful base calling for 24-capillary DNA sequencing was achieved to 450 bp with 99% accuracy.

Finally, we addressed the sample preparation for the high-throughput DNA sequencing. An integrated system of nanoreactor for cycle-sequencing reaction coupled with online CZE purification and CGE DNA sequencing is presented. Less than 100 nl of premixed reagent solution, which includes dye-labeled terminator pre-mix, BSA, and template, was hydrodynamically injected into a fused-silica capillary (75 μm i.d.) inside a homemade microthermocycler for cycle sequencing reaction. In the same capillary, the reaction products were purified by CZE followed by online injection of the DNA fragments into another capillary for CGE. Over 540 bp of DNA can be separated and the bases called for ss-DNA with 0.9% error rate. The total time was about 3.5 h, or a cycle time of 2 h with staggered operation. For ds-DNA, a longer reaction time was required and base calling up to 490 bp with 1.2% error rate was achieved. The whole system is readily adaptable to automated multiplex operation for DNA sequencing or PCR analysis.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation Organization

This dissertation begins with a general introduction. The following chapters are presented as four complete scientific manuscripts. General conclusions summarize the work and provide some prospective for future research. Appendices include the information of hardware and software in detail.

Capillary Electrophoresis

For no other biochemical separation method nowadays does one find so many new developments and methods as for electrophoretic separation techniques. From a historical perspective, the pioneering experiments of Tiselius in 1937[1] that showed the electrophoretic separation of blood plasma proteins provided the first practical demonstration of the use of electrophoresis as a separation technique. For this remarkable work, Tiselius was awarded the Nobel Prize in 1948. One of the early problems encountered when utilizing the Tiselius' approach was severe band broadening due to the convection currents generated by Joule heating. This effect was partly resolved by efficient fast water-circulating cooling of the electrophoresis cell. However, an important solution to this problem has been carrying out electrophoresis in buffer utilizing anticonvective supporting media such as paper,[2] starch,[3] agarose,[4] and most recently and importantly, polyacrylamide.[5] Today, polyacrylamide gel electrophoresis remains one of the most popular techniques for separation and characterization of proteins and nucleic acids in virtually every biochemistry laboratory.

A historical landmark advance in electrophoresis technique came in 1967, when Hjerten[6] first recognized that carrying out electrophoresis in narrow diameter tubes reduced thermal effects and demonstrated that it was possible to carry out electrophoretic separations in a 300-μm internal diameter glass tube and to detect the separated compounds by ultraviolet (UV) absorbance detection, which he called "free zone electrophoresis". Unlike the traditional electrophoresis, no supporting medium was necessary. Although other researchers used electrophoresis in tubes, glass, and Teflon[7,8,9], electrophoresis in a tube did not become popular until 1981 when Jorgenson and Lukacs[10] published their work in which they demonstrated the high resolving power of capillary zone electrophoresis (CZE). The simple and efficient instrumental setup, which is the basis of most commercial instruments on the market today, used a narrow ID fused-silica capillary, less than 100 μm, high voltage, 30 kV, and on-column UV detection for the separation of ionic species. The large surface-to-volume ratio of the narrow-bore capillary columns allowed for very efficient dissipation of Joule heat generated from the applied high electric fields. Separation efficiencies as high as 4×10^5 number of theoretical plates were achieved by these authors. Complex mixtures of analytes can be resolved and recorded as sharp peaks simply due to the lower risk of zone broadening.

Ever since that, tremendous efforts from many different research groups throughout the world have continued to focus on the new advances associated with this technique, which greatly expanded the horizons of CE. Today, "capillary electrophoresis" has become a collective name that is constituted of a family of specialized separation techniques using capillaries as separation channels and electric field as separation driving force, including CZE, capillary gel electrophoresis (CGE), micellar electrokinetic capillary chromatography

(MEKC), capillary isotacophoresis (CITP), capillary isoelectric focusing (CIEF), and capillary electrochromatography (CEC).

In analogy to chromatography, the zonal mode of operation is by far the most important technique in capillary electrophoresis. The reason is probably because of its simplicity and separation power. Basically, the 30-150 μm i.d. capillary, uniformly filled with a free buffer solution, is dipped into buffer reservoirs at both ends of the tube. Separations are achieved as a result of the unequal rate of migration of different solutes under the influence of an externally applied electric field. During its over two decades of practice, CZE has been successfully utilized to separate a wide range of charged simple molecules[11,12], organic and inorganic ions[13,14], peptides[15,16], proteins[17,18,19], carbohydrates[20,21], and nucleic acids[22] based on their difference in mass to charge ratio. Various addictives such as cyclodextrins[23] as well as other chiral discriminating agents[24] have been added to the separation buffers as chiral selectors to perform chiral separations by CZE.

Gels, defined as cross-linked networks swollen with a fluid component or sol, were introduced in electrophoresis originally as anti-convective media. But the viscous mesh with characteristic pore sizes turned out to be a very good sieving matrix, which provided unique size separation for analytes with similar charge to mass ratios, especially for large biological molecules like oligonucleotides, DNA restriction fragments, carbohydrates and proteins. Compared to conventional slab gel DNA analysis or SDS-PAGE protein analysis methods, CGE techniques usually provide the benefits of shorter analysis time, higher separation efficiency, lower sample consumption, and full automation of the analysis process including sample introduction, separation, and data reduction. Replaceable linear polymers generally used in CGE such as linear polyacrylamide (LPA) and its derivatives,[25,26] cellulose and its

derivatives,[27] polyvinyl alcohol[28], poly(ethylene oxide) (PEO),[29] and polyvinylpyrrolidone (PVP)[30] also provide unique possibilities of reusing the same capillary over many runs.

MEKC was unveiled by Terabe and co-workers in 1984[31] by adding surfactant SDS into the running buffer, where hydrophobic micelles were formed. These micelles provided a secondary phase other than the running buffer, which was usually called "pseudo stationary phase". Partitioning of the solutes between the buffer and pseudo stationary phase imparts a chromatographic mode of separation to CZE. By choosing ionic surfactant, even neutral compounds could be well separated based on their hydrophobicity,[32,33] which greatly extended the field of applicability of CE. Compared with its competing technique, reversed phase HPLC, much higher efficiency was obtained using only minute amounts of sample.[34] The "plug-like" profile of electroosmotic flow (EOF) and the very uniform distribution of the colloidal-sized micelles provide for less dispersion due to mass transfer problem. Some of the common surfactants developed for various MEKC applications include sodium dodecyl sulfate (SDS), dodecyltrimethylammonium bromide (DTAB), N-doecylsultaine (SB-12), polyoxyethlene-t-ocylphenol (Triton-X-100)[35] and polyoxyethylene(4)laurylether (Brij30).

To date, CITP and CIEF have been the least explored modes of CE operation. The former technique has been primarily used as an on-capillary preconcentration method for dilute mixture of analytes.[36] Approximate 100-fold enrichment can be achieved prior to CZE separations. This is especially useful when the analyte's concentration is very low (e.g., in biological media), thus UV detection is inadequate. In CIEF, a pH gradient under the influence of an electric field is set up along a capillary using ampholytes. Within the pH gradient, analytes, usually polypeptides, are focused to the position where their net charge is zero, i.e., where pH = pI. Focused analyte bands are usually transported slowly to the detect

window to be detected. CIEF is particularly useful for characterization of protein mixtures and determination of protein isoelectric points.

Another separation technique that is related to CE is capillary electrochromatography (CEC), which is a hybrid method between capillary HPLC and CE. In CEC, solvent transport is achieved by by electroosmotic flow (EOF) instead of hydraulic flow in HPLC. EOF arises in the electrical double layer, which exists at all solid-liquid interfaces. The main advantages of using EOF include a gain in separation efficiency through the plug flow profile and the usage of particles much smaller than in column HPLC. The latter is possible in CEC because of the absence of column back pressure when the solvents are moved by EOF. Column efficiency of 150,000 to 200,000 plates/meter or reduced plate heights of 1.7 to 2.2 were demonstrated by Erni et al with 50 μm i.d. capillaries packed with 3 μm ODS-Hypersil.[37]

As a brief summary, the most appealing advantage of CE over traditional separation techniques such as gas chromatography (GC), high performance liquid chromatography (HPLC), thin-layer chromatography (TLC), and slab gel electrophoresis (SGE) is its applicability for the separation of widely different compounds, inorganic ions, organic molecules and large biomolcules, using the same instrument and in most cases the same column while changing only the composition of the running buffer. This cannot be said about any of the other separation techniques. In addition, CE possesses the highest resolving power of any liquid separation technique, due to its plug flow and minimal diffusion. The amount of material needed for a CE experiment is minute, nanoliters of sample and microliters of buffer, compared to the other liquid separation techniques, which require microliters of sample and milliliters of solvent. The resulting CE waste, mostly an aqueous buffer, but

sometimes with a small percentage of organic modifier, is environmentally safe and can be discarded without any danger to the environment.

Capillary Array Electrophoresis

Some other advantages of CE, which were intentionally neglected in the previous section, such as the simplicity and small physical size of the instrumentation, took the center stage of the focus of many electrophoresis research groups in the past decade. Along with its other appealing features such as ruggedness of the columns, freedom from high-pressure pumps, and suitability for automation, CE turned out to be the most suitable technique in view of the trend towards running separations in parallel in order to greatly enhance the speed and throughput of chemical analysis in applications such as environmental monitoring, drug screening, clinical diagnosis, proteomics and industrial processing. A variety of elegant approaches were therefore proposed to achieve multiplexed capillary electrophoresis (CAE) separations. Dozens to hundreds of capillaries were bundled together to serve as independent separation channels with, generally, only one common detector, monitoring all different separation channels. The great success of these parallel CE separations revolutionized the separation field with their amazingly high throughput, high speed and low cost. It became the main workhorse for the completion of the 15-year global Human Genome Project (HGP) and greatly facilitated lots of new high-throughput screening techniques, which were out of imagination before the emergency of the CAE.

The pioneering work for capillary array electrophoresis was definitely the planar array of 25 capillaries with confocal scanning laser induced fluorescence (LIF) detection introduced by Mathies *et al.*[38,39] in 1992. The optical system utilized a planar array of 25

capillaries attached to a precision translation stage. As the translation stage was swept back and forth beneath the stationary microscope objective, the individual capillaries were interrogated in an epi-illumination format. In this format, the laser excitation beam was focused onto the capillaries by a microscope objective, and the emitted fluorescence was collected by the same objective using a 180-degree geometry, followed by detection using a photomultiplier tube. The small local illumination region enabled the use of a modest laser power and elimination of cross talk among adjacent capillaries. High repetition rate scan systems with sensitive closed feedback loops were required and therefore limited the number of capillaries that may be scanned. Constant improvement in illumination, detection and associated optical design were addressed since the debut and quite several commercial CAE instruments were presented.

Commercial Instruments.

Molecular Dynamics' instrument, MegaBASE 1000, was based on Mathies's confocal detection as described previously. The detection system utilized a set of four photomultiplier tubes with proper filers and dichroic beam splitters to achieve four-color detection for high-throughput DNA sequencing. Perkin-Elmer's system, ABI PRISM 3700 DNA Analyzer, employed post-column detection with liquid sheath flow. [40,41] Briefly, the capillary bundle is aligned inside a quartz cuvette. Along the dead space between the columns and the walls of the cuvette, a buffer solution is pumped through the cell. The liquid sheath flowing outside the capillary drags down the electrophoretic bands eluting from the column, tapering them to a small diameter. A laser beam crosses all flow streams and excites the fluorescent molecules. Light collection was made at 90° from the laser plane, which

reduced the scattered light. The fluorescent light is dispersed through a diffraction grating and imaged onto a cooled CCD camera. On-column detection is the approach of the SpectruMedix instrument, SCE 9600, which was first developed in our group.[42,43] The laser beam crosses all 96 capillaries that are immersed in a refractive index-matching liquid between glass plates. Due to the glass plates, the laser light is confined, and losses are minimized. The fluorescent light is collected at a right angle from the laser axis by an f-1.4 lens and detected by a CCD camera able to perform multicolor detection.

More interestingly, CombiSep Inc. recently developed the first 96-capillary array electrophoresis system based on UV-Vis absorption detection, MCE 2000.[44] The approach involves the use of a linear photodiode array on which a capillary array is imaged by a camera lens. Either a tungsten lamp or a mercury lamp can be used as the light source such that all common wavelengths for absorption detection are accessible by simply interchanging narrow-band filters. Zinc lamps can also be used for UV detection specifically at 214 nm. Each capillary spans several diodes in the photodiode array for absorption measurements. Over 100 densely packed capillaries can be monitored by a single photodiode array element with 1024 diodes. Since only about 10 percent of all compounds fluorescence naturally, this absorption approach provided unique simple solution for detecting a much wider range of analytes comparing with all the other commercial instruments based solely on laser induced fluorescence.

Homemade Capillary Array Systems.

Other than the listed commercial instruments, several other groups are still continuing to seek new advances on the development and design of CAE, mainly schemes concerning illumination and detection with LIF.

Kambara's group further tested side illumination with detection on column.[45] For this design, the number of capillaries in an array is generally limited by laser-power attenuation along the array due to reflection and divergence. Just recently, they improved the lens effect approach by placing the capillaries in water and intercalating each capillary with a glass rod of the same external diameter as the capillaries.[46] As a result, up to 45 capillaries could be simultaneously irradiated with a single laser beam and the fluorescence from all the capillaries could be detected with high sensitivity.

Quesada and Zhang took another approach for a multiple capillary instrument by the use of optical fibers for illumination and collection of the fluorescence in a 90° arrangement.[47] A more recent version of this instrument utilized cylindrical capillaries as optical elements in a wave guide, where refraction confined a focused laser beam to pass through 12 successive capillaries in a flat parallel array.[48] However, larger capillary arrays limited the refractive effects that spread the light along the length of capillaries.

Heller et al has designed a new CAE instrument and they also compared it with the existing systems in terms of detection limit.[49] In their instrument, the illumination of all 96 capillaries was provided by a laser line generator to produce a uniform intensity profile over the array, yet using fairly lower power laser (~30 mW). The instrument can also perform spectral analysis from 96 different channels with a single spectrograph. The sensitivity of 1×10^{-11} M (S/N = 40), which can be extrapolated to a detection limit in the very low pM range,

is similar to that achieved with fiber optic detection (3.7×10^{-12} M) or confocal scanning system (2×10^{-12} M).

In order to break the actual limit of 96 capillaries, several groups have attempted to address this problem by modifying their existing design. The expansion to 384 capillaries is foreseen for the SpectruMedix instrument, followed by an exchange in the camera lens.[50] Dovichi's group used sheath flow detection and a novel 2-D arrangement, which can hold up to 576 capillaries.[51] Mathies et al. has also continued to push the limit of the confocal system. They proposed a rotating-mirror version instrument with a microscope objective spinning inside a drum to work with up to 1000 capillaries.[52]

Extending the capability of CAE from a minute volume separation technique to a micro-preparation method also emerged as the focus of a couple of research groups. In Kambara's sheath flow detection scheme, they tried to set the 16-sampling-tray fraction collector beneath each sampling capillaries within the sheath flow cell to collect the flow-through DNA fragments.[53] Certain DNA fragments were automatically sorted by controlling the movement of the sampling trays according to the detected fluorescent signals. DNA fragments differ by one in length (363 and 364 bases) were successfully separated and further amplified by the followed PCR reaction, which opened the door for a new approach for gene hunting in research fields such as drug discovery and DNA diagnostics. A similar approach by mutli-well collection plate made of a solvent permeable gel was also demonstrated by Karger et al.[54]

Microfabricated CAE (μCAE).

A totally different platform to perform CAE is by microchip. Relatively well known in electronics, the microelectromechanical systems (MEMS) are being brought to chemistry as a compact platform to perform chemical analysis in the last decade.[55] For electrophoresis applications, while sharing a great deal of commonality with general fused capillaries in speaking of analytical properties and separation mechanism as general fused silica capillaries, microchips show some additional benefits. They have better heat dissipation efficiencies than do capillaries, so higher electric fields can be applied without causing significant Joule heating problems. When band-broadening in CE separations are dominated by axial diffusion, which is usually true when Joule heating can be neglected and the injection plug is small, CE separation efficiency is not a function of separation length but of applied voltage. Therefore, when very high electric field strength is used, the separation length and thus the analysis time can be substantially shortened without sacrifice of the separation efficiency. For example, Ramsey *et al.*[56] reported that electrophoretic separation of a binary mixture was achieved with baseline resolution in less than 150 ms with an electric-field strength of 1.5 kV/cm and a 0.9-mm long separation channel. Another advantage that could be easily seen is that a very large number of capillaries could be etched into a single wafer at low cost, making this technology especially amenable to highly parallel operation.

The first demonstration of 96-channel CAE in microchip was by Mathies' group for genotyping.[57] A microplate that can analyze 96 samples in less than 8 minutes have been produced by bonding 10-cm diameter micromachined glass wafers to form a glass sandwich structure. The microplate had 96 sample wells and 48 separation channels with an injection unit that permitted the serial analysis of two different samples on each capillary. An

elastomer sheet with an 8 by 12 array of holes was placed on top of the glass sandwich structure to define the sample wells. Samples are addressed with an electrode array that makes up the third layer of the assembly. Detection of all lanes with high temporal resolution was achieved using a laser-excited confocal fluorescence scanner as the authors used before. High-speed genetic sizing of DNA restriction fragments, PCR products and short tandem repeats were demonstrated.

DNA Sequencing and Related Sample Preparation

Up to now, one of the most important applications of CAE is high-throughput DNA sequencing. Actually, the initial driving force of the CAE was also the Human Genome Project (HGP), which was an international scientific collaboration aimed at sequencing the human genetic code with three billion base pairs in 15 years.

The basic strategy of DNA sequencing is similar to that of protein sequencing, which generally involves three steps: first, the large DNA molecule has to be specifically degraded and fractionated to fragments small enough to be fully sequenced; then, individual fragments are sequenced; the final step would be the ordering or resembling of fragments by repeating the preceding steps using a degradation procedure that yields a set of polynucleotide fragments that overlap the cleavage points in the first such set.[58] Before 1975, however, DNA sequencing techniques lagged far behind those of protein sequencing largely because there were no available endonucleases that were specific for sequences greater than a nucleotide. Dramatic progress was made in the past two decades owing to the several important advances in the nucleic acid research area: the discovery of restriction endonucleases, enzymes that cleave duplex DNA at specific sequences; the development of various DNA sequencing

techniques; and the development of molecular cloning and polymerase chain reaction (PCR), which permit the acquisition of any identifiable DNA segment in the amounts required for sequencing. Several completely different approaches were successfully used to characterizing a DNA molecule such as endonucleases restriction maps,[59,60] chemical cleavage method,[61] etc. But the most popular method for today's vast number of DNA sequencing tasks is the chain-terminator approach first developed by Sanger.[62]

The chain-terminator method, which is also usually called "Sanger reaction", utilizes the E. coli enzyme DNA polymerase to make complementary copies of the DNA being sequenced. The DNA fragment is first cloned into a plasmid or a vector. Then, the plasmid or vector DNA is extracted and used as template. Under the direction of the strand being replicated (the template strand), the DNA polymerase assembles the four deoxynucleoside triphosphates (dNTPs), dATP, dCTP, dGTP and dTTP, into a complementary polynucleotide chain that it elongates in the 5' $\rightarrow$ 3' direction. A primer, which is the 5' end of the chain being synthesized, is required to define the start position of replication and to initiate the reaction. In addition, a small amount of the 2',3'-dideoxynucleoside triphosphate (ddNTP) is added into the reaction mixture. Once the ddNTP is incorporated in the growing polynucleotide in place of the corresponding normal nucleotide, chain growth is terminated because of the absence of a 3'-OH group. Due to the competition between the dNTP and ddNTP, the replicated DNA fragment could stop at any position. A series of truncated fragments is generated, each of which is terminated by the ddNTP at one of the positions occupied by the corresponding base. Usually, either the primer or the ddNTP is uniquely labeled with distinct fluorescent dyes for emission spectra identification. After some cleanup

procedure, the Sanger fragments are separated by gel electrophoresis according to their lengths and therefore indicates the associated genetic sequence.

So generally, DNA sequencing usually involves five steps: (1) preparation of template DNA; (2) Sanger reaction to produce sequencing fragments; (3) purification of Sanger reaction products; (4) separation of DNA fragments; (5) base calling. In speaking of analytical technology development, people usually refer the latter two steps as DNA sequencing process while call the previous steps as sample preparation procedures.

DNA Sequencing.

For many years, slab gel electrophoresis (SGE) has been used as the workhorse for DNA sequencing, gene mapping and disease diagnosis. Basically, cross-linked polyacrylamide gel is sandwiched between two plates of glass. When the Sanger fragments (~5 μL) are loaded into wells at the top of the gel, an electric potential is applied between the top and bottom of the gel, causing the fragments to migrate through the gel and separate owing to length-dependent viscous drag. In general, 20 or more lanes could run simultaneously with about 12 hours separation time. The most recent slab gel sequencer, ABI 377, provided by Applied Biosystems, is capable of processing up to 96 samples per 9 – 11 h run with average read length of 650 - 750[63], which has the potential of producing sequencing data on the order of 100,000 bases/day.

However, the inevitable heat dissipation problem associated with the sandwiched gel geometry prevented SGE from shortening the run time. Only very low electric field could be applied. More recently, MJ Research Inc. and some other several groups tried to further push the throughput of SGE by using thinner slab gels[64] (<100 um in thickness) and multiplex

labeling.[65,66] But the labor-intensive steps of gel casting, gel loading, sample loading, as well as the difficulty of lane tracking limited their success.

Instead, the aforementioned CGE appears to be the most suitable alternative to address the persistence drawbacks of the SGE. The principal advantages associated with CGE is that the surface-to-volume ratio is much larger than in conventional SGE, and, as a result, higher electric field strengths (hundreds of volts per centimeter) can be used to drive the separation without excess Joule heating, resulting in very rapid, high-resolution separations. For example, efficiencies for separation of single-stranded fragments as high as 3×10^7 plates/m were sound in under 30 min. Nearly 3-fold improvement in resolution and 25-fold increase in the speed of the separation were achieved for individual separation lanes.[67,68] Mean while, the amount of sample required for analysis dramatically dropped to the nanoliter regime, which could potentially offer significant savings in the use of costly reagents required for preparing the sequencing fragments.

To compete with the parallel nature of SGE sequencer, CAE development were greatly spurred to achieve multiplexed CGE sequencing. Almost all the CAE instruments introduced in the previous section are originally designed for high-throughput sequencing. Automated 96-capillary gel filling and sample loading were easily developed for 24-hour unattended run. Less than 2 hours run usually is required for each 96-capillary parallel sequencing with read length up to 800 bases. More recently, Liu et al reported much faster μCAE sequencing by a microchip device.[69] The system routinely yielded more than 450 bases in 15 min in all 16 channels. In the best case, using an automated base-calling program, 543 bases have been called at an accuracy of >99%. Separations, including automated chip loading and sample injection, normally are completed in less than 18 min. In

Mathies's similar approach, 500 bases were resolved at over 99% accuracy in less than 20 min.[70] A 96-channel device based on these principles would be capable of 150,000 bases/h. Shi et al. described the possibility of building such a device based on a previous elegant 96-channel radial microplate design and high-speed rotary confocal scanner.[71] Cost in the separation and the identification (detection) tasks of DNA sequencing is substantially reduced as a direct result of automation and highly multiplexed operation.

Sample Preparation.

Unfortunately, the protocols for sample preparation and Sanger reaction did not keep pace with the low-cost high-speed high-throughput separation techniques. While capillary or microchip separations are scaled down to the nanoliter regime, the standard Sanger reaction and subsequent purification and injection are still performed in the microliter range because of difficulties in manipulating and transferring very small amounts of sample. Recently, miniaturization of PCR or cycle-sequencing reaction in capillaries or chips has been demonstrated. Burns and colleagues developed an integrated nanoliter DNA analysis device that is capable of metering a small, accurate volume of fluid by means of a hydrophobic patch and injected air.[72] Soper et al. made use of the biotin/streptavidin/biotin linkage to tether biotinylated DNA sequencing templates to the wall of the capillary for solid-phase cycle sequencing.[73,74,75] A single-dye amplification was accomplished in a volume of 62 nl in an (aminoalkyl)silane-derivatized fused-silica capillary. The capillary reactor was connected to the gel column via a zero-dead-volume fused-silica connector for on-line analysis. However, that approach involved a time consuming and labor intensive capillary coating process, which took more than 24 h. After a couple of runs, noticeable loss of anchored

streptavidin was observed and capillary regeneration was required.

Compatible miniaturized thermal-cycling equipment is also important for microscale sample preparation. Traditional metallic thermal blocks require a fairly long temperature transition time because of their high thermal mass. Several novel heating methods have been demonstrated for fast temperature change in the PCR or cycle-sequencing reaction. Hot-air thermocycler was first commercialized by Idaho Technology, which achieved rapid cycle sequencing within 25 min with dye-labeled primers.[76] Infrared-mediated heating was also successfully applied to microchamber thermal cycling.[77] Resistive thin films have been used to heat up individual capillaries with very fast ramping rate, although they may not be readily adapted for multiplex operation.[78]

Finally, to fully profit from the high throughput of such miniaturized systems, the integration of all the different parts are required. Several reports have shown a variety of coupling schemes for fluid handling with capillaries and valves in order to produce, purify and inject the sequencing sample.[79,80,81,82] Other reports showed total integration on microchips, performing PCR reactions, sample purification and separation,[83] although the protocol requires operator intervention.

Our Goal

Capillary array electrophoresis demonstrated very promising prospective in high throughput DNA sequencing and other genomic related analysis such as genotyping, mutation detection single nucleotide polymorphism (SNP) et al. But further improvement in performance and reduction in cost are imminent to make it a true state-of-art technique. Our goal is to develop a robust highly sensitive fluorescence detection system with simple design

for CAE sequencer. Automated nano-scale cycle sequencing sample preparation is also addressed to keep pace with the fast developing sequencing technology. Besides that, we also want to make full use of the high throughput of the 96-capillary array system by widening the application of such capillary arrays beyond the genomic regime.

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CHAPTER 2. MULTIPLEXED CAPILLARY ZONE ELECTROPHORESIS AND MICELLAR ELECTROKINETIC CHROMATOGRAPHY WITH INTERNAL STANDARDIZATION

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ABSTRACT

Although liquid chromatography and gas chromatography are the main workhorses in the analytical laboratory, samples can only be analyzed consecutively in an instrument. In this study, capillary zone electrophoresis and micellar electrokinetic chromatography separations are performed in a 96-capillary array system with laser-induced fluorescence detection. Migration times of four kinds of fluoresceins and six polyaromatic hydrocarbons (PAHs) are normalized to one of the capillaries using two internal standards. The relative standard deviations (RSD) after normalization are 0.6-1.4% for the fluoresceins and 0.1-1.5% for the PAHs. Quantitative calibration of the separations based on peak areas is also performed, again with substantial improvement over the raw data. This opens up the

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possibility of performing massively parallel separations for high-throughput chemical analysis for process monitoring, combinatorial synthesis and clinical diagnosis.

INTRODUCTION

Due to the continuous efforts of many research groups, for example, Jorgenson and Lukacs,[1, 2] capillary electrophoresis (CE) has been firmly established as a high efficiency and high speed analytical technique in recent years. Special techniques such as micellar electrokinetic chromatography (MEKC)[3] and capillary gel electrophoresis (CGE)[4] have extended the field of application to the analysis of neutral compounds and of important biological species. Some of the attractive features of CE include its small sample requirement and almost zero solvent consumption. Along with the small physical size of the columns, freedom from high pressure pumps and valves, ruggedness of the columns and suitability for automation, CE has many inherent advantages over liquid chromatography (LC) and gas chromatography (GC). These features are particularly important in view of the trend towards running separations in parallel in order to greatly enhance the speed and throughput of chemical analysis in applications such as environmental monitoring, drug screening, clinical diagnosis, proteomics and industrial processing. While it is necessary to run samples consecutively in a given instrument for GC and for LC, it is relatively easy to run CE in a massively parallel operation in one instrument.

Huang and Mathies demonstrated successful DNA sequencing in a 24-capillary array in a confocal geometry.[5, 6] More recently, a rotating-mirror version has been proposed to work with 1000 capillaries.[7] Our group has developed multiple capillary schemes based on

excitation through optical fibers[8] and through line focusing and simultaneous detection in a charge-coupled device (CCD) camera.[9] Other groups have reported multiple sheath-flow geometries for excitation and detection.[10-12] Various commercial versions of these concepts with 96 capillaries are already available. Other multiplexed CE schemes such as micromachined multichannels also have been suggested.[13, 14]

However, up to now, all the applications of multiplexed CE is limited to DNA sequencing and genetic analysis. The driving force is clearly the Human Genome Project. The combination of CZE or MEKC with multiplexed CE would greatly widen the application of such capillary arrays. This would provide a powerful tool to the chemical laboratory in addition to the molecular biology laboratory. Also, the fact that everything could be developed first for a single capillary in existing commercial instruments makes it convenient and extremely cost effective for scaling up to highly parallel runs in capillary array electrophoresis.

On the other hand, in order to receive wide acceptance in these areas, CE needs to provide comparable reproducibility as other established techniques such as GC and LC. Unfortunately, this remains the most difficult issue. Small variations in injection voltage, injection time, temperature, ionic strength and pH of the sample and buffer solutions and even the surface chemistry of the capillary can result in substantial changes in the migration times and peak areas in CE. These variations are not of concern in DNA sequencing, where the order of migration of the Sanger fragments rather than the absolute migration times is relevant and where the relative intensities at several wavelengths rather than the absolute intensities are used for base-calling. In genetic typing, DNA size standards provide for

reasonable calibration in a given capillary column. For critical applications where minor mobility shifts resulting from different base compositions or different fluorescence labels can lead to misinterpretations, the use of an absolute standard has been successfully demonstrated.[15]

Although several kinds of normalization methods exist, external standards have not been entirely satisfactory because of run-to-run variations. Internal standardization is frequently used in CE identification and quantitation. The reason is that the internal standard provides the possibility of multiple corrections to eliminate various discriminations mentioned above, such as injection, electroosmotic flow, temperature and the response factor of the detector. One internal standard[16-20] as well as two internal standards[21] have been used for normalization. The use of a single internal standard puts large constraints on the selection of the species used as the internal standard. In addition to criteria similar to those in HPLC, the optimum internal standard in CE requires that it is electrophoretically similar to the analytes, since in CE the linear velocities of each separated component at the detector is different. Alternatively, measuring the conductivity of the sample solution[22] or monitoring the electrophoretic current during separation and during injection[23, 24] have been shown to provide suitable correction factors for various experimental biases.

In this article, we present the CZE separation of four kinds of fluoresceins and the MEKC separation of six polyaromatic hydrocarbons (PAHs) on a 96-capillary array by using a simple but truly parallel detection scheme with laser-induced fluorescence. The first and the last peaks for each sample mixture are selected as internal standards to normalize the migration times and the peak areas of the other components in the multiple

electropherograms. Good reproducibility is thus achieved among capillaries in the same run and in between runs. The results show that reliable high-throughput analyses can be performed based on CE or MEKC.

EXPERIMENTAL SECTION

96-Capillary Array Electrophoresis System.

The experimental arrangement for fluorescence detection in a capillary array is shown in Figure 1. 96 capillaries (Polymicro Technologies Inc., Phoenix, AZ), 75 μm i.d. and 150 μm o.d., with 35 cm effective length and 55 cm total length were used. The detection windows of all 96 capillaries were close-packed to each other and clamped in between two flat surfaces of a plastic mount. The mount was attached to a magnetic base under the CCD camera. Since there are no moving parts involved in this setup, the image of each capillary was stable throughout many separations. At the ground end, the capillary array was bundled together. At the negative (injection) end, each capillary was inserted into a Peek tubing (1/16" o.d., 0.001" i.d.) with 4 mm of capillary tip extending out for injection. The Peek tubings were mounted on a copper plate in an 8 × 12 format with 9 mm spacing to fit standard microtiter plates. Gold-plated pins were mounted on the copper plate near each capillary tip to serve as individual electrodes, with the capillary tips slightly extended (~0.5 mm) beyond the electrodes to guarantee contact with small-volume liquid samples.

Commercial Capillary Electrophoresis System.

Single-capillary CE separations were optimized on an ISCO 3140 Electropherograph system (ISCO, Lincoln, NE) before the multiplexed CE runs. Uncoated fused-silica

capillaries (Polymicro Technologies Inc., Phoenix, AZ) were used with 75 μm i.d. and 60 cm length (35 cm effective length). Direct UV absorption detection was performed at 254 nm. A voltage of 12 kV was applied. The electrokinetic injection time was from 2 to 5 s.

Reagents and Chemicals.

All chemicals were of analytical reagent grade, and organic solvents acetonitrile and 2-propanol were HPLC grade. Fluorescein and 5(6)-carboxyfluorescein were obtained from Sigma (St. Louis, MO); 2,7-diacetate, dichloro-fluorescein was obtained from Acros. Brij-S was made by sulfonation of Brij-30 ($CH_3(CH_2)_{11}(OCH_2CH_2)_4OH$) with chlorosulfonic acid according to the procedure in ref. 25. 9,10-diphenylanthrancene and benzo[ghi]-perylene are kindly supplied by Dr. J. S. Fritz (Iowa State University, Ames, IA). All other chemicals were used as obtained from Aldrich (Milwaukee, WI), J. T. Baker (Phillipsburg, NJ), and Sigma (St. Louis, MO).

Preparation of Sample Solutions and Buffers.

The sample solutions of fluoresceins were prepared by dissolving appropriate amounts of these fluoresceins in 1 × TBE buffer with 0.2% (w/w) polyvinylpyrrolidone (PVP). Stock solutions of PAHs were prepared in acetonitrile. The sample solutions were prepared by adding appropriate aliquots of stock solutions to the buffer, resulting in solute concentrations of about 10 μM. The running buffer was prepared by adding appropriate aliquots of 1.0 M HCl and 250 mM Brij-S stock solutions into a mixture of deionized water and organic solvents. The pH was adjusted as required by adding 0.1 M HCl or 0.1 M NaOH and confirmed by a pH meter. Between separations, we used a pump to flush the capillaries

with the buffer solution. It takes no more than 1-2 min for column reconditioning for both CZE and MEKC.

Data Treatment.

The data acquired from the CCD camera per run is so voluminous that it is almost impossible to manipulate them manually. With the help of LabView (National Instruments) and Grams386 (Galactic Industries) software, we were able to program routines as macros and in array-Basic to convert the image files into multiple electropherogram files and analyze them in the batch mode. Migration times, peak areas and peak heights are picked out automatically for normalization.

RESULTS AND DISCUSSION

Normalization Methods.

In the multiplex capillary electrophoresis system we are using, the lengths of the 96 capillaries are similar. When voltage is applied on the two ends of the capillaries, they act as a parallel circuit and the field strength in each capillary is the same. The buffers (specifically the pH and the viscosity) are also identical in each capillary. Provided that the operating temperatures are the same, the electrophoretic migration velocity (v_{ep}) of each analyte should be identical from capillary to capillary.

$$(v_{ep,istd})_1 = (v_{ep,istd})_j \quad (1)$$

$$(v_{ep,i})_1 = (v_{ep,i})_j \quad (2)$$

Here, istd stands for the internal standards. The index i refers to each analyte and the index j refers to each capillary among the 96. However, as discussed before, electroosmotic flow (EOF) could vary greatly in different capillaries due to different surface chemistry, temperature and ionic strength in the sample zone. To a first approximation, it remains constant in a given capillary during the whole run and the migration of every analyte will be affected identically. That is to say, the electroosmotic velocity (v_{eo}) is the same for all the analytes in a capillary.

$$(v_{eo,istd})_j = (v_{eo,i})_j = (v_{eo})_j \tag{3}$$

The total migration of each analyte would be the sum of the two processes:

$$(v_{total,i})_j = (v_{eo})_j + (v_{ep,i})_j \tag{4}$$

In the normalization of the migration time in CE, we define the normalized velocity of analyte i ($v'_{total,i}$) as

$$(v'_{total,i})_j = (v_{total,i})_j - \Delta(v_{eo})_{j,1} \tag{5}$$

where $(v_{eo})_{j,1} = [(v_{eo})_j - (v_{eo})_1]$ and is the EOF difference between capillary j and capillary 1. From the definition, we can see that

$$(v'_{total,i})_j = [(v_{eo})_j + (v_{ep,i})_j] - [(v_{eo})_j - (v_{eo})_1]$$
$$= (v_{ep,i})_j + (v_{eo})_1$$
$$= (v_{total,i})_1 \quad (6)$$

In this way, the total velocity of the analyte in any capillary could be normalized to that in the first column, which then becomes independent of the electroosmotic flow difference among different capillaries. We may use an internal standard to measure the EOF difference:

$$\Delta(v_{eo})_{j,1} = [(v_{eo})_j + (v_{ep,istd})_j] - [(v_{eo})_1 + (v_{ep,istd})_1]$$
$$= (v_{total,istd})_j - (v_{total,istd})_1 \quad (7)$$

Also based on the relationship between v_{total} and t, the migration time per unit length:

$$(t_i)_j = 1/(v_{total,i})_j \quad (8)$$

We can define the normalized migration time for unknown analyte i (t'_i) as

$$(t'_i)_j = 1/(v'_{total,i})_j \quad (9)$$

So Equation (6) could be written as

$$1/(t'_i)_j = 1/(t_i)_1 \quad (10)$$

and Equation (5) becomes:

$$1/(t'_i)_j = 1/(t_i)_j - \Delta(v_{eo})_{j,1}$$

$$= 1/(t_i)_j - [1/(t_{istd})_j - 1/(t_{istd})_1] \quad (11)$$

Combining Equations (10) and (11) we can see that

$$(t'_i)_j = 1/\{1/(t_i)_j - [1/(t_{istd})_j - 1/(t_{istd})_1]\}$$

$$= (t_i)_1 \quad (12)$$

Equation (12) is the basis for utilizing one internal standard for the normalization of migration times. In practice, however, the operating temperature is typically not constant throughout the run (for any capillary). Temperature affects v_{ep} and v_{eo} differently. The column surface can also change during the run to affect each analyte differently. Equations (1) through (3) no longer hold. An improvement will be to use two internal standards to normalize the differences among capillaries. Provided that the two internal standards span the range of migration times of all analytes, interpolation will account for variations during the run, at least to first order.

The peak areas of the fluoresceins and PAHs are calibrated with internal standards following the two-internal-standard scheme introduced by Dose and Guiochon.[21] The electrokinetic injection volume was calibrated by the linear relationship between the effective

injection volume and the inherent mobility.

CZE in 96-Capillary Array.

Figure 2 depicts an image of a 96-capillary array electrophoresis separation of four kinds of fluoresceins. Over 10 runs were made with similar results. The horizontal direction is the capillary array arrangement while the vertical direction represents the migration time. This image shows that the four kinds of fluoresceins are well separated in all 96 capillaries, with minimal cross-talk between adjacent capillaries (< 0.8%). Each capillary produces a single electropherogram as shown in Figure 3. The detection limit at 488 nm is 10^{-10} M. From the image, we can see that the migration times of the 96 capillaries shifted substantially from one capillary to another. Even in this gray-scale representation, the intensities are also noticeably nonuniform. This particular packed capillary array has been used for 7 consecutive months in our laboratory for different tasks including genotyping and DNA sequencing. It represents a worst-case scenario for reproducibility evaluation for multiplexed CE runs.

In the fluorescein separation, we used the first and the last peaks in each electropherogram as the internal standards for normalization. Table 1 shows the relative standard deviation (RSD) of the migration times in the raw data as well as after normalization with one internal standard and with two internal standards respectively. It shows that the reproducibility of the migration times has been substantially improved from 6.3-9.7% to 0.6-1.4% by using two internal standards, or about 5-10 fold improvement. Actually, RSD does not describe the full range of correction that is possible. Figure 4(a) shows the individual migration times for 6-carboxyfluorescein before and after

normalization. The ruggedness of the normalization scheme is excellent.

We can see in Table I that although normalization of the migration times of 6-carboxyfluorescein by two internal standards is not as good as by using 5-carboxyfluorescein alone, the overall reproducibility is better with two internal standards than with either one internal standard. The similarity between the two isomers of carboxyfluorescein and their proximity in the electropherograms can explain the accidental superior normalization with only one internal standard. Unfortunately, this coincidence cannot be expected for the general case of components in a mixture and two internal standards are recommended.

It is interesting to note that inter-run statistics are about the same as intra-run statistics. When the raw data in Data Set 2 in Table I were normalized instead to capillary 1 in Data Set 1, the RSDs for the two analytes became 1.0% and 1.3% respectively, or nearly identical to those normalized to capillary 1 in Data Set 2. There is a weak correlation in migration times between the two runs ($r^2 = 0.58$ for peak 4), i.e. some capillaries always give faster migration times. These are not in any particular order in the array, as seen in the raw data in Figure 4(a). We can conclude that the capillary surface and/or diameter are responsible for variations in migration time rather than the running temperature. The latter is expected to be higher for the center capillaries.

Table II shows the reproducibility of the peak areas for the fluoresceins. There was 39-55% RSD in each set of raw data, which was much larger than the RSD of migration times. This mainly comes from the injection bias in each capillary and the Gaussian distribution of the power density of the laser beam over the 1.5 cm width of the capillary array detection window. Again the first and the last peaks were used as internal standards to perform the normalization. The relative standard deviation was lowered to 2.6-12%, or a 5-15

fold improvement. Quantitation based on peak heights produced similar results as in Table II. Figure 4(b) shows the individual peak areas for 6-carboxyfluorescein before and after normalization, confirming the utility of dual internal standards even for raw peak areas that are 10-fold different. However, unlike the case for migration times, there is no noticeable correlation between the peak areas from one run to the next for a given capillary. So, the RSDs are random measurement errors rather than from injection bias. We found that in Data Set 2 there is one electropherogram where the raw peak areas are unusually low. After normalization, the peak area was much lower than the other values in the array. When that electropherogram was omitted from the calculation of statistics, the RSDs became 3.1% and 7.7% respectively, or about the same as those in Data Set 1. This electropherogram also showed unusually long migration times, but those were properly corrected for as indicated in Table I.

MEKC in 96-Capillary Array.

In-house synthesized Brij-S was used as the surfactant in the MEKC separations.[25, 26] A very acidic buffer (pH = 2.4) was used to suppress EOF. Because of the high ionic strength, the current is fairly high. In single-capillary electrophoresis, we applied 200 V/cm on the 75 μm capillary and the current was about 60 μA. However, our power supply cannot safely provide enough current to sustain the high total current for all 96 capillaries (~6 mA). Some of the capillaries may even lose current during the separation because of the formation of bubbles in the capillary due to Joule heating. Since fast separations was not the main goal of this study, we lowered the applied voltage to 100 V/cm in the multiplexed runs and the migration time of the last PAH became quite long (~100 min). We expect that if a higher

capacity power supply is available and if liquid cooling of the capillaries is implemented, separations within 10-20 min should be obtained.[26]

Figure 5 shows an image of a 96-capillary array electrophoresis separation of six PAHs. More than 5 runs were made with similar results. Selected electropherograms are shown in Figure 6. Similar to above, the first and the last peaks were chosen as internal standards to normalize the migration times and peak areas of the other analytes. RSDs before and after normalization are listed in Table III. We can see that the RSD of the raw data is comparable to that of the fluoresceins, ranging from 2.6-13%, but the RSD of normalized migration times is substantially smaller, ranging from 0.1-1.5%. This is especially obvious in data set 2, where the RSD for all four normalized peaks are below 0.2%. This is because in MEKC the migration of the neutral species depends on the dynamic equilibrium of its association with the ionized surfactant. The equilibrium for each neutral species is independent of the electric field. Instead, the electric field affects only the migration of the surfactant which is a common parameter for all the PAHs. The EOF is also suppressed at these low pH conditions. Even if there is some residual EOF, again the effect is common to all PAHs.

RSD of the PAH peak areas are however larger than those for the fluoresceins. This might be due to the special surfactant Brij-S used in the separation. The background noise is much higher and the detection limit is only 10^{-7} M for most of the PAHs. Normalization results of the peak areas are listed in Table IV. The use of two internal standards reduced the RSDs of peak areas from 51-100% to 8-21%, which is also a 5-10 fold improvement.

Temperature affects the viscosity, pH, association equilibrium and the EOF. The fact that the normalized migration times are very reproducible in all cases implies that

temperature variations are quite well accounted for across the array despite the lack of liquid cooling and the high operating currents in MEKC. It is somewhat surprising that in both cases the RSDs for normalized peak areas is much larger than those for normalized migration times. After all, injection bias is related to the velocity of the analytes inside the capillary column.[23, 24] This can be partially rationalized by the inherent problems of fluorescence detection. A higher noise level exists due to stray light fluctuations, mechanical instability (which affects the excitation and collection efficiencies), photobleaching (which depends on the time the analyte molecules are within the laser beam) and intensity drift in the laser beam. This is supported by the fact that the fluorescein areas are more precise than the PAH areas, even though the latter group should suffer less bias in injection because they are all neutral molecules.

Computation Time.

Because we have programmed to manipulate the data in the batch mode, it is fairly easy to deal with the large amount of data. After the electropherograms for each of the capillaries in the array are extracted from the CCD image file, the most time consuming step in the whole normalization process would be testing the appropriate parameters for peak picking in the electropherograms. These parameters depend on the S/N of the data and drifts in the baseline. The Grams386 software provides several different kinds of methods to detect the beginning and ending points of the peaks as well as to smooth the raw data. Once these parameters are set, the program can complete the peak reports for each array run in 1 min, including migration time, peak area, peak height, etc. For this study, we are taking advantage of Excel 97 to do the rest of the calculations, but there should not be much difficulty to

incorporate this step into the analysis program. The total analysis time for one set of 96 capillary electrophoresis data for this manually-assisted batch mode is less than 15 min.

CONCLUSIONS

For the first time, CZE and MEKC were performed in capillary array electrophoresis. We simply used our in-house capillary array system originally developed for DNA analysis without much modification. Without the use of the sieving matrix and multiple color detection, the experiment is in fact much simplified. In this way, the throughput of CE was improved 100 fold. The particular experimental condition employed here for MEKC is applicable to many separations where LC is currently utilized.[25, 26] The small amounts of buffer solution needed for CE is essential for taking advantage of this exotic buffer additive. The use of two internal standards greatly improved the reproducibility of multiplexed CE and made it reliable enough for most industrial and pharmaceutical applications. RSD for the normalized migration times become comparable to those for GC and for LC. Peak area reproducibility are somewhat worse even after normalization. These are primarily due to mechanical instabilities and laser intensity fluctuations in the current prototype instrument. We expect that more sophisticated correction schemes such as those involving integrating the injection current and/or integrating the running current[23, 24] will provide additional improvements. One area that is of great interest is single-cell analysis[27, 28] with the ultimate goal to screen cell populations for disease markers. A single-capillary instrument simply cannot provide the required throughput. If the proper injection scheme could be achieved, such as by coupling a capillary array with flow cytometry, this concept may eventually be suitable for clinical diagnosis.

ACKNOWLEDGMENT

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Table I. Migration Time Reproducibility of Fluoresceins

		RSD, %			
Data Set 1		5-carboxy-fluorescein	6-carboxy-fluorescein	2,7-dAdCl-fluorescein	fluorescein
	raw data	6.2	6.3	7.8	8.4
	std A alone		0.2	1.8	2.1
	std B alone	1.4	1.3	1.3	
	two int std		0.6	1.2	

		RSD, %			
Data Set 2		5-carboxy-fluorescein	6-carboxy-fluorescein	2,7-dAdCl-fluorescein	fluorescein
	raw data	8.4	8.5	9.7	9.7
	std A alone		0.2	2.9	3.6
	std B alone	2.4	2.4	1.4	
	two int std		1.1	1.4	

Table II. Peak Area Reproducibility of Fluoresceins

		RSD, %			
Data Set 1		5-carboxy-fluorescein	6-carboxy-fluorescein	2,7-dAdCl-fluorescein	fluorescein
	raw data	39.1	38.7	38.6	38.5
	int std A		2.9	11.2	
	int std B		14.3	9.5	
	two int std		2.6	6.7	

		RSD, %			
Data Set 2		5-carboxy-fluorescein	6-carboxy-fluorescein	2,7-dAdCl-fluorescein	fluorescein
	raw data	52.1	52.9	52.6	55.2
	int std A		7.7	26.1	
	int std B		12.2	14.0	
	two int std		8.2	11.8	

Table III. Migration Time Reproducibility of PAHs

		RSD, %					
Data Set 1		BzPery	DPAnthr	BzPy	BzAnthr	Fluo	Anthr
	raw data	4.3	4.8	5.3	6.5	6.1	6.1
	std A alone		0.5	1.2	2.1	3.2	3.9
	std B alone	3.4	3.1	2.5	1.7	0.6	
	two int std		1.1	0.6	0.4	1.5	

		RSD, %					
Data Set 2		BzPery	DPAnthr	BzPy	BzAnthr	Fluo	Anthr
	raw data	4.0	4.3	4.6	5.2	4.9	2.6
	std A alone		0.6	0.9	1.6	1.1	0.7
	std B alone	0.5	0.5	0.4	0.3	0.1	
	two int std		0.2	0.2	0.1	0.2	

		RSD, %					
Data Set 3		BzPery	DPAnthr	BzPy	BzAnthr	Fluo	Anthr
	raw data	5.6	5.8	6.2	7.0	10.4	12.8
	std A alone		0.3	0.5	1.0	2.9	3.8
	std B alone	2.3	2.2	2.1	1.7	0.5	
	two int std		0.6	0.9	0.6	0.7	

Table IV. Peak Area Reproducibility of PAHs

		RSD, %					
Data Set 1		BzPery	DPAnthr	BzPy	BzAnthr	Fluo	Anthr
	raw data	62.6	71.6	59.4	59.8	62.4	56.6
	std A alone		20.7	18.1	20.6	18.4	
	std B alone		26.7	14.3	12.1	10.4	
	two int std		19.9	13.0	12.0	7.8	

		RSD, %					
Data Set 2		BzPery	DPAnthr	BzPy	BzAnthr	Fluo	Anthr
	raw data	93.2	80.4	75.2	84.6	99.7	85.2
	std A alone		18.2	23.9	28.1	26.6	
	std B alone		22.5	18.7	13.0	14.7	
	two int std		16.1	18.9	17.5	13.3	

		RSD, %					
Data Set 3		BzPery	DPAnthr	BzPy	BzAnthr	Fluo	Anthr
	raw data	56.1	58.5	57.2	51.0	62.7	54.6
	std A alone		11.4	15.2	16.9	21.3	
	std B alone		34.6	32.2	27.6	31	
	two int std		12.3	10.8	10.5	21.4	

FIGURE CAPTIONS

Figure 1. Experimental arrangement for fluorescence detection in a 96-capillary array.

Figure 2. Data for multicapillary electrophoresis of 4 fluoresceins. The horizontal direction represents locations across the capillary array. The vertical direction represents time from 0 to 30 min.

Figure 3. Selected electropherograms extracted from the data in Figure 3.

Figure 4. Comparison of raw data (open symbols) from Figure 3 vs. normalized data (solid symbols). (a) Migration times of 2 fluoresceins and (b) peak areas of 2 fluoresceins.

Figure 5. Data for multicapillary MEKC of 6 PAHs. The horizontal direction represents locations across the capillary array. The vertical direction represents time from 0 to 120 min.

Figure 6. Selected electropherograms extracted from the data in Figure 6.

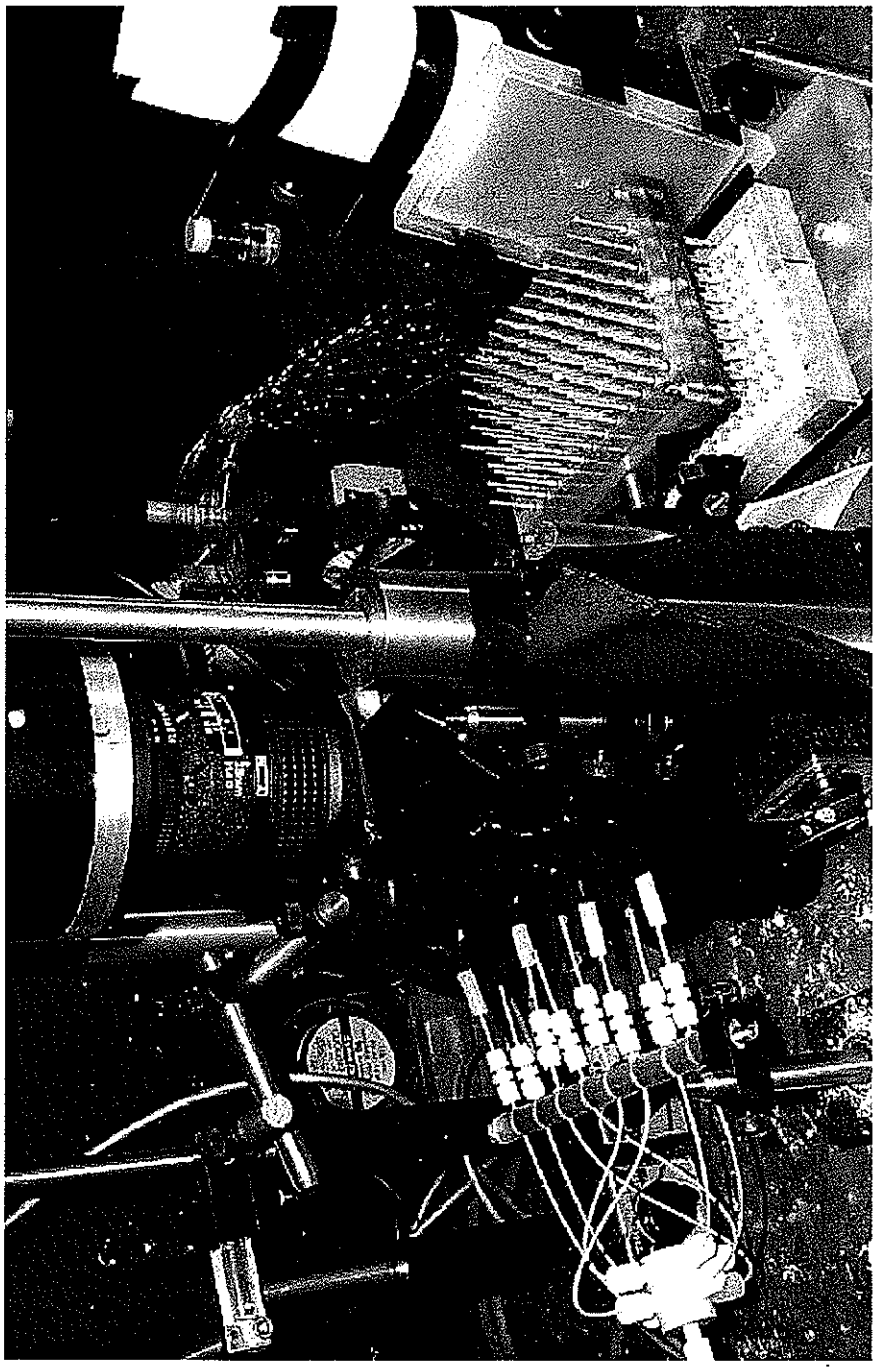

FIGURE 1

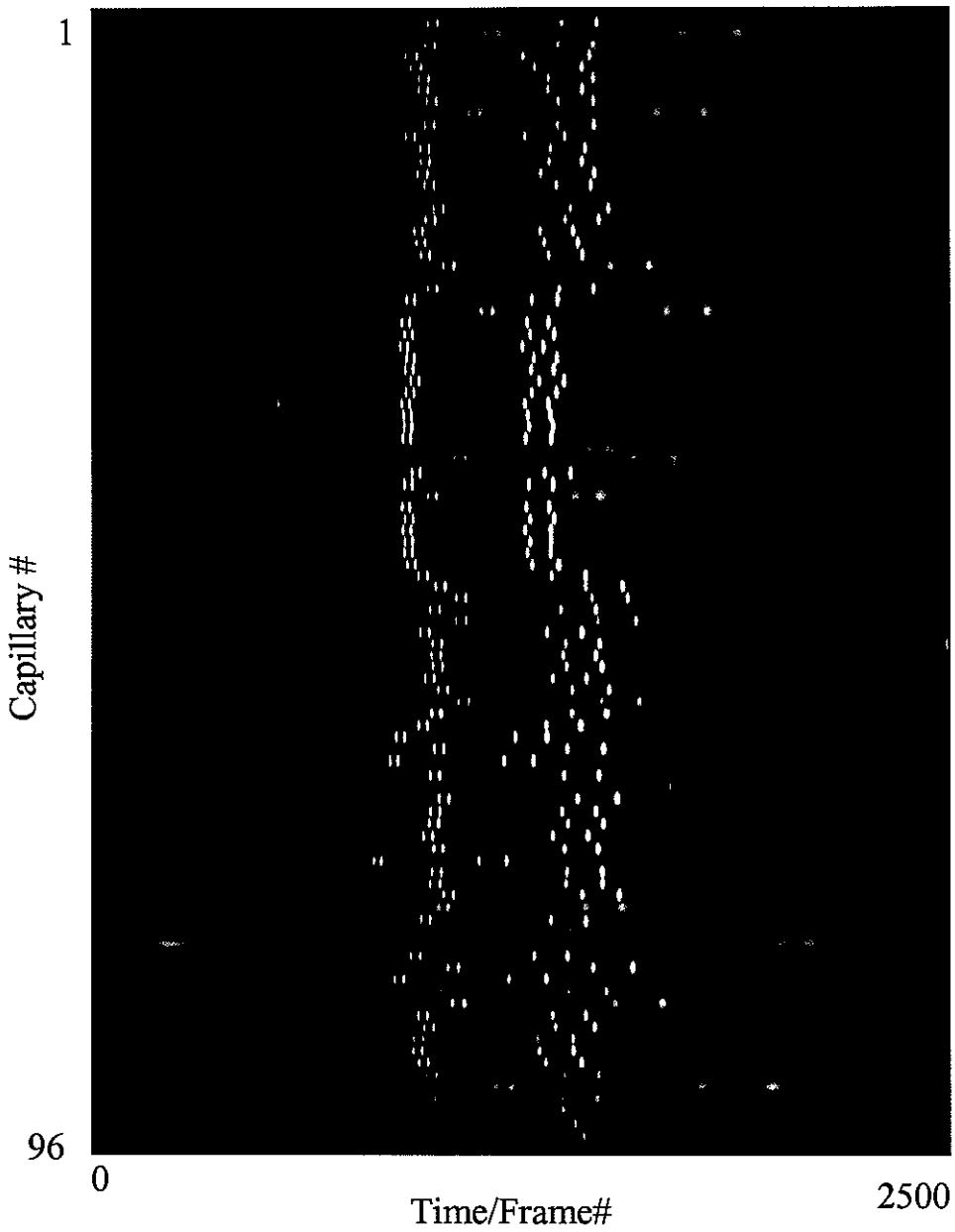

FIGURE 2

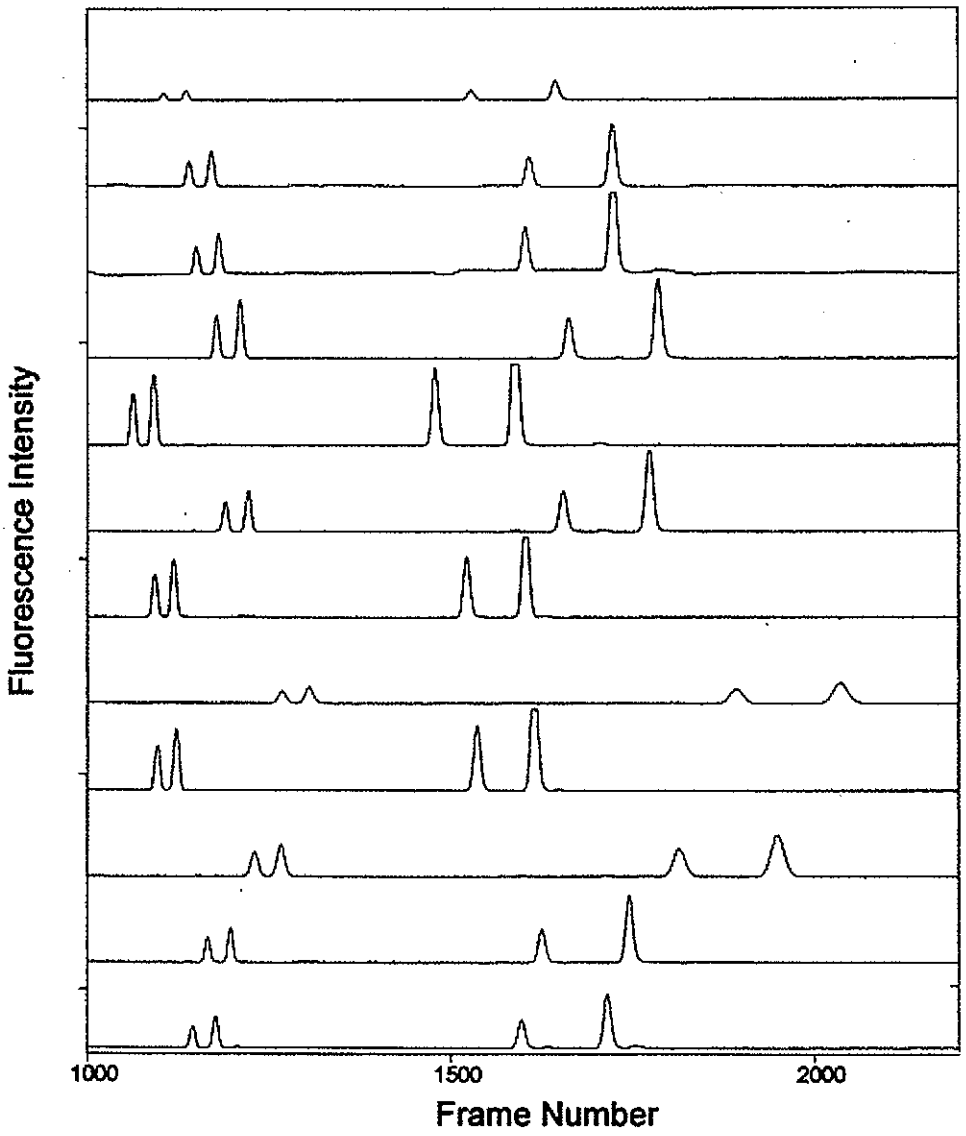

FIGURE 3

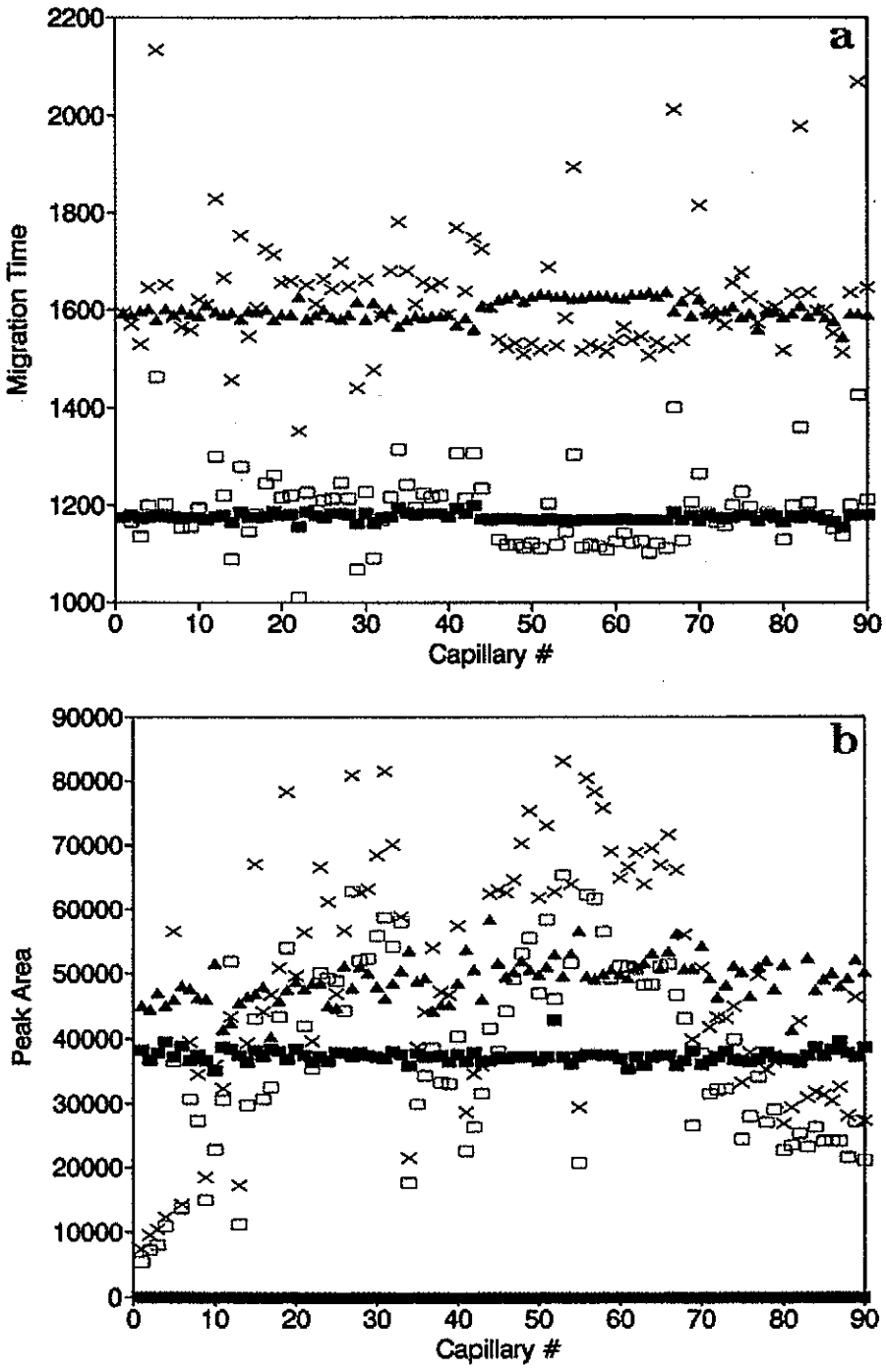
a
Migration Time
2200
2000
1800
1600
1400
1200
1000
0
10
20
30
40
50
60
70
80
90
Capillary #
b
Peak Area
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
0
10
20
30
40
50
60
70
80
90
Capillary #

FIGURE 4

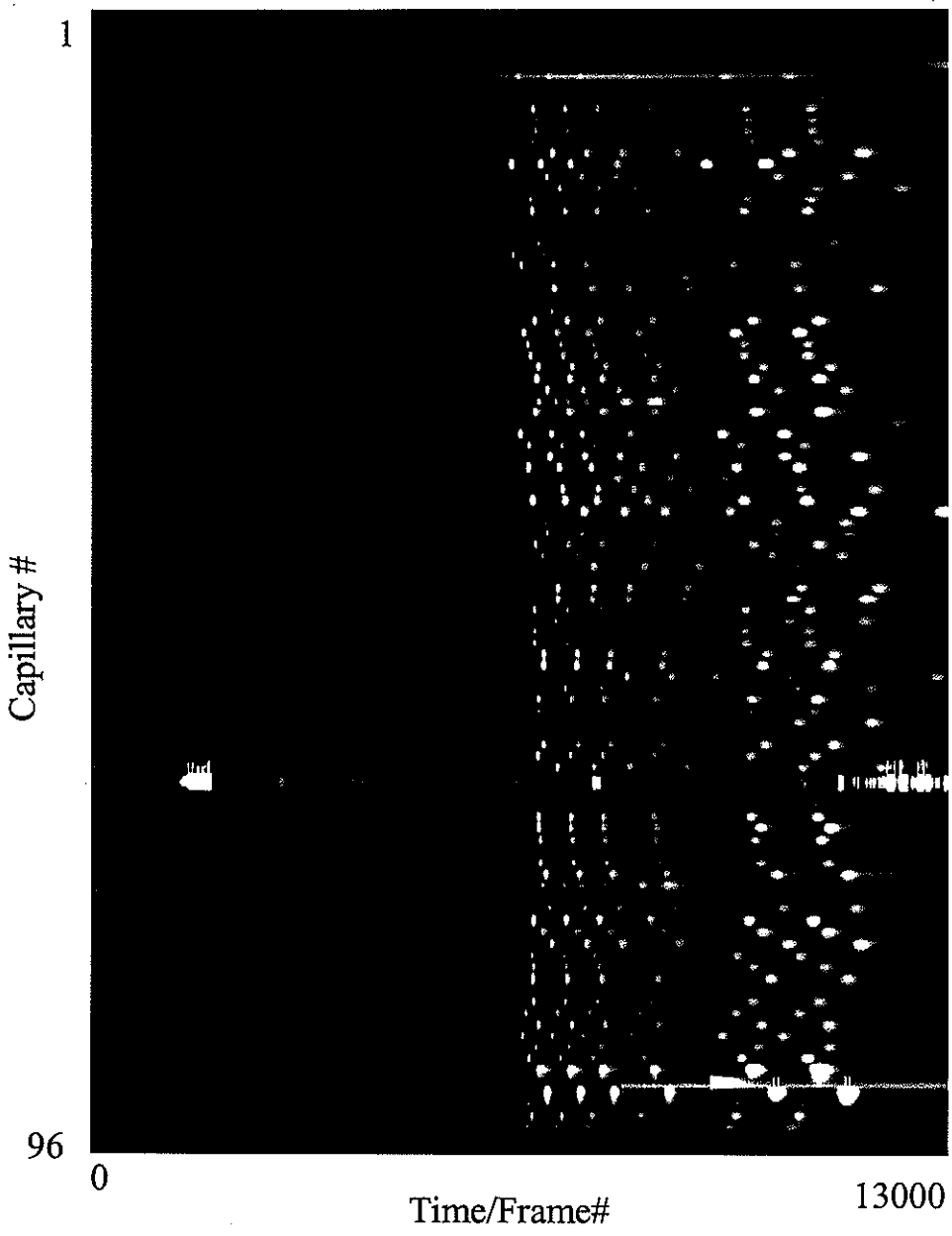

FIGURE 5

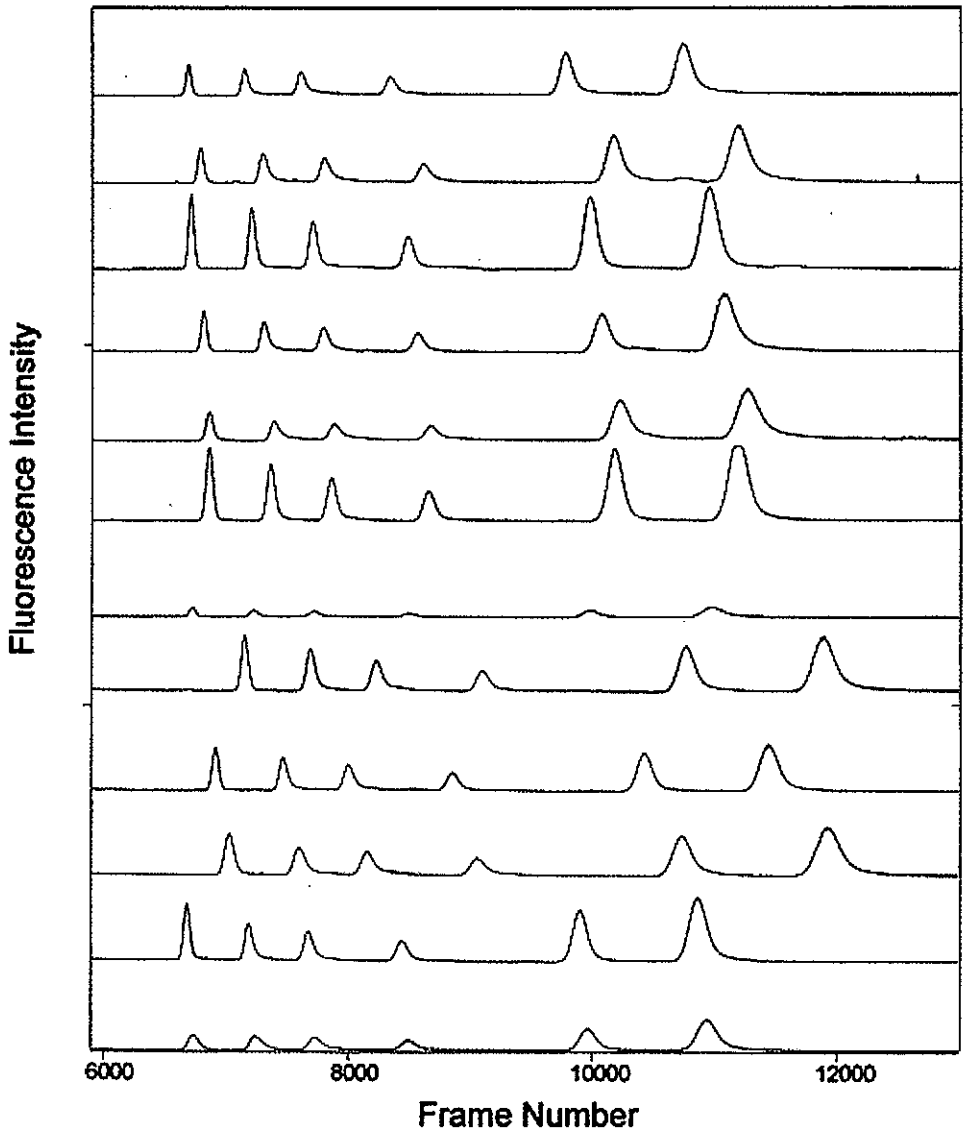
Fluorescence Intensity
6000
8000
10000
12000
Frame Number

FIGURE 6

CHAPTER 3. FLUORESCENCE DETECTION IN CAPILLARY ARRAYS BASED ON GALVANOMETER STEP SCANNING

A paper published in Electrophoresis *

Gang Xue and Edward S. Yeung

ABSTRACT

A computer-controlled galvanometer scanner is adapted for scanning a focused laser beam across a 96-capillary array for laser-induced fluorescence detection. The signal at a single photomultiplier tube is temporally sorted to distinguish among the capillaries. The limit of detection for fluoresceins is 3×10^{-11} M (S/N = 3) for 5-mW of total laser power scanned at 4 Hz. The observed cross-talk among capillaries is 0.2%. Advantages include the efficient utilization of light due to the high duty-cycle of step scan, good detection performance due to the reduction of stray light, ruggedness due to the small mass of the galvanometer mirror, low cost due to the simplicity of components and flexibility due to the independent paths for excitation and emission.

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INTRODUCTION

The rapid development of chemical, biological, medical and pharmaceutical technologies in recent years has posed a challenge for analytical science in terms of speed and throughput. Parallel processing (i.e., simultaneous multi-sample analysis) is a natural way to increase the throughput. Conventional chromatographic separation techniques such as high-performance liquid chromatography (HPLC) or gas chromatography (GC) are difficult to multiplex due to the column and sample size, pressure requirement and cost of instrumentation and consumables per channel, etc. Instead, with attractive features such as small physical size, low solvent consumption, simplicity in instrumentation, rapid analysis time and high separation efficiency, high-performance capillary electrophoresis (CE) has emerged as a highly promising solution for parallel analyses that require separation.

Many advances have been made in the development of various fluorescence-based capillary array electrophoresis (CAE) systems. In 1992, Huang and Mathies demonstrated DNA sequencing in a 24-capillary array in a confocal geometry.[1, 2] Taylor and Yeung[3] used on-capillary optical-fiber excitation and charge-coupled device (CCD) detection. Kambara and Takahashi[4] developed a sheath-flow system with CCD detection. Ueno and Yeung[5] employed on-capillary line excitation and CCD detection. Commercial versions of 96-capillary CAE instruments are now available for DNA sequencing with their actual designs originating from the above prototypes. Indeed, the rapid completion of the Human Genome Project is largely due to the availability of such instrumentation. Recently, a rotating-mirror version with a microscope objective spinning inside a drum has been proposed to work with 1000 capillaries.[6] Other multiplexed CE schemes such as micromachined channels have also been suggested.[7-10]

Referring to the detection schemes, array systems can roughly be divided into two main groups: (1) parallel systems, in which the capillaries are continuously irradiated and fluorescence from all capillaries is simultaneously imaged onto an array detector (e.g., CCD camera), and (2) sequential systems, in which the capillaries are irradiated one at a time and fluorescence is collected synchronously from each capillary. Different excitation schemes have been developed for parallel systems, including cross-beam relative to a multiple sheath flow,[11] on-column side-entry with or without focusing,[12-14] on-column line focusing[5, 15] and optical-fiber array.[3] Simplicity, robustness, high duty cycle (100%) and the lack of moving parts are the most appealing advantages of parallel systems. However, as the number of capillaries (channels) increases, it becomes more and more difficult to maintain a tight focus in the cross-beam or side-entry geometries. For the line-focusing and the optical-fiber geometries, dividing the laser beam to couple to multiple capillaries is rarely 100% efficient, particularly since one needs to produce a uniform intensity profile. In addition, since imaging detection is based on spatial discrimination among the channels, these systems are more sensitive to laser scattering from the capillary walls and stray fluorescence signals from the other channels. High quality imaging optics with a large numerical aperture are required. The sequential systems typically use a motor-driven scanning system to direct the laser beam onto the capillaries. The confocal geometry offers the best sensitivity because of the excellent spatial discrimination in both excitation and collection. However, the bidirectional mechanical movement of a microscope objective[1, 2] not only introduces spatial imprecision due to hysteresis but also limits the scanning speed. The use of a continuous radial scan alleviates part of the problem.[6] The duty cycle is determined by the scanning speed and the effective collection time per channel in the sequential mode. When the walls or the gaps in the array are ir-

radiated, time and laser energy are wasted. More recently, Neumann et al. proposed a bi-directional discontinuous scanning scheme.[16] Proper alignment and data collection are effected by software. Although the settling time was fairly long (~40 ms) because of the mass of the optical unit, that scheme increased the duty cycle and avoided the strong scattered light originating from the capillary walls. Imprecision due to hysteresis remains.

An acousto-optical deflection (AOD) based excitation system for fluorescence detection on microchips has been reported.[10] As the authors pointed out, the linear scan range of AOD is limited by the deflection angle and the working distance. The former is determined by the overlapping diffraction orders. The demonstrated scan range of 1 mm[10] allows for detection in only 8 channels. The angular resolution (positional accuracy) is further limited by the device size and the acoustic wavelength, in analogy to that of a diffraction grating. Diffraction also exhibits

angle-dependent efficiency (which must be renormalized for quantitative measurements), beam distortion and intensity loss into other diffraction orders. Local temperature gradients due to heating further limit the maximum laser power that can be used.

We present here a fast step-scanning 96-capillary electrophoresis system that employs a single photomultiplier tube (PMT) for fluorescence detection. The excitation laser beam is focused and directed sequentially to the centers of the individual capillaries by a high-performance galvanometer scanner. A camera lens collects light from all spatial locations simultaneously. By having independent excitation and fluorescence optical paths, stray light is minimized while flexibility (e.g., choice of wavelengths) is maximized. Since fluorescence only occurs when the laser irradiates a given capillary, the sequential signals can be sorted out as a function of time. Step scan maximizes the duty cycle and guarantees that the capil-

lary walls are not irradiated. Since no sophisticated or expensive optics are involved, it constitutes a simple, low-cost and high-efficiency fluorescence detection system for multiplexed capillary electrophoresis.

EXPERIMENTAL SECTION

96-Capillary Array Electrophoresis System.

The capillary array setup with independent injection electrodes is similar to what we have described before.[17] Briefly, 96 fused-silica capillaries (Polymicro Technologies Inc., Phoenix, AZ), 75-μm i.d. and 150-μm o.d., with 50-cm effective length and 70-cm total length were used. After detection windows were created by burning off the polyimide coating, the capillaries were close-packed to one another and clamped in between two flat surfaces of a plastic mount. The mount was attached to a magnetic base under the camera lens and the photomultiplier tube (PMT). Since there are no moving parts involved in the electrophoresis setup, the coupling of light to each capillary was stable throughout many separations over many days.

At the ground end, the capillary array was bundled together for filling and flushing. At the negative (injection) end, the capillaries were spread out and inserted into individual Peek tubing (1.5-mm o.d., 0.25-mm i.d.) with 4 mm of capillary tip extending out for injection. The Peek tubings were mounted on a copper plate in an 8 × 12 format with 9-mm spacing to fit standard microtiter plates. Gold-plated pins were mounted on the copper plate near each capillary tip to serve as individual electrodes, with the capillary tips slightly extended (~0.5 mm) beyond the electrodes to guarantee contact with small-volume liquid samples.

Laser Step-Scanning System.

Figure 1 depicts the schematic of the laser step-scanning system for capillary-array laser-induced fluorescence (LIF) detection. An Ar^+ ion laser (Coherent I-90) with 10-mW output at 488 nm was used for excitation. The laser beam was passed through a laser-line filter (488 nm) and reflected to a 10× beam expander (Oriel, Stamford, CT), where the beam was expanded and apertured to 9-mm in diameter for better focusing. A 10-cm focal length best-form lens (Esco Products Inc., Oak Ridge, NJ) was used to refocus the laser beam. A galvanometer optical scanner (Cambridge Technology Inc., Model 6210) was placed ~ 4 cm away from the capillary array to deflect the focused laser beam onto the array window. The scanner is capable of 20° mechanical deflection, which is more than sufficient to cover the entire array of 1.5 cm.

Since the galvanometer scanner is a moving-magnet actuator, theoretically it has no saturation torque limit and very little electrical inductance. Thus, extremely high torque can be generated very quickly. In our application, we tested the scanner and found a settling time of less than 100 μs, which is negligible compared to the 2.5-ms dwell time at the center of each capillary during the selected 4-Hz unidirectional step scan of the 96-capillary array. This ensures efficient use of the available laser power. The fraction of the total laser intensity received by each capillary per unit time (duty cycle) is better than that of the on-column illumination systems.[1, 5] This is because no light is wasted on the capillary walls or the gaps between capillaries[1, 5] and because the laser does not have to be overexpanded[5] to provide uniform intensity.

The servo amplifier accepts an analog command voltage and maps it into a mechanical position. By feedback from the scanner's position-error signal, the galvanometer strives

to force the error signal to zero by rotating the scanner's shaft. Thus, real-time scanner control can be achieved by programming the feedback in the analog signal.

Detection System.

Fluorescence from all capillaries were simultaneously collected by a 28-mm Nikon camera lens and focused onto a single side-on PMT (Hamamatsu, Model 928). Since fluorescence only occurs when the laser irradiates a given capillary, the sequential signal could be sorted out unambiguously as a function of time. A black painted slit was placed between the camera lens and the PMT to block out the laser light reflected by other optical parts. A 488-nm notch filter (Kaiser Optical System, Inc.) and a 515-nm long-pass filter were located in front of the PMT window to filter out the excess laser scattering. The camera lens used here had a nominal magnification factor of 1.4 and the image of the 1.5-cm long array window was ~ 2.1 cm. The minimum photosensitive area of the PMT is 2.4 cm in the corresponding dimension. This ensures approximately uniform detector response. The PMT signal was passed through an 800-Hz low-pass RC filter to filter out the high-frequency noise before it was converted to a 16-bit digital signal and stored in the computer.

Scanner Control and Data Acquisition.

A single multifunctional DAC board (National Instruments, 6030E) was used for synchronized scanner control and data acquisition. For synchronization purposes, 100 positions, including the centers of 96 capillaries and 4 positions outside of the capillary array, were stored in a position table in the computer memory and repeatedly sent to the servo amplifier at 4 cycles/s. Meanwhile, data acquisition was configured at 10 kHz. Since both analog out-

put and analog input were triggered by the same timer, they are easily synchronized such that 25 data points were collected at each scanner position. Because of the (approximate) 0.4-ms time constant of the RC filter, the first 7 data points at each step were rejected and the remaining 18 data points were averaged to further reduce the random noise in the DAC board.

All code used to control the scanner and to acquire the data were written in-house using National Instruments LabView 5.0 software. Signals from up to 12 of the 96 capillaries could be displayed in real time in our LabView program. Real-time monitoring of more capillaries was limited by the video speed of our computer. However, the program could freely display different sets of capillaries during the electrophoresis run. The raw data sets were converted to a WinView (Roper Scientific) image by another in-house LabView program. Data treatment and analysis were performed using WinView32 and GRAMS/32 5.05 (Galactic Industries). The root-mean-squared (rms) noise in the electropherograms was obtained using a section of baseline near one of the analyte peaks. This baseline section has about the same width as the peaks of interest.

Separation Conditions.

For the capillary zone electrophoresis (CZE) experiments, the capillary array was flushed with deionized water for clean-up. pH 8.6 10-mM Tris buffer with 0.1% PVP (MW = 1,000,000) was filled into the capillary array from the ground (bundled) end while the injection end was immersed into the buffer tray. After the buffer filling, the ground end was immersed into the second buffer tray. Two seconds of electrokinetic injection was performed at 70 V/cm for sample introduction.

Reagents and Chemicals.

All chemicals were of analytical reagent grade. Fluorescein (F) and 5(6)-carboxy-fluorescein (5CF, 6CF) were obtained from Sigma (St. Louis, MO), while 2,7-dichlorofluorescein (DCF) was obtained from Acros. Tris, HCl and all other chemicals were used as obtained from Aldrich (Milwaukee, WI) and Sigma. The sample solutions were prepared by dissolving the appropriate amounts of these fluoresceins in 10-mM Tris buffer.

RESULTS AND DISCUSSION

Unidirectional Step Scanning.

The advantage of step scan over continuous scan is quite obvious. With the fast response of the galvanometer scanner, the transit time is almost negligible, which leads to a very high duty cycle. Besides that, the PMT picks up signal only when the laser beam is stabilized at the center of each capillary. This avoids the strong scattering of the capillary walls, which, even if temporally discriminated against,[1, 2] can saturate the PMT. Since only one capillary was illuminated at a time, there should be no contribution in the fluorescence signal from the neighboring capillaries even when the array window is not perfectly flat or when the image of the capillary window is not well focused. That means a reduced risk of cross-talk.

Before the electrophoresis experiment, a 1000-step scan across the entire array was performed to locate each capillary. Figure 2A is an image of such a scan of the 96-capillary array filled with 10^{-8} M fluorescein. As can be seen in Figure 2B, the center of each capillary corresponds to a "plateau" in the image. For most of the capillaries, there are two "peaks" which correspond to the capillary walls. When the laser hits a capillary wall, the strong specularly scattered light will illuminate both the capillary that is hit and the neighboring

capillaries (which are filled with fluorescein) as well. The fluorescence signals thus generated is larger than that from an individual capillary alone. However, when the laser is well focused at the center of a capillary there is virtually no contribution from the neighboring capillaries because the laser passes cleanly out of the array. Between two adjacent capillaries, there is normally a spacing that shows up as a "valley" (Figure 2B). When the capillary array is well aligned with respect to the scanning system, the signals at these valleys became minimized. This feature was used to produce the best alignment and to locate the capillaries which are not necessarily evenly packed. The positions corresponding to the centers of the capillaries were saved in a position table in the computer. This full-array positioning scan only needs to be performed once after optical alignment and the saved position table becomes the reference for the subsequent step-scanning detection.

Because of the optical geometry here, there was a slight difference in the optical pathlengths to the focusing lens between the capillaries in the middle of the array and those at the edges. The laser beam was therefore focused at about the 1/4 width position of the array. However, most of the capillaries showed similar signal intensities (< 2× here compared to 5× in Ref. 17).

During detection, 100-step unidirectional scan was initiated at the same time as the data acquisition. Usually, a 4-Hz sampling rate is sufficient for most chromatographic separations. However, this scanning system is capable of up to 20-Hz 100-step scan if desired. AOD systems are capable of 180-ns access times but were not used at those speeds in multiplexed detection so far because of limitations on data-acquisition rates and on random-address control.[10]

Detection Limit.

To determine the limits of detection (LOD) achievable using this laser step-scanning system, electrophoresis of fluorescein, at the injected concentration of 1.47×10^{-10} M in 10 mM Tris buffer, was performed using 10-mM Tris as the running buffer. The sample was electrokinetically injected for 3 s at 70 V/cm. No sample stacking is expected under these conditions. Capillary electrophoresis was run at 200 V/cm. Detection was performed at 5-mW laser power at the detection window. The electropherogram from one capillary in the array is shown in Figure 3A. In the raw data, the S/N for the fluorescein peak was ~3 (based on a peak height of 10.3 mV and a rms noise between frames 3400 and 3500 of 3.0 mV). After 16-point binning and boxcar smoothing, the S/N ratio was improved to ~13, as shown in Figure 3B.The resulting 3.2×10^{-11} M LOD (S/N = 3) for fluorescein injected was only slightly worse compared to what single-capillary LIF usually could achieve.[14] This level is several orders of magnitude better than that achieved in an AOD system.[10] Lower stray light, better focusing, higher efficiency for laser utilization and better collection efficiency are the contributing factors.

Cross-talk.

As discussed earlier, since only one capillary is illuminated at a time and the laser is always cleanly focused at the center of each capillary, this setup eliminates cross-talk quite efficiently. The measured cross-talk, defined as the signal at neighboring channels when only one channel is irradiated, was less than 0.2% at optimum alignment, which is negligible for multiplexed analysis. Careful selection of the step positions is very important to avoid hitting the capillary walls. This is especially true at either side of the capillary array where the laser

focusing was not optimal. If better focusing is desired, especially for the case of larger arrays, f-theta lenses that are used in printers and copy machines for flat-field focusing can be substituted for the best-form lens here.

Cost.

Another advantage here is the low cost of the whole system. With the efficient use of laser light, 50-mW output will be enough for exciting 1000 capillaries. The galvanometer scanner costs less than $1000 and the PMT is another $1000. Since the collection lens does not need to produce a good image, even single-element lenses should be adequate. This feature will become important when working in the UV region. Independent focusing and collection lenses further permit having a large collection efficiency while maintaining a narrow laser beam waist. The optics are all standard components. So the whole setup is truly a simple, low cost but rugged system. This is in contrast to the use of scientific CCD cameras[5] or the use of translational stages for moving the optics or for moving the capillaries.[1]

Array Electrophoresis.

Figure 4 shows the electropherograms of CZE separation of four visible dyes at concentrations of 1 nM in a 96-capillary array. The vertical direction represents the packed capillary array arrangement while the horizontal direction represents the migration time. The data demonstrates relatively uniform separation resolution and S/N distribution across the array. No cross-talk between adjacent capillaries was observed at this scale expansion, as expected for this analyte concentration range. All four analytes were well resolved within 15 min in all 96 capillaries. Baseline separation of the 5(6) carboxy-fluorescein isomers was achieved with

a resolution of 2.4.

Migration times of the four dyes varied from 4.1-5.7% relative standard deviation (N = 96, Table I and open symbols in Figure 5). The signal intensities of identical analytes separated on the array were also relatively consistent from capillary to capillary. The relative standard deviations for the peak areas of the four dyes are around 20% (N = 96, Table I) without using any calibration. The lack of systematic bias across the array confirms the uniform excitation of the laser scanning scheme. This is in contrast to the Gaussian profile typical of line-focusing systems.[5, 17] These RSD values are similar to those in the raw data of a line-focusing CCD detection system.[17] Appropriate normalization by two internal standards[17] greatly improves the precision of migration times (Table I and solid symbols in Figure 5), again similar to our previous study.[17] The capillary-to-capillary intensity variations most likely arose from the non-uniform injected sample amount as result of differences in electroosmotic flow (EOF) in different capillaries during electrokinetic injection and from intensity fluctuations in the laser. The former can be improved by using two internal standards[17] (Table I). The latter can be improved by further stabilizing the laser output.

CONCLUSIONS

A novel approach to laser-beam step-scanning fluorescence excitation and single-element detection for multiplexed capillary electrophoresis was demonstrated. Uniform separation efficiency and good S/N can be obtained. The LOD of the array almost matches that of single-capillary LIF on-column detection and is several orders of magnitude better than AOD scanning. The speed, accuracy, high-resolution positioning and simplicity of galvanometer step-scanning provide unique possibilities for simple fluorescence detection with large capil-

lary arrays or microfabricated devices. Successful high throughput CZE separation was demonstrated. With slight modification of the setup, two-dimensional laser scanning excitation should be easily achieved to accommodate, e.g., different excitation wavelengths for multi-color excitation or multi-location monitoring in microfabricated devices.

ACKNOWLEDGMENT

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Table I. Migration time and peak area reproducibility in the raw data and normalized data for fluoresceins

	RSD, %			
	5-carboxy-fluorescein	6-carboxy-fluorescein	2,7-dCl-fluorescein	fluorescein
Raw Data				
Migration Time	4.1	4.2	5.2	5.7
Peak Area	20.8	19.9	22.1	23.8
Normalized Data*				
Migration Time	—	0.7	0.6	—
Peak Area	—	3.9	5.4	—

*The first and the last peaks were used as internal standards for normalization based on the scheme presented in ref. 17.

FIGURE CAPTIONS

Figure 1. Schematic diagram of laser step-scanning system for multiplexed fluorescence detection in a 96-capillary array. F1, laser-line filter; M, mirror; BEX, laser beam expander; P, pinhole; L1, 10-cm focal length best-form lens; S, galvanometer scanner; SA, servo amplifier; L2, camera lens; and F2, 488-nm notch filter.

Figure 2. (A) 1000-step full-scan image of the entire 96-capillary array. (B) Image of one region of the 96-capillary array: (a) a capillary wall where specular scattering is substantial; (b) a "plateau" corresponding to the center core of the capillary; and (c) a "valley" associated with gaps in-between capillaries.

Figure 3. Electropherograms of fluorescein injected at 1.47×10^{-10} M. (A) raw data and (B) data with 16-point binning and smoothing. Each frame corresponds to 0.25 s. The two plots were offset for clarity.

Figure 4. Stacked electropherograms of CZE separation of four visible dyes in the 96-capillary array. In order, these are 5CF (1.35×10^{-9} M), 6CF (1.35×10^{-9} M), DCF (1.33×10^{-9} M), and F (1.48×10^{-9} M). The horizontal direction represents migration time from 0 to 22 min.

Figure 5. Migration time reproducibility of 6-carboxyfluorescein (triangles) and 2,7-dichlorofluorescein (circles). Open symbols represent raw data and solid symbols represent normalized data based on two internal standards.

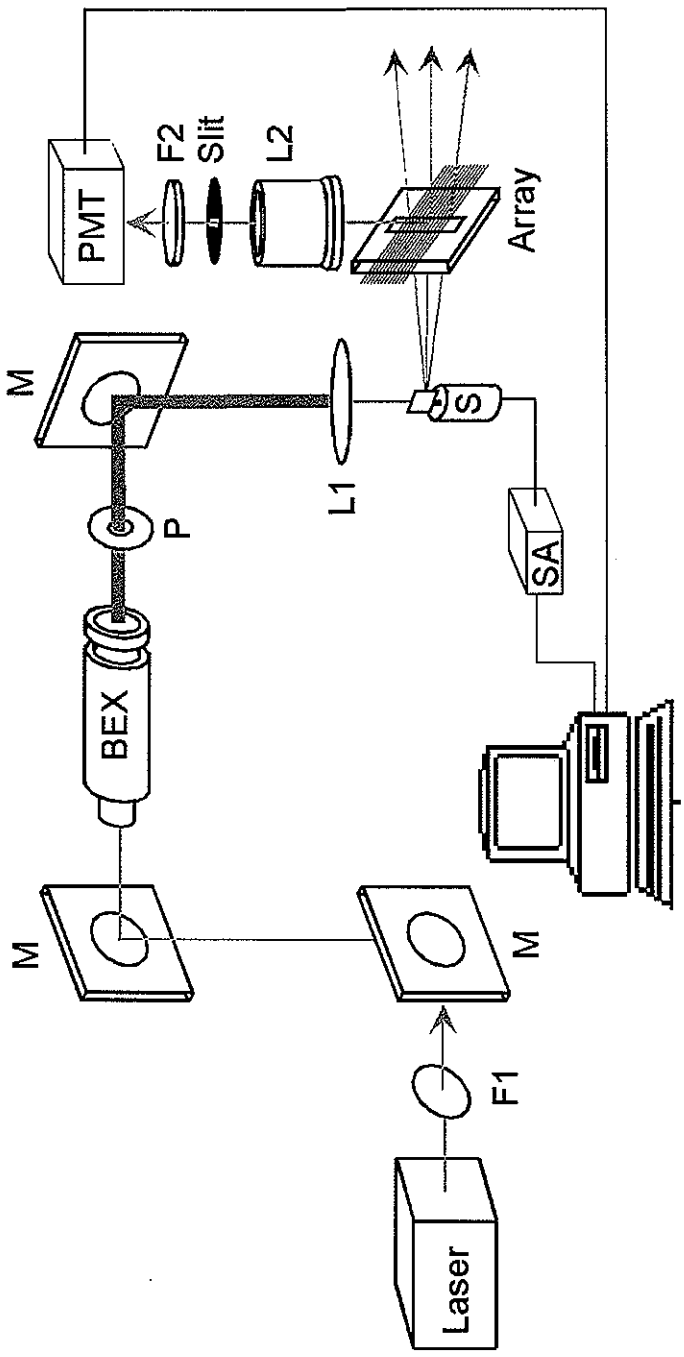

FIGURE 1

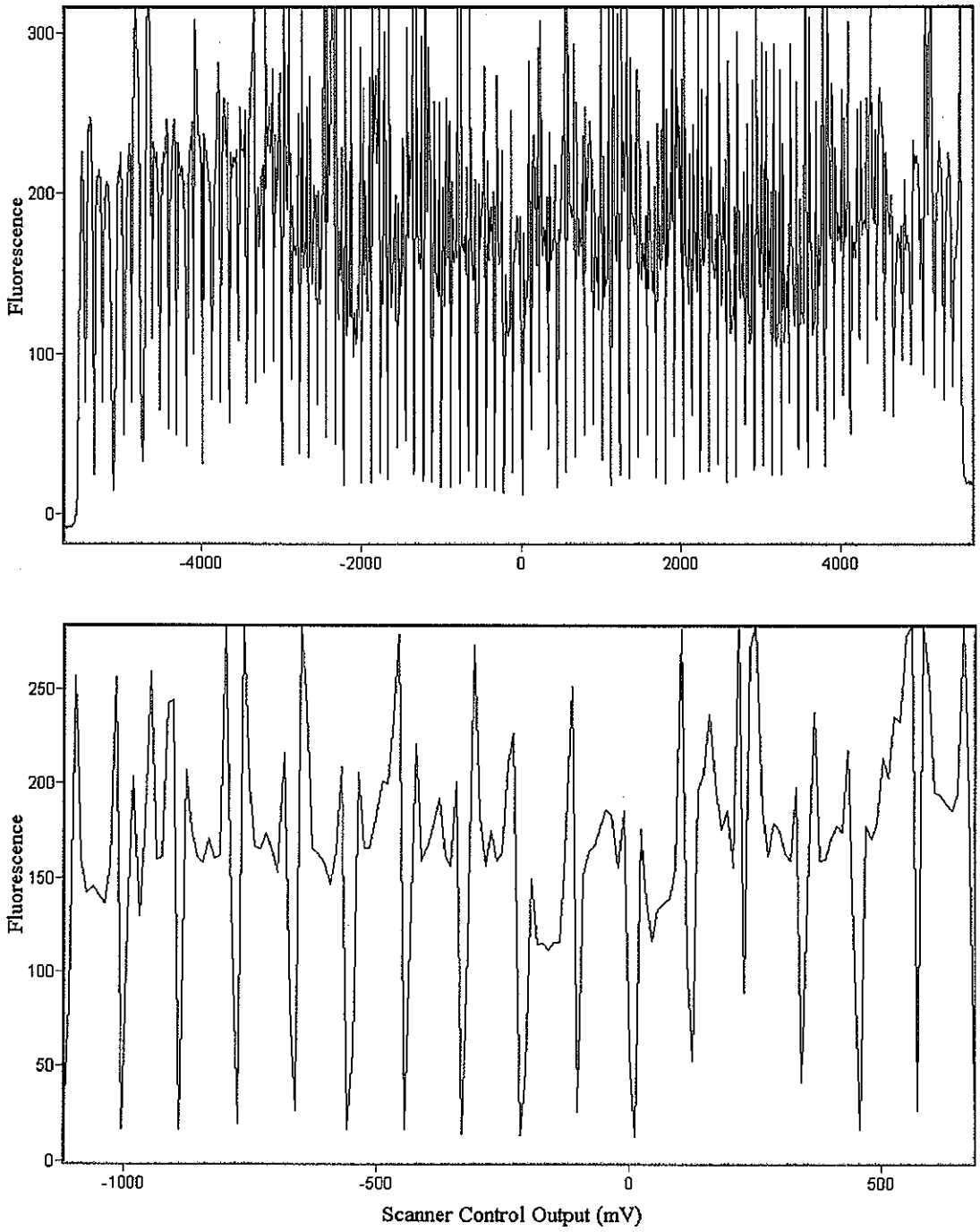
300
200
100
0
Fluorescence
-4000
-2000
0
2000
4000
250
200
150
100
50
0
Fluorescence
-1000
-500
0
500
Scanner Control Output (mV)

FIGURE 2

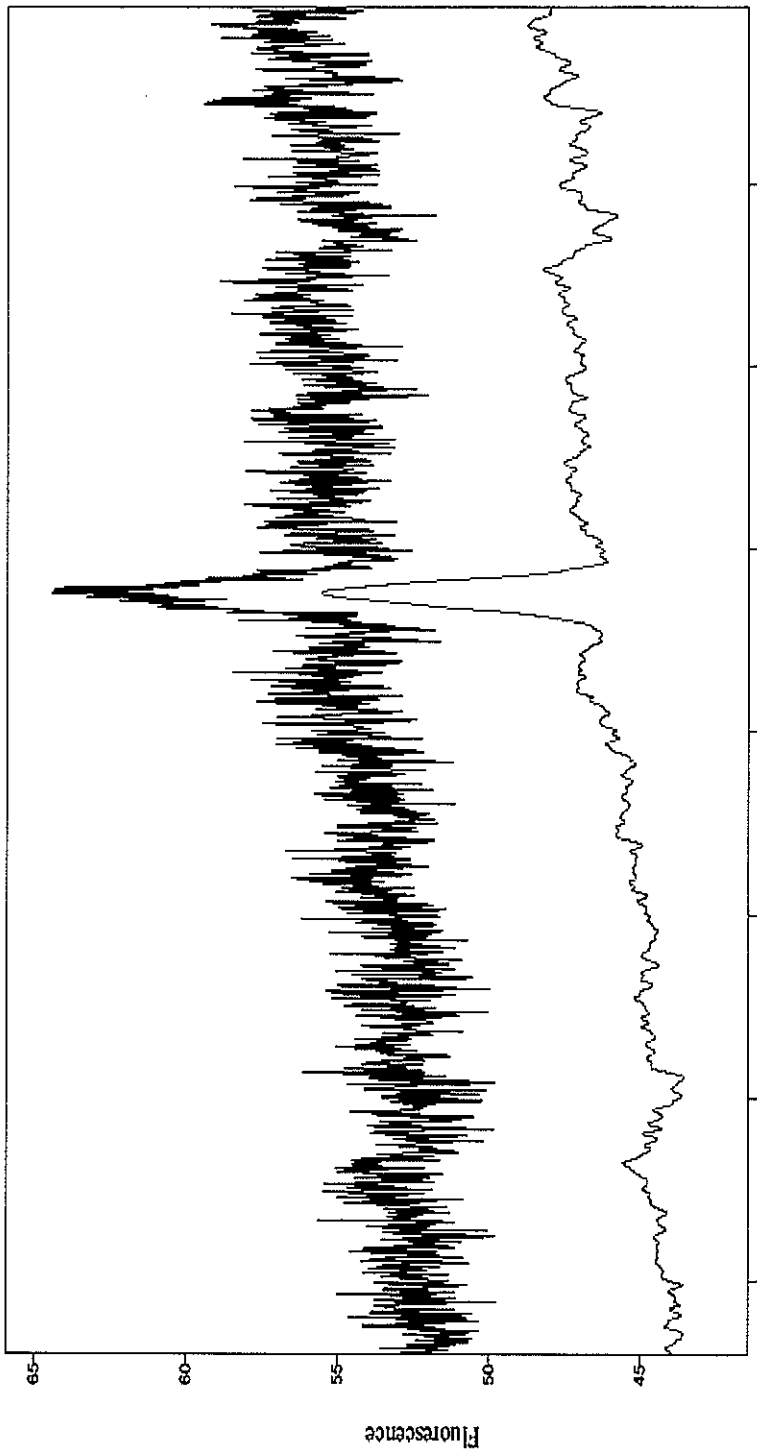
Fluorescence
65
60
55
50
45
2600
2800
3000
3200
3400
3600
3800
Frame Number

FIGURE 3

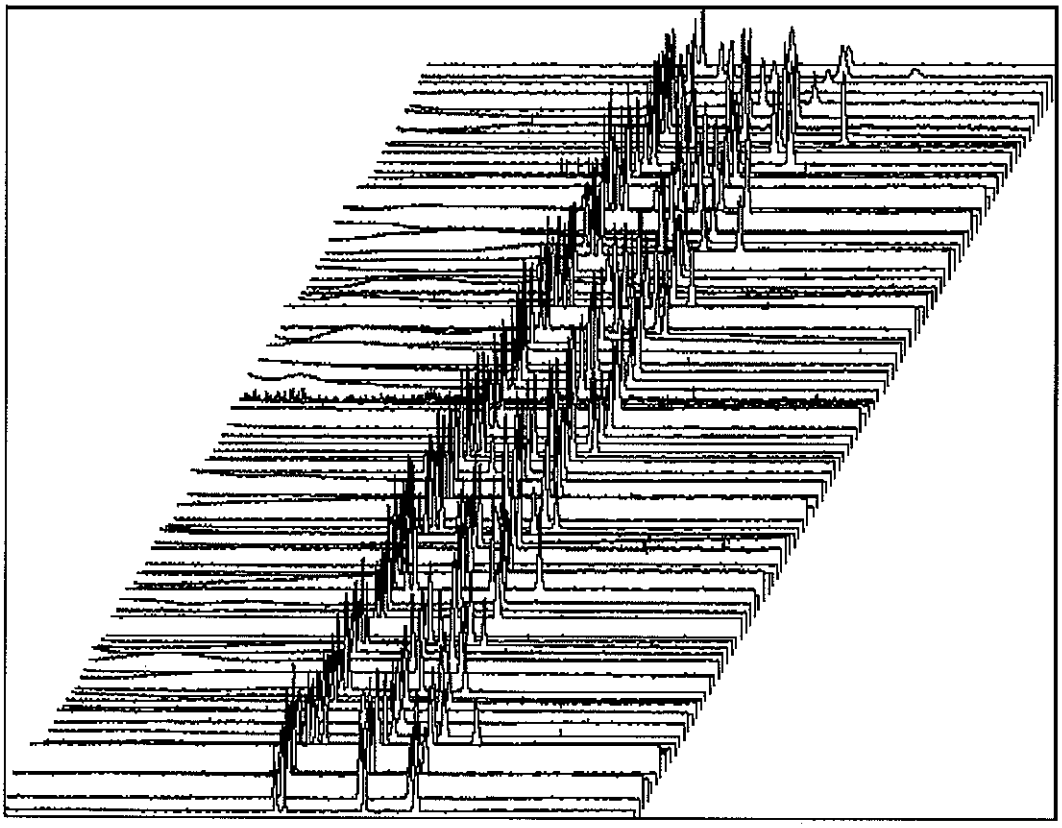

FIGURE 4

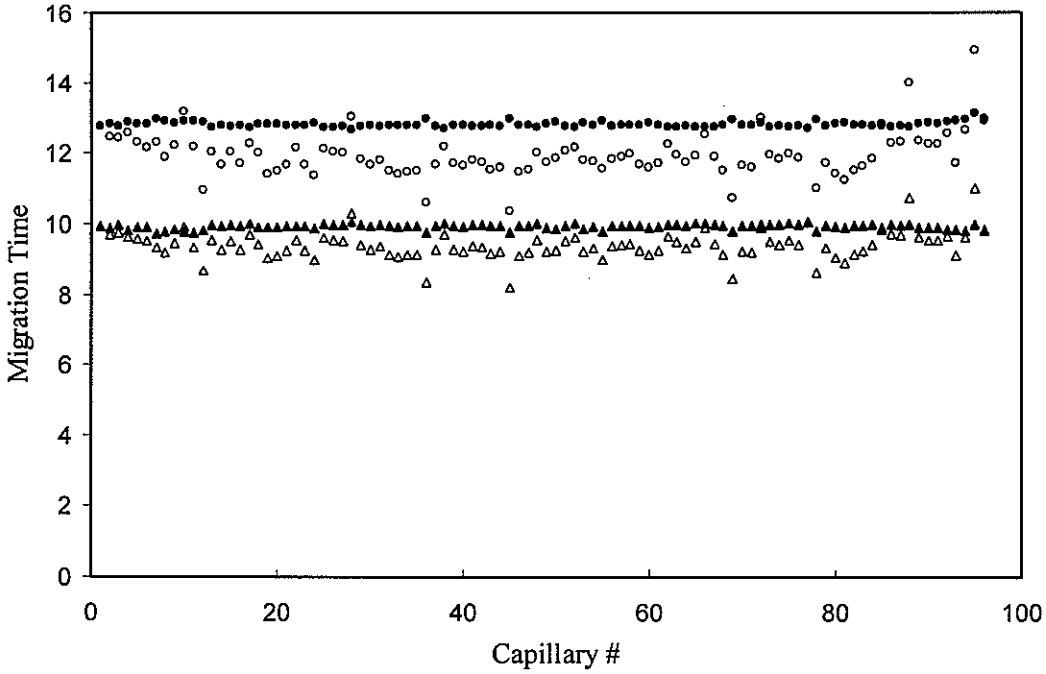

FIGURE 5

CHAPTER 4. TWO COLOR EXCITATION SYSTEM FOR FLUORESCENCE DETECTION IN DNA SEQUENCING BY CAPILLARY ARRAY ELECTROPHORESIS

A paper submitted to Electrophoresis

Gang Xue and Edward S. Yeung

ABSTRACT

Two computer-controlled galvanometer scanners are adapted for two-dimensional step scanning across a 96-capillary array for laser-induced fluorescence detection. 488-nm and 514-nm laser lines from the same Ar^+ laser were alternately coupled for two-color excitation in each capillary. The signal at a single photomultiplier tube is temporally sorted to distinguish among the capillaries and the excitation wavelengths. Based on the differences in absorption spectra for the dyes, the peak-height ratios in the 488-nm and 514-nm excitation electropherograms were used for peak identification for multiplexed capillary electrophoresis. Successful base calling for 24-capillary DNA sequencing was achieved to 450 bp with 99% accuracy. Advantages include the efficient utilization of light due to the high duty-cycle of step scan, good detection performance due to the reduction of stray light, ruggedness due to the small mass of the galvanometer mirror, low cost due to the simplicity of components

and flexibility due to the independent paths for excitation and emission.

INTRODUCTION

Due to the continuous efforts of many research groups, such as Mathies,[1] Kambara,[2] Dovichi,[3] and Yeung,[4] capillary array electrophoresis (CAE) has been firmly established as a high throughput, high resolution and high accuracy technique for DNA sequencing. Several types of CAE DNA sequencers [1-4] were successfully commercialized by Molecular Dynamics (Sunnyvale, CA, USA), Applied Biosystems (Foster City, CA, USA), SpectruMedix (State College, PA, USA) and Beckman Coulter (Fullerton, CA, USA) respectively. These fully automated systems greatly facilitated the Human Genome Project (HGP) in accomplishing its mission to sequence the human genome (with three billion base pairs) in 15 years. Genome maps of several types of organisms have already been completed, and a working draft of the entire human genome was announced in June 2000,[5] with an expected completion date of 2003. However, the human genome is only one of almost one hundred genomes being sequenced around the world.[6] Also, comparative genomics, single nucleotide polymorphism (SNP), and other genome-related issues are examples of applications that will continue to demand new advances in high-throughput sequencing technologies with higher performance and lower cost.

The current concerns of CAE sequencer development were mostly focused on illumination and detection. Besides the liquid sheath flow approach,[7] Kambara's group began testing side illumination with detection on column. Recently, they improved the self-focusing approach by placing the capillaries in water and interleaving each capillary with a glass rod of the same external diameter as the capillaries.[8] With this design, improved light transmis-

sion was achieved for side illumination. In order to accommodate more than 96 capillaries, Scherer et al. proposed a rotating-mirror instrument with a microscope objective spinning inside a drum to illuminate up to 1000 capillaries.[9] More recently, an acousto-optical deflection (AOD) based excitation system for fluorescence detection on microchips has been reported.[10] However, as the authors pointed out, the linear scan range of AOD is limited by the deflection angle and the working distance. The former is determined by the overlapping diffraction orders. The demonstrated scan range of 1 mm allows for detection in only 8 channels. The angular resolution and positional accuracy is further limited by the device size and the acoustic wavelength, in analogy to that of a diffraction grating. Diffraction efficiency is typically lower than other scanners.

Although different dye schemes have been proposed for base calling including one-color, four-intensity and two-color two-intensity, the most robust technology is still the original four-color scheme. The most widely used four-channel detection method differentiates the four different dye labels by their differences in emission spectra. Data is recorded through four different narrow-band-pass filters coupled with four photomultiplier tubes (PMTs)[11] or by using transmission gratings for spectral discrimination coupled with CCD detection. In our previous work, we proposed a simple two-channel base calling approach by analyzing peak-height ratios in two different emission bands for the four dye labels, which greatly simplified the detection system design.[12] With this method, successful base calling was demonstrated in many different high-throughput DNA sequencing applications.[13-16]

Recently, we demonstrated an approach to step scan a focused laser beam across a 96-capillary array for laser-induced fluorescence detection by a computer-controlled galvanometer scanner.[17] The signal at a single PMT was temporally sorted to distinguish among

the capillaries. Advantages of the new system include the efficient utilization of light due to the high duty-cycle of step scan, good detection performance due to the reduction of stray light, ruggedness due to the small mass of the galvanometer mirror, low cost due to the simplicity of components and flexibility due to the independent paths for excitation and emission.

We present here a two-dimensional fast step-scan 96-capillary electrophoresis system for DNA sequencing with two-color excitation. Two lines from the multi-line Ar^+ laser, 488 nm and 514 nm, were alternately directed by the first scanner to the second scanner through the focusing lens, while the second scanner sequentially reflected the focused laser beams to the centers of the individual capillaries. A single PMT is used to collect the fluorescence signals from all the capillaries. By the difference in absorption spectra of the four labeling dyes, peak ratios of the two-color excited fluorescence signals are used for base calling. Without sophisticated or expensive optics, this constitutes a simple, low-cost and high-efficiency fluorescence detection system for high-throughput DNA sequencing.

EXPERIMENTAL SECTION

Capillary Array Electrophoresis System.

The capillary array setup with independent injection electrodes is similar to what we have described before[17]. Briefly, 96 or 24 fused-silica capillaries (Polymicro Technologies Inc., Phoenix, AZ), 75-μm i.d. and 150-μm o.d., with 50-cm effective length and 70-cm total length were used. After detection windows were created by burning off the polyimide coating, the capillaries were close-packed to one another and clamped in between two flat surfaces of a plastic mount. The mount was attached to a magnetic base under the camera lens

and the photomultiplier tube (PMT). Since there are no moving parts involved in the electrophoresis setup, the coupling of light to each capillary was stable throughout many separations over many days.

At the ground end, the capillary array was bundled together for filling and flushing. At the negative (injection) end, the capillaries were spread out and inserted into individual Peek tubing (1.5-mm o.d., 0.25-mm i.d.) with 4 mm of capillary tip extending out for injection. The Peek tubings were mounted on a copper plate in an 8 × 12 format with 9-mm spacing to fit standard microtiter plates. Gold-plated pins were mounted on the copper plate near each capillary tip to serve as individual electrodes, with the capillary tips slightly extended (~0.5 mm) beyond the electrodes to guarantee contact with small-volume liquid samples.

Laser Step-Scan System.

Figure 1 depicts the schematic of the two-color laser step-scan system for capillary-array laser-induced fluorescence (LIF) detection. An Ar^+ ion laser (Coherent I-90) with multi-line emission was used for excitation. The 488- and 514-nm lines were dispersed and separated with a glass prism. Each laser beam was passed through a laser-line filter (488 nm and 514 nm respectively) and reflected to a galvanometer optical scanner (Cambridge Technology Inc., Model 6210). By controlling the angle of the scanner, the two beams were alternately redirected to a 10× beam expander (Oriel, Stamford, CT), where the beam was expanded and apertured to 9-mm in diameter for better focusing. A 10-cm focal length best-form lens (Esco Products Inc., Oak Ridge, NJ) was used to refocus the laser beam. A second galvanometer optical scanner (Cambridge Technology Inc., Model 6220) was placed ~ 6 cm away from the capillary array to deflect the focused laser beam onto the array window. The

scanner is capable of 20 mechanical deflection, which is more than sufficient to cover the entire array of 1.5 cm.

As described earlier[17], the moving-magnet actuator property of the galvanometer scanner ensures its extremely fast scan speed. A settling time of less than 100 μs was observed, which is negligible compared to the 2.5-ms dwell time at the center of each capillary during the selected 4-Hz unidirectional step scan of the 96-capillary array. This ensures efficient use of the available laser power. The fraction of the total laser intensity received by each capillary per unit time (duty cycle) is better than that of the on-column illumination systems[1,4]. This is because no light is wasted on the capillary walls or the gaps between capillaries[1,4]. and because the laser does not have to be over-expanded to provide uniform intensity.

The servo amplifier accepts an analog command voltage and maps it into a mechanical position. By feedback from the scanner's position-error signal, the galvanometer strives to force the error signal to zero by rotating the scanner's shaft. Thus, real-time scanner control can be achieved by programming the input analog signal.

Detection System.

Fluorescence from all capillaries were simultaneously collected by a 28-mm Nikon camera lens and focused onto a single side-on PMT (Hamamatsu, Model 928). Since fluorescence only occurs when the laser irradiates a given capillary, the sequential signal could be sorted out unambiguously as a function of time. A black painted slit was placed between the camera lens and the PMT to block out the laser light reflected by other optical parts. A 488-nm notch filter (Kaiser Optical System, Inc.), a 514-nm notch filter (Kaiser Optical System, Inc.), and a 530-nm long-pass filter were located in front of the PMT window to filter out the

excess laser scattering. The camera lens used here had a nominal magnification factor of 1.4 and the image of the 1.5-cm long array window was ~ 2.1 cm. The minimum photosensitive area of the PMT is 2.4 cm in the corresponding dimension. This ensures approximately uniform detector response. The PMT signal was passed through a two polar 800-Hz low-pass RC filter to filter out the high-frequency noise before it was converted to a 16-bit digital signal and stored in the computer.

Scanner Control and Data Acquisition.

A single multifunctional DAC board (National Instruments, 6030E) was used for synchronized scanner control and data acquisition. For synchronization purposes, 100 positions, including the centers of 96 capillaries and 4 positions outside of the capillary array, were stored in a position table in the computer memory and repeatedly sent to the servo amplifier of the second scanner at 4 cycles/s. The first (wavelength-selecting) scanner was flipped back and forth at 4 Hz between two positions corresponding to 488 and 514 nm respectively. In this way, the capillary array was first step scanned by 488 nm, followed by a cycle of 514 nm, and then 488, 514, and so on. Meanwhile, data acquisition was configured at 10 kHz. Since both analog output and analog input were triggered by the same timer, they are easily synchronized such that 25 data points were collected at each scanner position. Because of the (approximate) 0.4-ms time constant of the RC filter, the first 7 data points at each step were rejected and the remaining 18 data points were averaged to further reduce the random noise in the DAC board.

All code used to control the scanner and to acquire the data were written in-house using National Instruments LabView 6i software. Signals from up to 12 of the 96 capillaries

could be displayed in real time in our LabView program. Real-time monitoring of more capillaries was limited by the video speed of our computer. However, the program could freely display different sets of capillaries during the electrophoresis run. The raw data sets were converted to a WinView (Roper Scientific) image by another in-house LabView program. Data treatment and analysis were performed using WinView32 and GRAMS/32 5.05 (Galactic Industries).

Separation Conditions.

For the capillary zone electrophoresis (CZE) experiments, the capillary array was flushed with deionized water for clean-up. pH 8.6 10-mM Tris buffer with 0.1% PVP (MW = 1,000,000) was filled into the capillary array from the ground (bundled) end while the injection end was immersed into the buffer tray. After the buffer filling, the ground end was immersed into the second buffer tray. 2 s of electrokinetic injection was performed at 70 V/cm for sample introduction while the separation field strength was kept at 200 V/cm.

The sieving matrix for DNA sequencing was made by dissolving 1.5% (w/v) of 8,000,000 MW PEO and 1.4% (w/v) of 600,000 MW PEO in 1× TBE buffer with 7 M urea. Between runs, the capillary array was flushed with 2% 1,000,000 MW PVP to dynamically coat the capillary walls.

Reagents and Chemicals.

All chemicals were of analytical reagent grade. Fluorescein (F) and 5(6)-carboxyfluorescein (5CF, 6CF) were obtained from Sigma (St. Louis, MO), while 2,7-dichlorofluorescein (DCF) was obtained from Acros. Tris, HCl and all other chemicals were

used as obtained from Aldrich (Milwaukee, WI) and Sigma. The sample solutions were prepared by dissolving the appropriate amounts of these fluoresceins in 10-mM Tris buffer.

pGEM/U DNA samples were obtained from the local DNA facility. The DNA samples were prepared according to the commercial protocol (ABI Prism Dye Terminator cycle sequencing kit with AmpliTaq Polymerase, FS) without modification. For each DNA sample, 10 μl of low conductivity formamide (Aldrich) was used as denaturing reagent to resuspend the dried sample powder. Then 5 μl of the sample was pipetted into each tube of the 8 × 12 microtiter plate. The sample plate was heated to 96 C for 3 min and then immediately placed on a plastics coolant (–20 C) to prevent sample renaturing. The sample tray with the plastic coolant was then placed under the injection end of the capillary array for simultaneous electrokinetic injection at 75 V/cm for 60 s. A 150 V/cm field strength was employed for separation.

RESULTS AND DISCUSSION

Base Calling Methods.

In laser-induced fluorescence (LIF) detection, the relationship between the fluorescence signal detected and the sample concentration was illustrated in Eqn. 1:

$$E_{FL}(\lambda, Cap, Dye) = k \cdot E_0(\lambda) \cdot L(\lambda, Cap) \cdot \varepsilon(\lambda, Dye) \cdot c(Dye) \qquad (1)$$

where k is a constant, $E_0(\lambda)$ is the laser power, $L(\lambda,Cap)$ is the pathlength of excitation and observation for the individual capillary, $\varepsilon(\lambda,Dye)$ is the molar absorptivity while c(Dye) is the sample concentration.

For two-color excitation, the fluorescence signal ratio of the same dye would be

$$\frac{E_{FL}(\lambda_1, Cap, Dye)}{E_{FL}(\lambda_2, Cap, Dye)} = \frac{E_0(\lambda_1) \cdot L(\lambda_1, Cap) \cdot \varepsilon(\lambda_1, Dye)}{E_0(\lambda_2) \cdot L(\lambda_2, Cap) \cdot \varepsilon(\lambda_2, Dye)} \tag{2}$$

For the dye signal within the same capillary, since the laser power and the optical paths were kept constant throughout the separation, $E_0(\lambda)$ and $L(\lambda,Cap)$ would be constant for all different dyes. So,

$$\frac{E_{FL}(\lambda_1, Dye)}{E_{FL}(\lambda_2, Dye)} = k' \frac{\varepsilon(\lambda_1, Dye)}{\varepsilon(\lambda_2, Dye)} \tag{3}$$

i.e., the fluorescence ratio is proportional to the molar-absorptivity ratio of a given dye, which is distinct for different fluorophores. Some of the absorptivity ratios are listed in Table 1.

For comparison of fluorescence ratio between different capillaries, at least one internal standard is needed to compensate for the optical path difference, $L(\lambda,Cap)$:

$$\frac{E_{FL}(\lambda_1, Cap, Dye) / E_{FL}(\lambda_1, Cap, IS)}{E_{FL}(\lambda_2, Cap, Dye) / E_{FL}(\lambda_2, Cap, IS)} = \frac{\varepsilon(\lambda_1, Dye) / \varepsilon(\lambda_1, IS)}{\varepsilon(\lambda_2, Dye) / \varepsilon(\lambda_2, IS)} = k'' \frac{\varepsilon(\lambda_1, Dye)}{\varepsilon(\lambda_2, Dye)} \tag{4}$$

Again, this ratio would be distinct for the different dyes detected here. When the histograms for the ratios were plotted, appropriate thresholds could be assigned to differentiate among the different dyes.

Separation of Fluoresceins.

We first tested this base-calling algorithm by 96-capillary CZE separation of 1×10^{-9} M fluorescein mixtures. Fig. 2(a) shows the result of parallel separation excited by a 488-nm laser. Excellent signal-to-noise ratios are obtained. The horizontal direction represents the migration time while the vertical direction is the capillary arrangement. Each capillary would produce an electropherogram as shown in Fig. 2(b). Four fluorescein derivatives were well separated in all 96 capillaries. There is some shift in the migration time among different capillaries, but the signal intensity was fairly uniform across the array.

For two-color excitation, there are two such images for each run, one for 488 nm excitation and one for 514 nm. Electropherograms from two different capillaries excited by two different wavelengths were overlaid in Fig. 3. Peak-height ratios were noticeably different for the four dye peaks. For 5CF, 6CF and Fluorescein, 488 nm is clearly a much better excitation wavelength, while for 2,7-DCF, fluorescence excited by 514 nm is stronger than that excited by 488 nm. All the other capillaries showed similar results. The first 5CF peak in each electropherogram was chosen as the internal standard to normalize the peak-height ratios across the array. The normalized peak-height ratios fall into three distinct clusters with no overlap as shown in Fig. 4. The averaged relative peak-height ratios were 0.79 ± 0.04 for 6CF, 1.27 ± 0.15 for F, and 0.21 ± 0.01 for DCF. Thus, choosing 0.46 and 0.95 as thresholds, confident peak identification was achieved. No error was observed for all 96 capillaries.

DNA Sequencing with Dye-Labeled Terminators.

Two-color excitation DNA sequencing was performed in a 24-capillary array. As

shown in Fig. 5(a), the lower part of the image represents the 24-capillary fluorescence signal excited by 488 nm while the upper half represents the same 24-capillary fluorescence excited by 514 nm. Since, for the ABI old dye set used here, 514 nm is a better excitation wavelength, the upper half of Fig. 5(a) showed stronger signals than the lower half.

Peak-height ratios up to 460 bp were calculated for each capillary. Fig. 6 shows the ratio histogram for one of the capillaries. Since it constitutes comparisons within a capillary, no internal standard was required. As shown in the histogram, the peak-height ratios fell into four distinct clusters, centered at 0.9, 1.7, 2.4, 3.3 respectively. There is a small overlap in C and T, which may introduce some ambiguity in base calling.

Fig. 7 shows the unsmoothed reconstructed traces from the 514-nm channel for 12 capillaries, all on the same scale. Other lanes show similar results, as can be seen in Fig. 5. A relatively uniform resolution and intensity distribution was obtained across the array. The migration times of a given DNA fragment are slightly different from capillary to capillary due to variations in internal diameter, capillary wall properties, heat distribution, etc. However, such variations do not affect base calling.

Base calling results based on two-color peak-height ratios were shown in Fig. 8. With TTHE buffer in the sieving matrix, the ABI old dye-labeled terminator pGEM fragments were well resolved starting from base number 1. No error was observed on base calling up to 400 bp and four errors (0.9%) were obtained within 450 bp. All of the mistakes were C miscalled as T. The reason is that 488 nm is not a very good wavelength to excite the corresponding TAMRA (T) and ROX (C) labeling dyes. The signals are fairly low. This introduces much uncertainty in the peak-height distribution, especially for the long DNA fragments, whose concentration were much lower than the shorter fragments due to the in-

herent nature of the Sanger reaction. These uncertainties are clearly seen in the wider distributions in the histogram (Fig. 6). However, single-base resolution could be observed up to 550 bp. We can expect still better base calling accuracy for this data set with software that provides improved baseline correction and peak deconvolution, or in a modified system with more than two excitation wavelengths. For the entire array, 22 capillaries showed similar performance, i.e. 99% accuracy for base calling up to 450 bp. Two capillaries showed slightly lower separation resolution such that 99% accuracy could be achieved up to 360 bp.

Cost.

Simplicity, robustness and high performance are the most appealing features of the two-dimensional laser scanning system. With the dispersed multi-line Ar^+ laser beam, this system differs from our previous one-dimensional step scan system by just adding one more galvanometer scanner while inheriting all of its advantages. By using only standard optics and one single-element photon detector, this setup provides unique possibilities for simple, low-cost fluorescence detection with large capillary arrays or microfabricated devices for high-throughput DNA sequencing and other CE analysis.

CONCLUSIONS

A novel approach for two-dimensional laser step-scan fluorescence excitation and single-element detection for multiplexed capillary electrophoresis was demonstrated. Uniform separation efficiency and good S/N were obtained for both multiplexed CZE and CGE separations. A simple two-color excitation method was successfully applied for peak identification and DNA base calling with only one laser and one single-element detector. Since the

Ar^+ laser has several fairly strong laser lines, the system is fully capable of four-color excitation for DNA sequencing. If the appropriate labeling dye set is developed, whose absorption spectra maximize at those corresponding wavelengths, practical four-color base calling based on excitation could be easily developed for high-throughput DNA sequencing.

ACKNOWLEDGMENTS

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Table 1. Relative absorbance (A) and absorptivity (ε) ratios of the dyes tested.

	$A_{488\ nm}$	$A_{514\ nm}$	$\varepsilon_{488}/\varepsilon_{514}$		$A_{488\ nm}$[a]	$A_{514\ nm}$[a]	$\varepsilon_{514}/\varepsilon_{488}$
5(6)CF[b]	83.24	15.35	5.42	R110	83.56	34.86	0.42
F	53.07	8.09	6.56	R6G	45.75	83.02	1.81
DCF	49.47	48.90	1.01	TAMRA	12.39	39.93	3.22
				ROX	6.65	15.81	2.38

[a]Absorption spectra were provided by Molecular Probes.

[b]Carboxyfluorescein came as a 1:1 isomer mixture. So the absorption spectrum was determined as a mixture.

FIGURE CAPTIONS

Figure 1. Schematic diagram of 2-dimensional laser step-scan system for multiplexed fluorescence detection in a 96-capillary array. F1, laser-line filter; M, mirror; BEX, laser beam expander; P, pinhole; L1, 10-cm focal length best-form lens; S, galvanometer scanner; SA, servo amplifier; L2, camera lens; and F2, 488-nm notch filter.

Figure 2. (a) Data for 96-capillary separation of 1×10^{-9} M fluorescein mixtures. The horizontal direction represents migration time (data points at 4 Hz). The vertical direction represents the capillary array arrangement. (b) Extracted electropherogram from the data in (a). The four peaks correspond to 5CF, 6CF, F, DCF respectively (from left to right).

Figure 3. Overlaid two-color electropherograms of fluorescein separation by two-color excitation. (a) Capillary #18. (b) Capillary #57.

Figure 4. Histogram of normalized peak-height ratios across the 96 capillary array. 5CF was used as the internal standard to normalize the ratios across the array.

Figure 5. DNA sequencing electropherograms from a 24-capillary array. (a) The horizontal direction represents migration time (data points at 4 Hz). The vertical direction represents the capillary array arrangement. The lower half of the im-

age is the fluorescence signal excited by 488 nm while the upper half is the corresponding 514 nm excited electropherogram. (b) Extracted electropherogram from one capillary.

Figure 6. Histogram of peak-height ratios for one capillary. The red lines correspond to the set threshold for base calling.

Figure 7. Reconstructed electropherograms from 12 capillaries in the 24-capillary array. All traces are on the same scale. Other capillaries show similar results.

Figure 8. Two-color excitation base calling of dsDNA pGEM. Errors are marked and corrected by the inserted alphabets. Red: 488 nm excitation. Blue: 514 nm excitation.

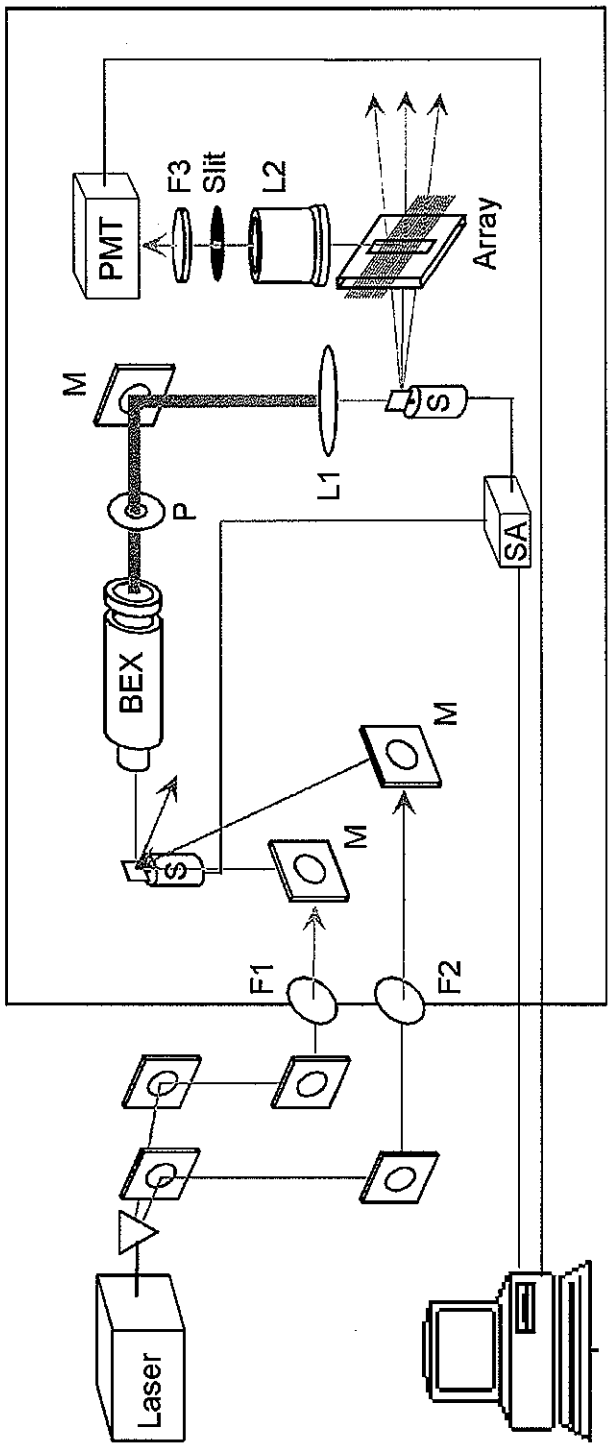

FIGURE 1

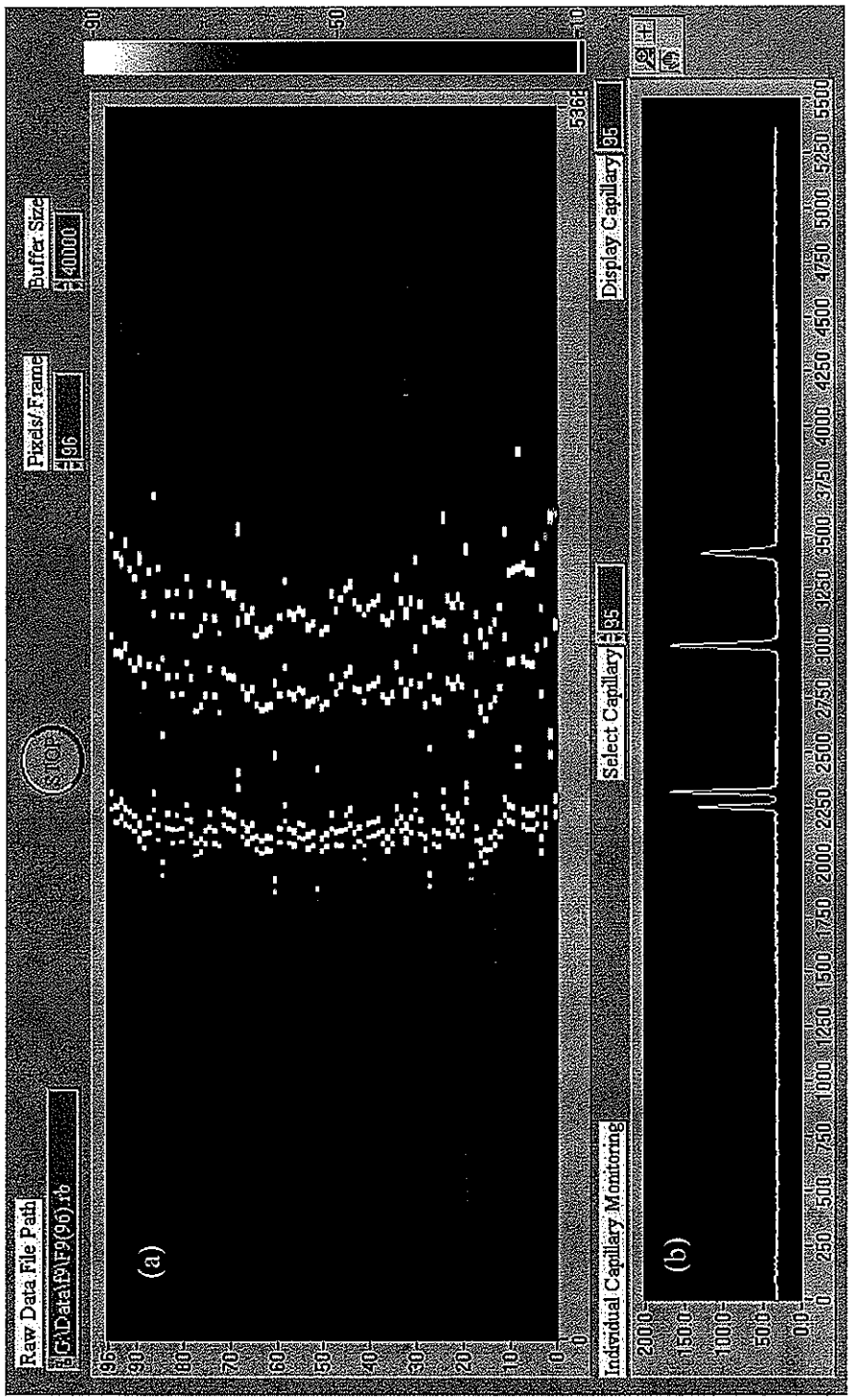
Raw Data File Path
G:\Data\MF9(96).fs
Pixels/Frame
96
Buffer Size
40000
Stop
(a)
(b)
Select Capillary
95
Display Capillary
95
Individual Capillary Monitoring

FIGURE 2

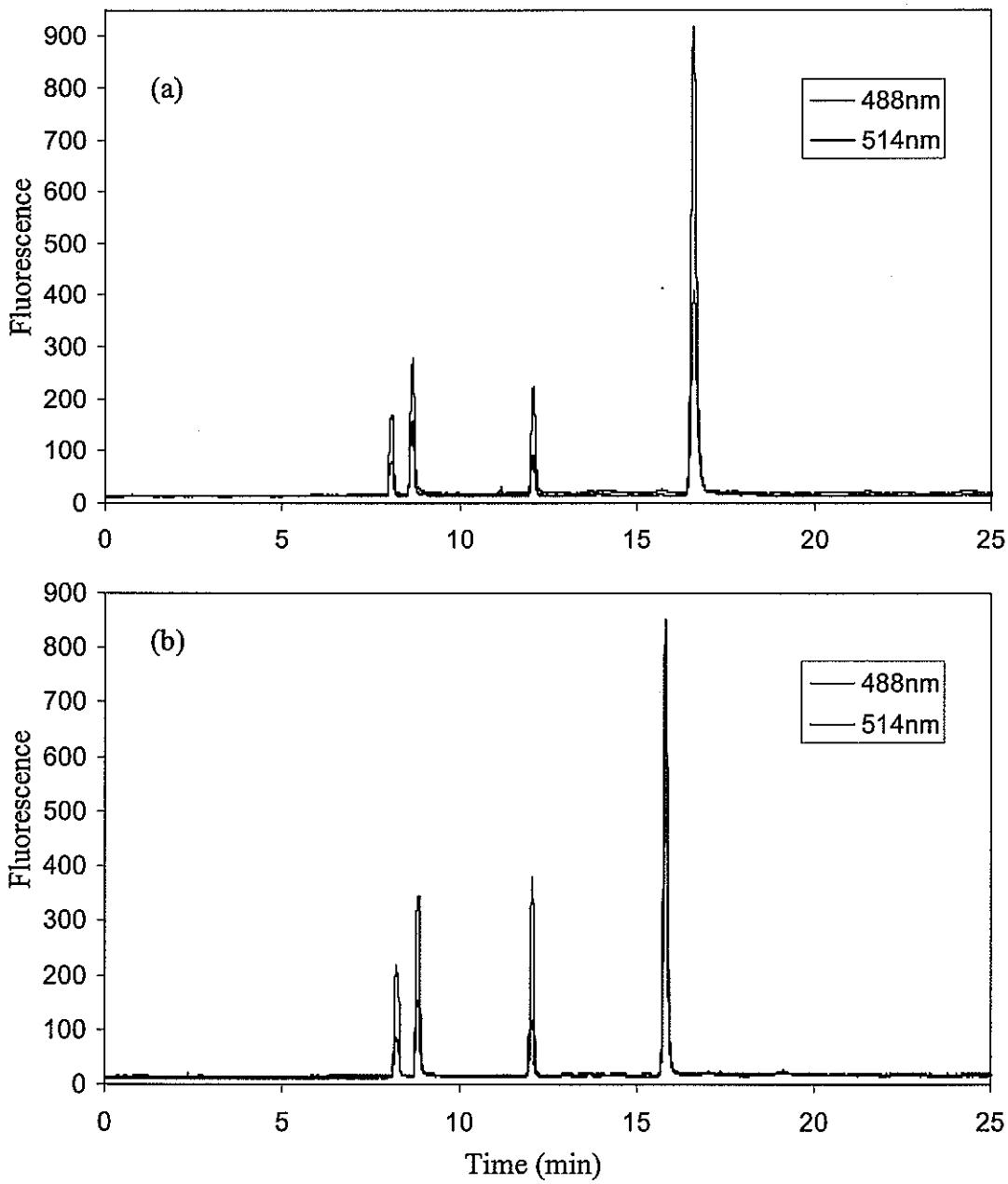
(a)
488nm
514nm
Fluorescence
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
(b)
488nm
514nm
Fluorescence
900
800
700
600
500
400
300
200
100
0
0
5
10
15
20
25
Time (min)

FIGURE 3

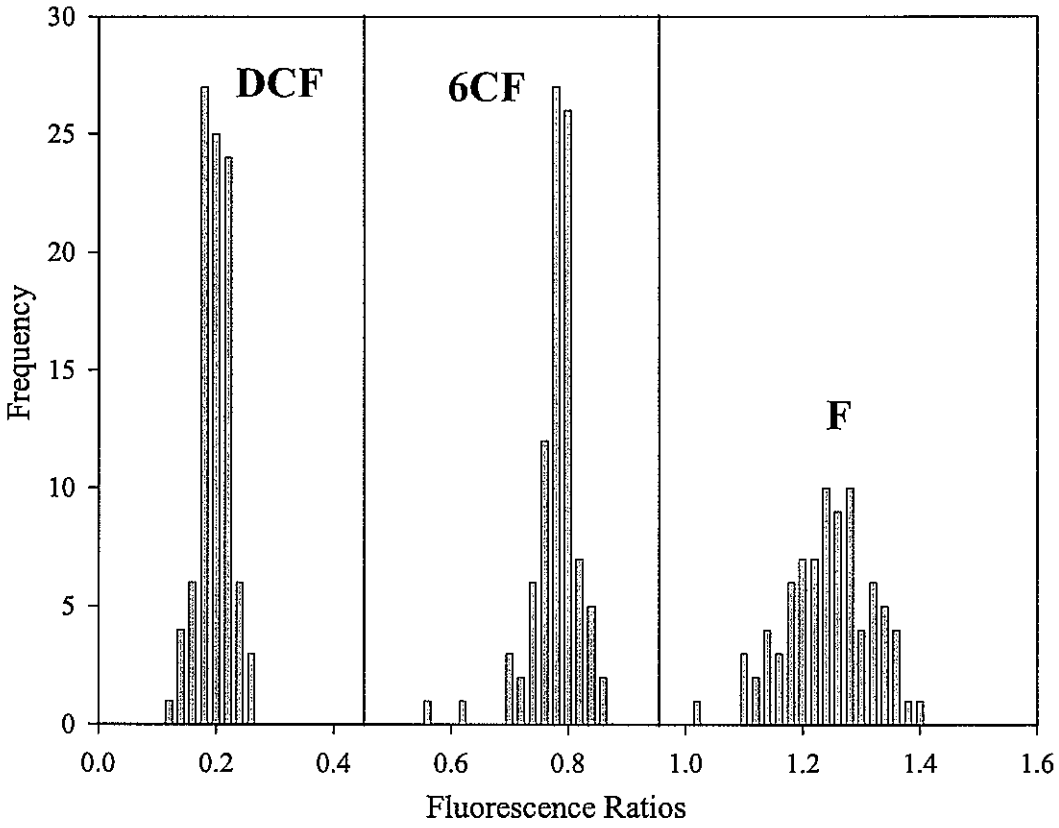
DCF
6CF
F
Frequency
Fluorescence Ratios
0
5
10
15
20
25
30
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6

FIGURE 4

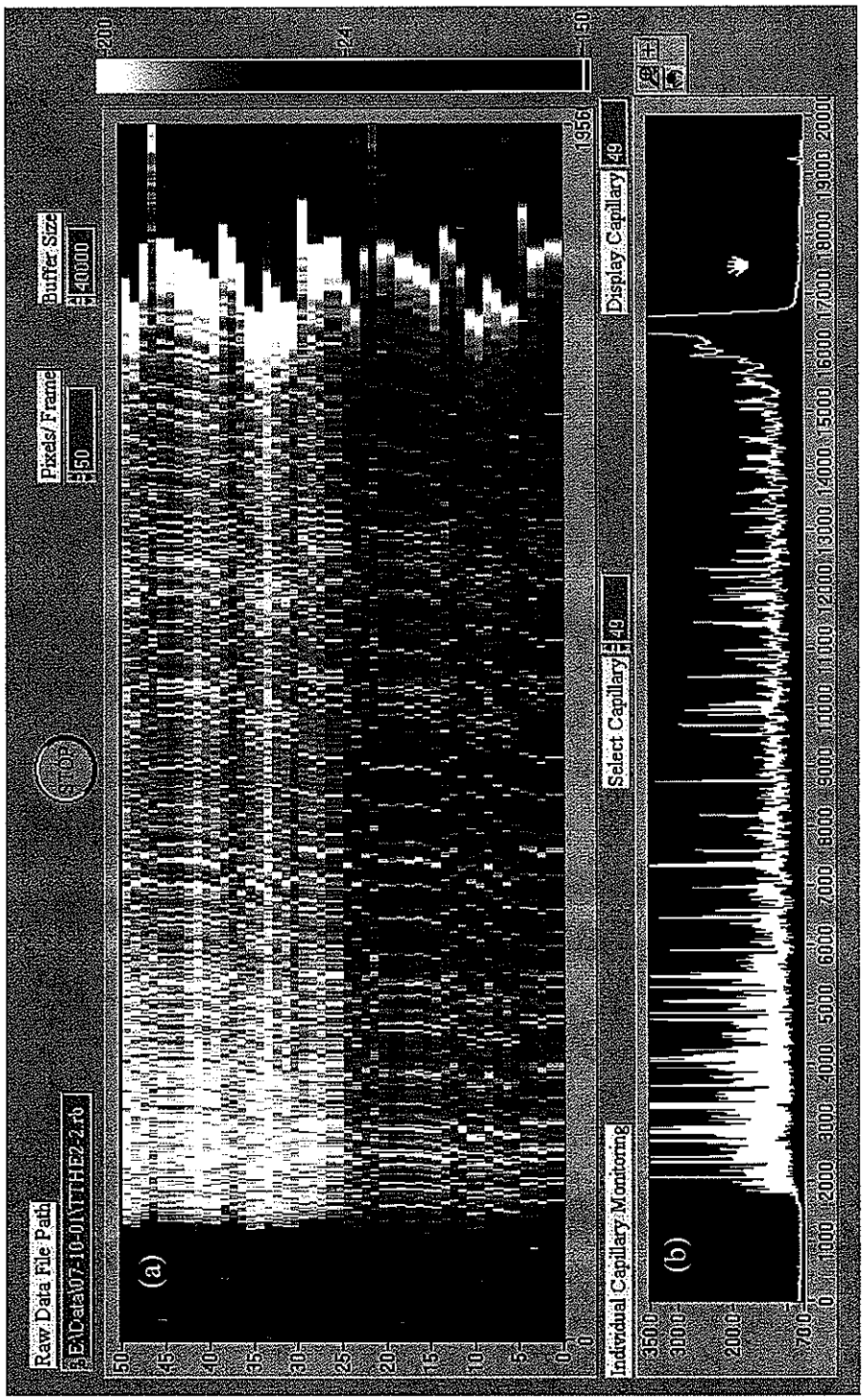
Raw Data File Path
Pixels/ Frame
Buffer Size
STOP
Select Capillary
Display Capillary
Individual Capillary Monitoring
(a)
(b)

FIGURE 5

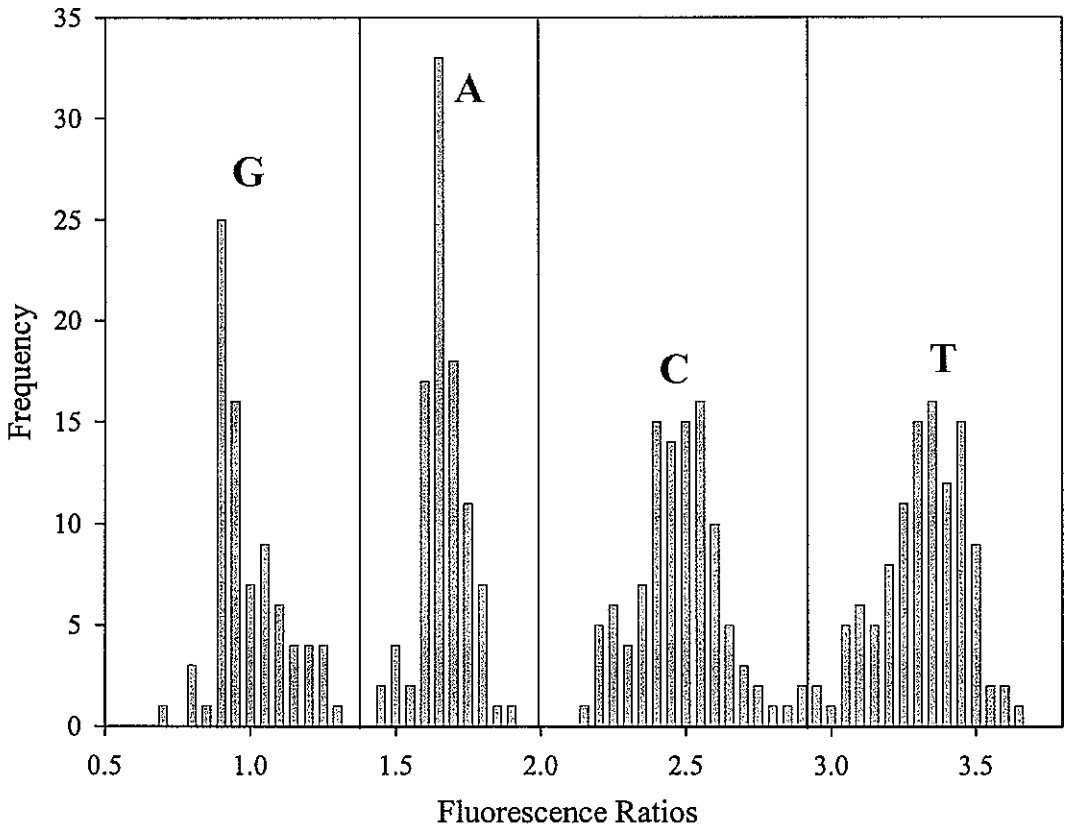
35
30
25
20
15
10
5
0
Frequency
G
A
C
T
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Fluorescence Ratios

FIGURE 6

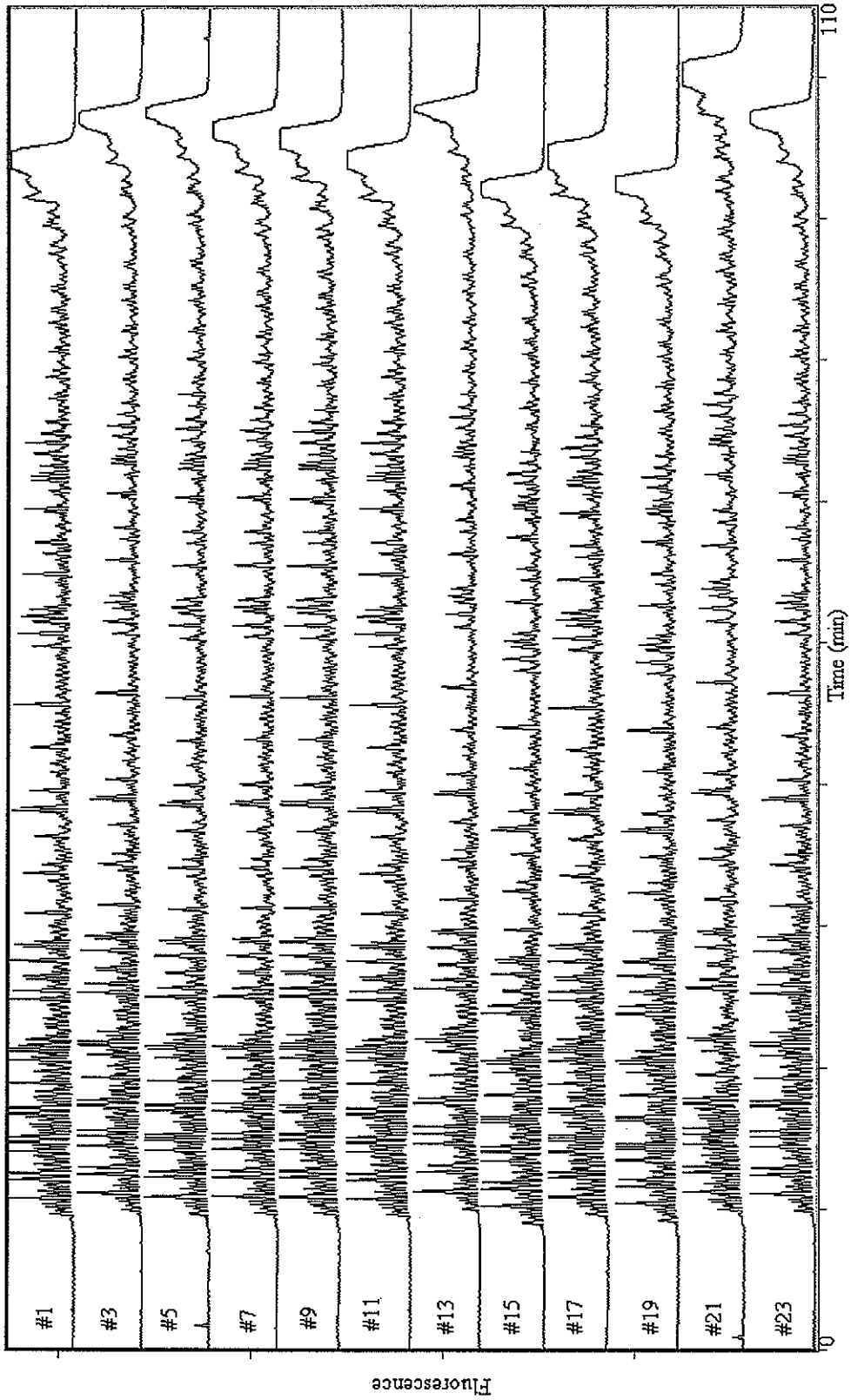

FIGURE 7

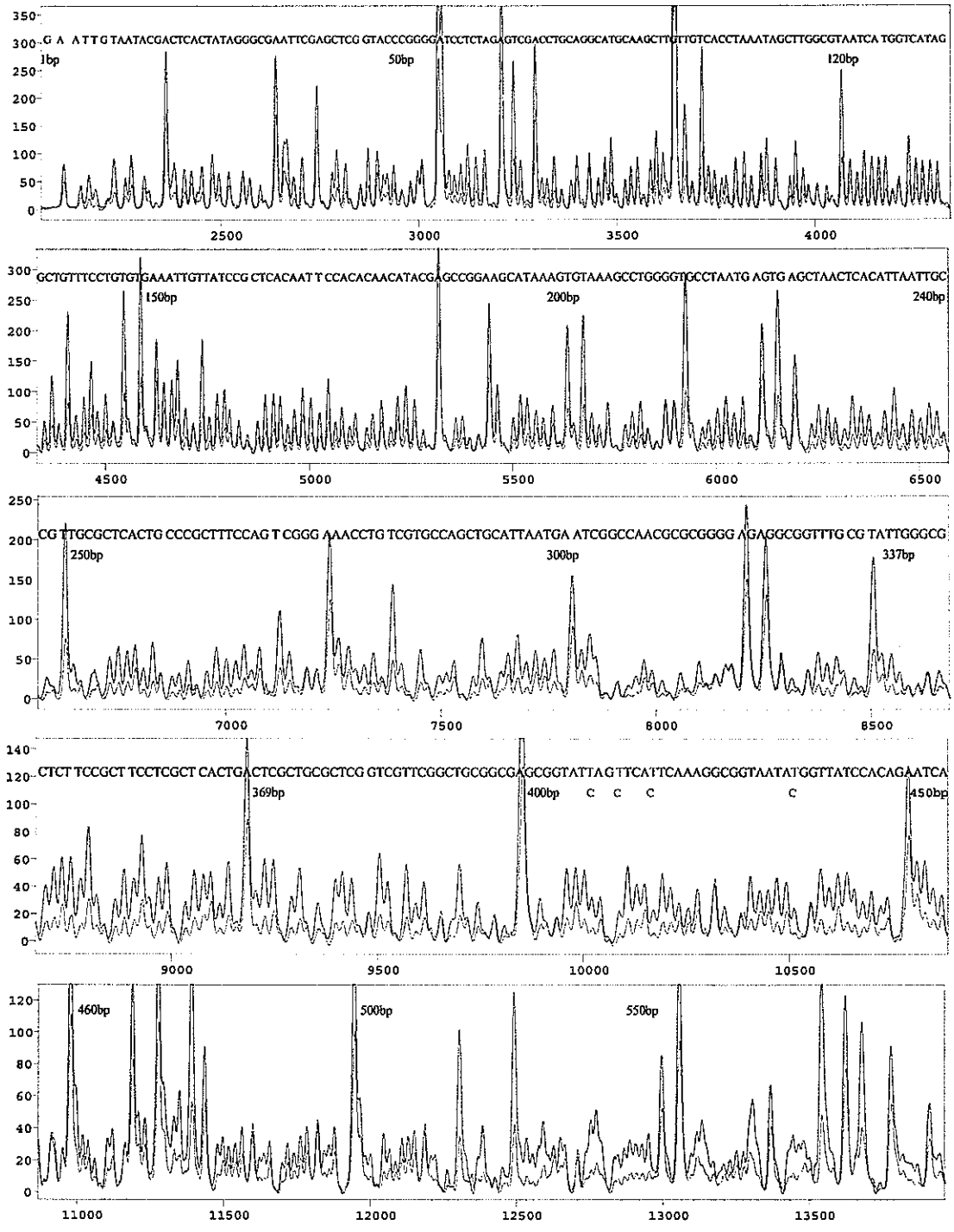
G A ATTG TAATACGACTCACTATAGGGCGAATTCGAGCTCG GTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTGTTGTCACCTAAATAGCTTGGCGTAATCA TGGTCATAG
1bp
50bp
120bp
GCTGTTTCCTGTGTGAAATTGTTATCCG CTCACAAT T CCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATG AGTG AGCTAACTCACATTAATTGC
150bp
200bp
240bp
CG TTGCGCTCACTG CCCGCTTTCCAG T CGGG AAACCTG TCGTGCCAGCTGCATTAATGA ATCGGCCAACGCGCGGGG AGAGGCGGTTTG CG TATTGGGCG
250bp
300bp
337bp
CTCT TCCGCT TCCTCGCT CACTGACTCGCTGCGCTCG GTCGTTCGGCTGCGGCGAGCGGTATTAG TTCATTCAAAGGCGGTAATATGGTTATCCACAGAATCA
369bp
400bp
450bp
460bp
500bp
550bp

FIGURE 8

CHAPTER 5. ONLINE NANOLITER CYCLE SEQUENCING REACTION WITH CAPILLARY ZONE ELECTROPHORESIS PURIFICATION FOR DNA SEQUENCING

A paper published in Journal of Chromatography A*

Gang Xue, Ho-ming Pang and Edward S. Yeung

ABSTRACT

An integrated system of nanoreactor for cycle-sequencing reaction coupled with online capillary zone electrophoresis (CZE) purification and capillary gel electrophoresis (CGE) DNA sequencing is presented. Less than 100 nl of premixed reagent solution, which includes dye-labeled terminator pre-mix, BSA and template, was hydrodynamically injected into a fused-silica capillary (75 μm i.d.) inside a homemade microthermocycler for cycle sequencing reaction. In the same capillary, the reaction products were purified by CZE followed by online injection of the DNA fragments into another capillary for CGE. Over 540 bp of DNA can be separated and the bases called for ss-DNA with 0.9% error rate. The total

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time was about 3.5 h, or a cycle time of 2 h with staggered operation. For ds-DNA, a longer reaction time was required and base calling up to 490 bp with 1.2% error rate was achieved. The whole system is readily adaptable to automated multiplex operation for DNA sequencing or PCR analysis.

INTRODUCTION

DNA sequencing usually involves five steps: (1) preparation of template DNA; (2) Sanger reaction to produce sequencing fragments; (3) purification of Sanger reaction products; (4) separation of DNA fragments; (5) base calling. Driven by the Human Genome Project, lots of effort has been applied to developing DNA sequencing methods that are more rapid, accurate and cost-effective than traditional sequencing methodologies.

Recent advances came from the development of capillary gel electrophoresis (CGE) separation[1,2,3,4] and the laser-induced fluorescence (LIF) detection of dye labeled primers or dideoxy nucleotides.[5,6,7] As a direct result, the cost in the separation (electrophoresis) and identification (detection) tasks is substantially lowered with greatly improved through-put.[8,9,10] CGE and the more recent microchip electrophoresis[4] took advantage of the small volume of the separation column, which allowed the use of much higher electric fields. Nearly 3-fold improvement in resolution and 25-fold increase in the speed of the separation were achieved in CGE compared to traditional slab gel electrophoresis (SGE).[11] Meanwhile, the amount of sample required for analysis dramatically dropped to the nanoliter regime, which could potentially offer significant savings in the use of costly reagents required for preparing the sequencing fragments.

Unfortunately, the protocols for sample preparation and Sanger reaction did not keep

pace with the low-cost high-speed high-throughput separation techniques. While capillary or microchip separations are scaled down to the nanoliter regime, the standard Sanger reaction and subsequent purification and injection are still performed in the microliter range because of difficulties in manipulating and transferring very small amounts of sample. Recently, miniaturization of PCR or cycle-sequencing reaction in capillaries or chips has been demonstrated. Burns and colleagues developed an integrated nanoliter DNA analysis device which is capable of metering a small, accurate volume of fluid by means of a hydrophobic patch and injected air.[12] Soper et al. made use of the biotin/streptavidin/biotin linkage to tether biotinylated DNA sequencing templates to the wall of the capillary for solid-phase cycle sequencing.[13,14,15] A single-dye amplification was accomplished in a volume of 62 nL in an (aminoalkyl)silane-derivatized fused-silica capillary. The capillary reactor was connected to the gel column via a zero-dead-volume fused-silica connector for on-line analysis. However, that approach involved a time consuming and labor intensive capillary coating process which took more than 24 h. After a couple of runs, noticeable loss of anchored streptavidin was observed and capillary regeneration was required.

Compatible miniaturized thermal-cycling equipment is also important for microscale sample preparation. Traditional metallic thermal blocks require a fairly long temperature transition time because of their high thermal mass. Several novel heating methods have been demonstrated for fast temperature change in the PCR or cycle-sequencing reaction. Hot-air thermocycler was first commercialized by Idaho Technology, which achieved rapid cycle sequencing within 25 min with dye-labeled primers.[16] Infrared-mediated heating was also successfully applied to microchamber thermal cycling.[17] Resistive thin films have been used to heat up individual capillaries with very fast ramping rate although they may not be readily

adapted for multiplex operation.[18]

Finally, to fully profit from the high throughput of such miniaturized systems, the integration of all the different parts are required. Several reports have shown a variety of coupling schemes for fluid handling with capillaries and valves in order to produce, purify and inject the sequencing sample.[19,20,21,22] Other reports showed total integration on microchips, performing PCR reactions, sample purification and separation,[23] although the protocol requires operator intervention.

In this report, we describe an integrated system of capillary nanoreactor (reaction volume ~100nL) for cycle sequencing reaction with capillary zone electrophoresis (CZE) purification coupled with CGE for DNA separation. No complicated accessories such as high-pressure pumps or valves were needed. The closed system is capable of continuous operation from the introduction of reagents to called bases. The advantage of this system are its simplicity, reliability, cost effectiveness, high speed and suitability for highly multiplexed operation.

EXPERIMENTAL SECTION

Reagents, Buffers and Separation Matrix.

10× Bovine serum albumin (BSA) was obtained from Idaho Technology Inc. (Idaho Falls, ID). 1× TBE with 7 M urea buffer was prepared by dissolving pre-mixed TBE buffer powder (Amresco, Solon, OH) and urea (ICN Biomedicals, Inc. Aurora, OH) in deionized water. The dynamic coating matrix was made by dissolving 2% (w/v) of 1,300,000 MW poly(vinylpyrrolidone) (PVP) (Sigma, St. Louis, MO) into de-ionized water. Tris, HCl, $MgCl_2$, TritonX-100, and poly(ethylene oxide) (PEO) was received from Aldrich Chemical

(Milwaukee, WI). 1× PCR buffer consisted of 10 mM Tris, 50 mM KCl, 1.5 mM Mg_2Cl and 0.1% TritonX-100. The CZE separation buffer was prepared by dissolving 10 mM Tris, 1.5 mM $MgCl_2$, 2 mM KCl and 0.3% (w/v) PVP in de-ionized water. The buffer was shaken for 30 s and left to stand for 1 h to remove bubbles. The CGE sieving matrix was an entangled polymer solution made by dissolving 1.5% (w/v) of 8,000,000 MW PEO and 1.4% (w/v) of 600,000 MW PEO in 1× TBE buffer with 7 M urea. The solution was stirred vigorously overnight until all material was dissolved and no bubbles can be observed. The bare fused-silica capillary was purchased from Polymicro Technologies (Phoenix, AZ). FC-coated capillary, PEG-coated capillary, and DB-WAX-coated capillary were purchased from J&W Scientific (Folsom, CA). PVA-coated capillary was received from Beckman Instruments (Fullerton, CA).

Sequencing Reaction Protocol.

ThermoSequenase old-dye terminator pre-mix kit (Amersham Life Science, Cleveland, OH) was used for online cycle sequencing. Modifications were made to fit the protocol to online capillary format since the original protocol was developed for the ABI Model 9600 or 2400 instrument.

The old-dye terminator premix included 125 mM Tris-HCl, 5 mM $MgCl_2$, 1.25 mM dITP, 0.25 mM dATP, 0.25 mM dCTP, 0.25 mM dTTP, 0.25 mM old-dye labeled ddNTP, ThermoSequenase, TAP, Nonidet P40, Tween 20 and 6.25% glycerol. A typical cycle-sequencing reaction mixture consisted of 8 μL of such premix and 1 μL 5.0 μM universal – 17 M13 primer. 2 μL of 10× BSA was added to prevent adsorption of the enzymes on the surface of the capillary tubes and an additional 1.0 μL 25 mM $MgCl_2$ was added to compen-

sate for the loss during the reaction due to diffusion. For ss-DNA sequencing, 2 μL of 0.2 μg/μL M13mp18 in 1× TE buffer (pH ~ 7.5, Amersham) was used as the template. For ds-DNA, 4 μL of 0.2 μg/μL pGEM was used since only one strand of DNA served as template during reaction. The total reaction volume was made up to 20 μL in both cases with an appropriate aliquot of deionized water.

The temperature protocol for ss-DNA cycle sequencing was adjusted to the following: the sample mixture was heated to 92 °C and held for 2 min; 30 cycles were performed with denaturation at 94 °C for 5 s, annealing at 50 °C for 5 s and extension at 63 °C for 2 min. When ds-DNA was amplified, the sample mixture was first heated to 92 °C and held for 2 min; 35 cycles were performed with denaturation at 96 °C for 5 s, annealing at 50 °C for 5 s and extension at 63 °C for 4 min.

Instrumentation.

Figure 1 shows the schematic diagram for the setup. A single 75 μm i.d. capillary served as both the cycle-sequencing reaction vessel and the CZE purification column. The entire system consisted of a microthermocycler, a CZE and a CGE electrophoretic system. A microtee (Upchurch Scientific, Oak Harbor, WA) was used to connect the CZE capillary, CGE capillary and a waste arm.

Microthermocycler.

A home-built microthermocycler was used for online reactions. Two major features were incorporated in the thermocycler design: rapid thermal cycling and good temperature

accuracy and stability.

The reaction capillary (75 μm i.d.) was sandwiched between two pieces of thin brass sheet (7.5 cm × 2.5 cm × 25 μm) (Small Parts Inc., Miami Lakes, FL) and a Kapton insulated flexible heater (7.5 cm × 2.5 cm × 0.25 mm, 10 W/in^2) (Omega, Stamford, CT) as shown in Figure 2. A thermal epoxy (Delta Bond 155, Wakefield Engineering, Inc, Wakefield, MA) was used to bond the brass sheet and the flexible heater together. This microthermocycler has a very small thermal mass since the total thickness excluding the capillary reactor is ~300 μm so that it allows rapid temperature changes. The length of the heater allowed the future adaptation for 8 capillaries (9-mm spacing was used for the standard 8 × 12-tray format) or 16 capillaries (4.5 mm spacing for the 384-tray format) for simultaneous reactions.

Thermal conductive silicone grease (Radio Shack) was applied onto the capillary surface and in between the brass sheets to ensure proper heat transfer. For 75 μm i.d. capillary, 23 mm reaction length corresponded to ~100 nl maximum reaction volume. One could change to different inner diameters to accommodate different reaction volumes.

In order to accurately measure and control the inner temperature of the reaction capillary, a miniature bare type K thermocouple (75 μm diameter, Omega Engineering, Inc.) was inserted into a 250 μm i.d., 360 μm o.d. capillary which was filled with water and sealed with epoxy on both ends. The thermocouple probe was placed in the middle of the thermocycler. Silicon thermal grease was applied to the surface of the probe capillary as well for better heat conduction.

A proportional-integral-derivative (PID) temperature controller (CN77300, Omega Engineering, Stamford, CT) was used to control the temperature profile for cycle sequencing.

This method of control varies the magnitude of heat applied to the sample in proportion to the difference between the actual and the set temperature. The advantages of PID control over traditional ON/OFF control is that the system can be carefully tuned to compensate for temperature overshoot and ringing.[24]

A 4-W aquarium air pump (Tetra Secondnature, Blacksburg, VA) was used to blow room air onto the heater to cool it down quickly during the ramp from denature to annealing steps. A faster cooling rate can be obtained if desired by using a higher gas flow rate offered by a more powerful pump or a compressed gas jet.[25]

An in-house Labview program was used control the microthermocycler. Standard RS-232 serial port was used to communicate with the temperature controller. The digital TTL-level parallel port output was fed to a solid-state relay (Omega Engineering) to control the ON/OFF of the air pump. The control system can be easily adapted to any PC computer for a variety of applications such as online digestion,[26] PCR, etc.

Microtee connection.

A peek microtee with 9 nL dead volume was used to connect together the 35 cm long CZE capillary (75 μm i.d.), 63 cm long CGE capillary (75 μm i.d.) and a 10 cm long waste arm. A much larger i.d. capillary (250 μm i.d.) was chosen as the waste arm because of its small flow resistance for rinsing and gel filling. Tight connection was confirmed by the stable current during CE separation.

Detection system.

Laser-induced fluorescence (LIF) detection was used both in CZE monitoring and

DNA sequencing. A 15-mW Ar-ion laser (Uniphase, San Jose, CA) was used for the excitation in both separation systems by splitting the laser beam 50-50. Both optical paths used an uncoated plano-convex lens with 12-mm focal length to focus the laser beams onto the capillaries. For CZE separation detection, a 10× microscope objective (Edmund Scientific, Barrington, NJ) was used to collect the fluorescence into a photomultiplier tube (PMT) with the use of a 560-nm long-pass filter to cut off the laser scattering. For CGE, another 10× microscope objective was used. A 514-nm notch-plus filter (Kaiser Optical System, Ann Arbor, MI) was used to block the laser scattering. Then a 80/20 beam splitter was used to split the fluorescence into two PMTs for simultaneous monitoring. One PMT (80% splitting ratio) had an additional RG630 long-pass filter in front of it. This arrangement allowed base calling for DNA sequencing.[9] All PMTs (R928, Hamamatsu Corp., Bridgewater, NJ) were terminated with 10-kΩ resistors before connecting to a 24-bit A/D interface (PC4350, National Instruments, Austin, TX). A Pentium II 266 MHz computer (Dell, Austin, TX) was used to control the system and for data acquisition.

Sample Injection and On-column Cycle Sequencing.

Before sample injection, the CZE capillary was filled with pH 8.2 separation buffer containing 10 mM Tris, 1.5 mM $MgCl_2$, 2 mM KCl and 0.3% PVP. Hydrodynamic injection of the cycle-sequencing mixture into the CZE capillary was performed by raising the microthermocycler by 11 cm. For the 75 μm i.d. capillary with total length of 35 cm, the reaction mixture was injected for 60 s and then the separation buffer was introduced for 60 s. Even though the sample vial contains a few microliters of reaction mixture, the injection process only takes up ~100 nL of sample. The premixed reaction mixture was reused more than 20

times without noticeable decrease in activity. This leads to a true saving in reagent costs. After injection, the microthermocycler was lowered to the original level and the capillary immersed into the vial containing separation buffer. 30 to 35 cycles was then performed by the microthermocycler. No additional denaturing was needed before injection into the CGE capillary.

CZE Purification.

The same 75 μm i.d. reaction capillary with effective length of 31 cm was used for CZE purification to get rid of the excess dye terminators and salt. 10 kV was applied to vial 1 and vial 2 (Fig.1) which contained the separation buffer. Purification was completed in 10 min. When the DNA fragments were detected at the detection window, an appropriate delay was estimated to allow for the fragments to move to the microtee. Electrokinetic injection of the DNA into the CGE capillary was initiated by switching the positive high voltage electrode from vial 2 to vial 3. After injection at 20 kV for 2 min, the CZE capillary was flushed with 1× TBE buffer with 7 M urea to remove the uneluted excess dye terminators and salts. Vial 2 was replaced by 1× TBE buffer with 7 M urea for sequencing separation.

CGE Sequencing.

The CGE capillary (63 cm long, 50 cm effective) was flushed with deionized water and 2% PVP, and filled with PEO gel (1.5% 8,000,000 MW and 1.4% 600,000 MW) before the CZE capillary was flushed with the separation buffer and loaded with the reaction mixture. 11 kV was applied to vial 2 and vial 3 to perform DNA sequencing. Two-channel detection was used for base calling.[9]

Base Calling.

Two-channel electropherograms were smoothed and baseline corrected by commercial Grams32 software (Galatic Industries) before peak picking. The software could export the peak table to an Excel worksheet. The peak-height ratios of corresponding peaks and absolute peak heights in the red channel were set as criteria for base calling in Excel.[9] Peaks were inserted as a G base when a gap was detected by calculating the distance between neighboring peaks.

RESULTS AND DISCUSSION

Microthermocycler Performance.

Figure 3 documents the reactor temperature as a function of time for the cycle-sequencing amplification of M13mp18. The lower panel shows the temperature profile of several cycles in greater detail. Because of the low thermal mass, very rapid temperature changes were achieved. Cooling from the denaturation temperature (94 °C) to the annealing temperature (50 °C) takes ~10s, corresponding to a cooling rate of 4.4 °C/s. The heating rates are even higher, ~10 °C/s. These fast heating and cooling rates are essential for speeding up sample preparation, increasing the yield and suppressing nonspecific binding and false priming problems that are normally associated with PCR amplification. The temperature profile also exhibited very good stability. The inner capillary temperature was regulated to within ±0.5 °C. We also moved the reaction capillary around and performed the sequencing reaction at different locations of the thermocycler. All experiments showed very similar yields. This level of homogeneity makes the current design suitable for adaptation to multiplexed capillary cycle-sequencing reactions.

Sample Introduction.

According to the Poiseuille equation, the linear flow rate (V) of an analyte in the column can be calculated from

$$V = \rho g r^2 \Delta h / 8 \eta L$$

where ρ is the solution density, g is the gravitational force constant, r is the internal radius of the capillary, Δh is the difference in height between the two ends of the capillary, η is the solution viscosity, and L is the capillary length. The reaction mixture was introduced for 10 s hydrodynamically. For a 75 μm i.d. capillary, when vial 1 was raised by 11 cm relative to vial 2, the flow rate (V) was around 2.3 cm/min. So, 1 min hydrodynamic injection introduced a 2.3-cm reaction mixture plug, which corresponded to ~100 nL, into the capillary. Then, separation buffer was hydrodynamically injected for 1 min to push the reaction mixture to the part of the capillary which was sandwiched in the microthermocycler. The sequencing results demonstrated that this process was highly reproducible.

Online Thermal Cycling.

The most convenient way to perform automated DNA sequencing today is by the dye-terminator chemistry since only one-tube sequencing reaction and single-capillary separation is needed for each DNA sample. Unfortunately, incorporating the dye terminators involves fairly slow kinetics. Usually, 4-min extension time is required. With our microthermocycler, only 2-min extension time was needed to produce enough DNA fragments to sequence M13mp18. Fig. 4 shows the relative signal of DNA fragments in CZE separation with different extension times. Only 15% signal loss was observed when the extension time was reduced from 3 min to 2 min. Further reduction of the extension time to 1

min would reduce the DNA signal by more than 35%, which was not sufficient for sequencing of the DNA fragments longer than 250 bp. So 2-min extension time was used for ss-DNA amplification and 30 cycles were completed within 80 min.

However, amplification of ds-DNA such as pGEM is more difficult than ss-DNA because the complementary strand of DNA may compete with the primer for annealing. Higher denaturing temperature (96 °C) and longer extension time (4 min) were needed to achieve comparable efficiencies. Also, increasing the BSA concentration to 0.5 μg/μL proved to be helpful for the cycle sequencing of pGEM.

No special pretreatment of the fused-silica capillary was needed. The reaction capillary was completely regenerated simply by rinsing the capillary with separation buffer. For over 1 month of experiments, the same capillary was used for over 40 reactions without noticeable loss of reaction efficiency.

CZE Purification with Suppressed EOF.

Since the excess old-dye terminators comigrate with ~60 bp and ~110 bp DNA fragments in CGE, they will mask several base pairs around those regions in the sequencing separation and introduce errors in base calling. Also the high salt content of the reaction mixture makes electrokinetic injection to the CGE capillary extremely difficult. Proper purification of the cycle-sequencing product to get rid of these interferences must be incorporated into the online system. In our previous work, several separation schemes have been devised such as size-exclusion chromatography in a 250 μm i.d. capillary[27] and CZE separation.[28] CZE separation is especially attractive because it does not require additional instrumental components such as a high-pressure pump. However, in bare fused-silica capillaries, since

electroosmotic flow (EOF) dominates the direction of migration, the dye terminators, which have smaller electrophoretic mobilities, migrate faster than the DNA fragments. The strong tailing of the terminator peaks, which is probably due to their strong hydrophobicity, makes the separation very difficult. Furthermore, the uncertainty in EOF due to variable surface conditions of the capillary inner wall makes the migration times in a capillary array very irreproducible.[22] This makes multiplexed CZE purification, where very precise timing and very short injection time to the CGE capillary is required, almost impossible.

Here, we use CZE with suppressed EOF to separate the dye terminators from the DNA fragments. Several separation systems were studied to optimize the online system. The most obvious idea would be to use the 1 × PCR buffer as the separation buffer (pH = 8.9), which is completely compatible with the cycle-sequencing reaction. Because of its high ion strength, the cations would shield the deprotonated silanol groups on the inner wall of the capillary to suppress EOF. Very good separation was achieved (Fig. 5a). The DNA fragments migrated as a narrow band because of their similar mass-to-charge ratios. However, when high voltage is applied across the CZE capillary and the CGE capillary during electrokinetic injection, most of the voltage will drop along the CGE capillary. Thus, the field strength in the CZE capillary will be much smaller than that in the CGE capillary, which is just the opposite scenario as in electro-stacking. Therefore, electrokinetic injection turned out to be very inefficient.

One of the other choices would be using coated capillaries. Different coated urface yies, such as fluorocarbon (FC), polyethleneglycol (PEG), polyvinylalcohol (PVA), DB-WAX coated capillaries, were tested as the integrated reaction vessel and separation column. 1× PCR buffer with low concentrations of KCl (pH = 8.9) was tested as the

separation medium. PVA-coated capillary proved to be the best choice in terms of suppressing EOF. Good separation from the dye terminators was achieved (Fig. 5b), although the DNA fragments appeared as a broader band. Different concentrations of KCl were tested for the CZE separation and electrokinetic injection efficiency (Table I). 2~4 mM KCl proved to be best separation condition as a tradeoff between DNA bandwidth and online injection efficiency, which was confirmed by good DNA signals and sequencing separation with base calling up to 500 bases. No dye terminator interference was observed. However, there are two inevitable problems inherent to coated capillaries. One is the cost in a multiplexed system and the other is degradation of the coating. After about 20 reaction and CZE separations, noticeable decrease in the reaction efficiency was observed. Also, EOF gradually increased. Attempts to regenerate the capillary by washing it with methanol and D.I. water were not successful.

In the previous studies, we demonstrated that EOF of bare fused-silica capillary could be substantially suppressed by the dynamic coating of PVP solution due to the strong hydrogen bonding between the hydrophilic carbonyl group of PVP and the residual hydroxyl group on the capillary wall.[29] Compatibility of the PVP coating with the cycle-sequencing reaction was therefore studied by flushing the long capillary loop with 2% PVP followed by placing the capillary in the commercial air thermocycler (Idaho Technology) for offline cycle-sequencing reaction. After reaction, about ~2 μL of reaction product was collected and purified by spin column. CGE separation of the purified DNA product showed similar signal strength as that without PVP coating, which confirmed that PVP did no harm to the reaction.

During CZE separation, PVP was added to the separation buffer to achieve more even coating. Different concentrations of PVP were investigated for efficiency for suppressing

EOF and for separation. At pH 8.9, 1.0% PVP was required to consistently suppress EOF. However, since the entanglement limit of 1,000,000 MW PVP is about 0.7%, at such a high PVP concentration the DNA fragment peak was substantially broadened by the sieving effect. An alternative approach was to lower the pH of the separation buffer. It was found that at pH 8.2, only 0.3% PVP was needed to suppress EOF. Different sized DNA fragments comigrated as a narrow band with half peak width of ~1.5 min (Fig. 5c). More important was that there was no compromise in the reaction efficiency in such a low pH buffer. The urfacetant, Triton X-100, was taken out from the buffer to avoid bubble formation around the microtee connection during CZE separation.

Very reproducible separation was achieved with PVP dynamic coating. The bare fused-capillary could be reused again and again by simply flushing the capillary with the separation buffer between runs. For over one month of experiments, more than 40 reactions and separations were performed on the same capillary. No decay in the online reaction efficiency was observed and the DNA fragments showed very reproducible migration times.

DNA Sequencing Separation.

Both ss-DNA and ds-DNA were successfully amplified online, purified and injected into the CGE capillary for two-channel detection. Fig. 6 shows the two-channel electropherograms of ss-DNA M13mp18.[9] No dye-labeled ddNTP peak was detected. As summarized in Table II, there were no errors in base calling up to 400 bp. The error rate was below 1% up to 540 bp.

Fig. 7 is the two-channel sequencing data for ds-DNA pGEM.[9] A shorter read length compared to that of M13mp18 was achieved. This may be partially due to the lower effi-

ciency in the amplification of ds-DNA. Other reasons might be the reannealing of the Sanger fragments with the complementary strand of the template, since the pGEM has a partial sequence which has a high content of G and C nucleotides. However, no error occurred till 460 bp and the error rate was kept below 1% up to 480 bp. If some heating element is placed before the tee or the tee itself is heated before injection into the CGE capillary, even better base calling result should be attainable because of additional denaturing.[20]

CONCLUSIONS

Here we demonstrated a simple but reliable microthermocycler which is capable of cycle-sequencing amplification of both ss- and ds-DNA. Fast nanoliter-scale reaction was accomplished in the same bare fused-silica capillary used for subsequent purification. PVP proved to be a reliable and efficient dynamic coating material in the CZE purification of Sanger fragments from the cycle-sequencing reaction. Purified DNA was successfully injected into the CGE capillary for sequencing separation by simply switching the high-voltage electrode. This closed system avoids external sample transfer and consumes only nanoliter volumes of expensive cycle-sequencing reagents in each run. It greatly reduces contamination and allows for faster execution of analysis at a substantially lower cost. The whole system is ready for conversion into a multi-capillary online reaction, purification and gel separation instrument for high throughput operation. Adaptation to a microchip format should also be possible, since no high-pressure fluidics is involved. Without much modification, the whole system could also be adapted to many other applications such as genotyping,[21] peptide mapping,[30] and combinatorial screening.[31]

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Table I. Effect of KCl concentration on CZE separation and electrokinetic injection.

KCl Conc. (mM)	Current in CZE (μA)*	Injection Current (μA)**	Field Strength during Injection**		FWHM (min)
			CZE Cap. (V/cm)	CGE Cap. (V/cm)	
0	6.8	6.8	300	146	2.9
2	9.5	8.0	220	170	2.4
4	12.2	8.8	190	190	1.5
6	15.5	9.5	160	200	1.3

*Voltage applied across the CZE (L_1= 35 cm) and waste arm (L_2= 10 cm) capillaries was 11 kV, corresponding to ~300 V/cm field strength in CZE capillary.

**Voltage applied across the CZE (L_1= 35 cm) and CGE (L_3= 64 cm) capillaries was 21 kV. The CGE capillary was filled with PEO gel while the CZE capillary was filled with 1 × PCR buffer.

When 150 V/cm was applied across the CGE capillary during sequencing separation, the current was ~7.0 μA.

Table II. Base-calling results.

ss-DNA M13mp18			ds-DNA pGEM		
bp number	Number of errors	Error Rate	bp number	Number of errors	Error Rate
400	0	0%	450	0	0%
500	2	0.4%	470	3	0.6%
540	5	0.9%	480	4	0.8%
590	15	2.5%	490	6	1.2%

Figure Captions

Figure 1. Schematic diagram of online DNA sequencing system.

Figure 2. Schematic diagram of microthermocycler.

Figure 3. Temperature profile of cycle-sequencing amplification of M13mp18.

Figure 4. Purified M13mp18 DNA fragments from cycle-sequencing amplification with different extension times.

Figure 5. CZE purification of cycle-sequencing product. (a) Bare fused-silica capillary, 1× PCR buffer with 50 mM KCl. (b) PVA-coated capillary, 1× PCR buffer with 4 mM KCl. (c) Bare fused-silica capillary, 1× PCR buffer with 2 mM KCl, 0.3% 1,000,000 MW PVP.

Figure 6. Online cycle sequencing of ss-DNA M13mp18 with 2-color base calling. Errors are marked by red alphabets.

Figure 7. Online cycle sequencing of ds-DNA pGEM with 2-color base calling. Errors are marked by red alphabets.

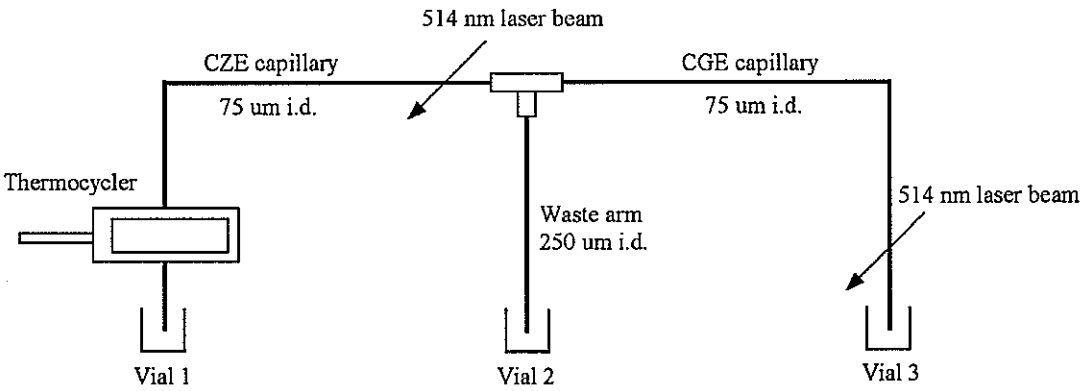
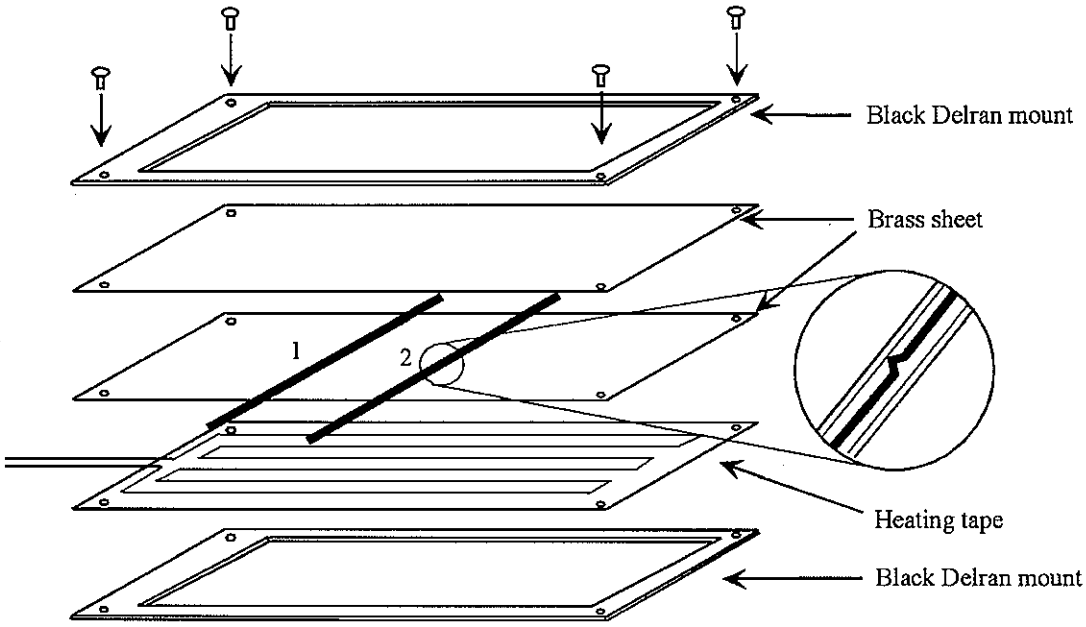

514 nm laser beam
CZE capillary
75 um i.d.
CGE capillary
75 um i.d.
Thermocycler
Waste arm
250 um i.d.
514 nm laser beam
Vial 1
Vial 2
Vial 3

FIGURE 1



1. Reaction capillary (75um i.d.)
2. Thermal couple inside capillary (250 um i.d.)

FIGURE 2

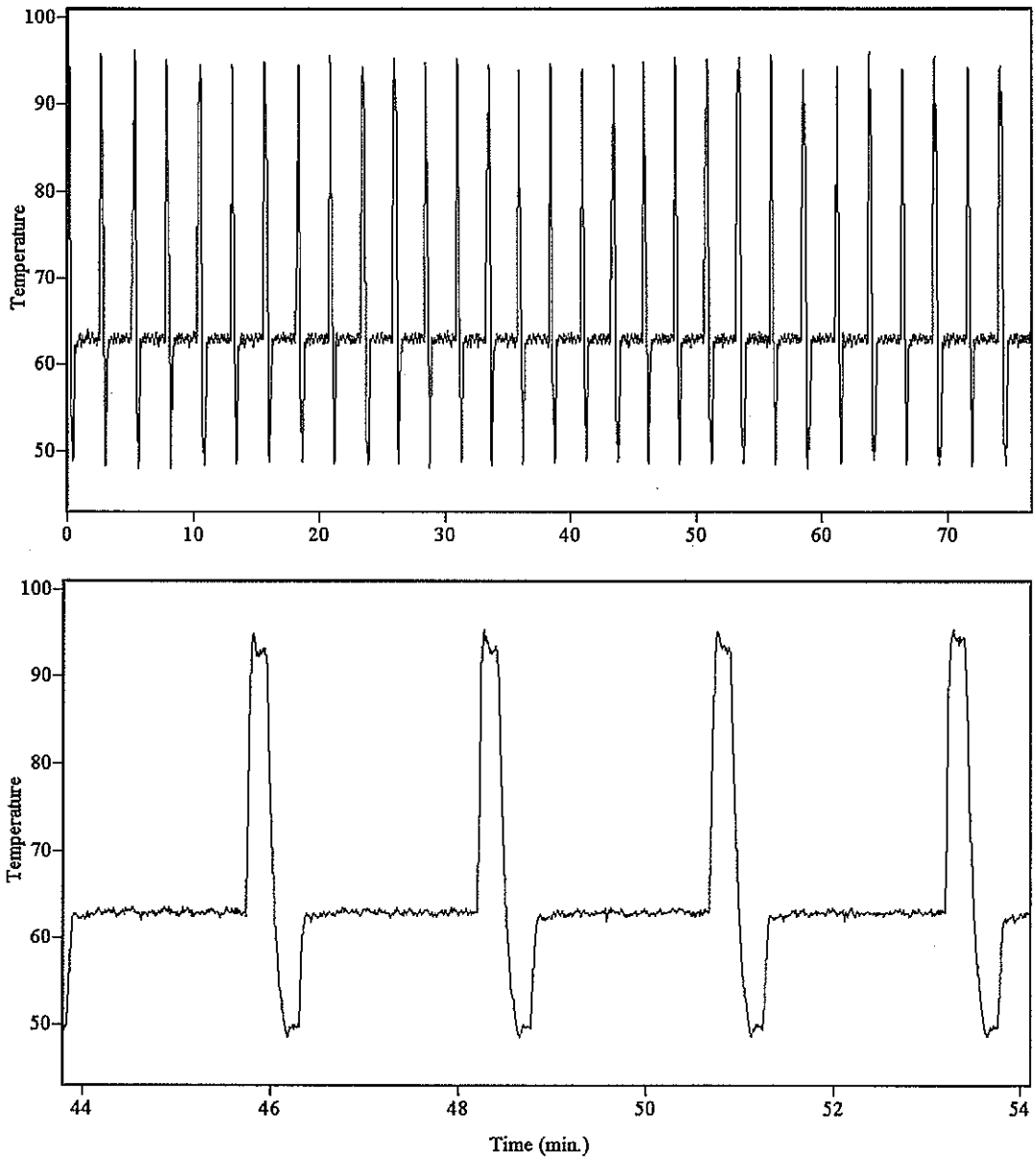

100
90
80
70
60
50
Temperature
0
10
20
30
40
50
60
70
100
90
80
70
60
50
Temperature
44
46
48
50
52
54
Time (min.)

FIGURE 3

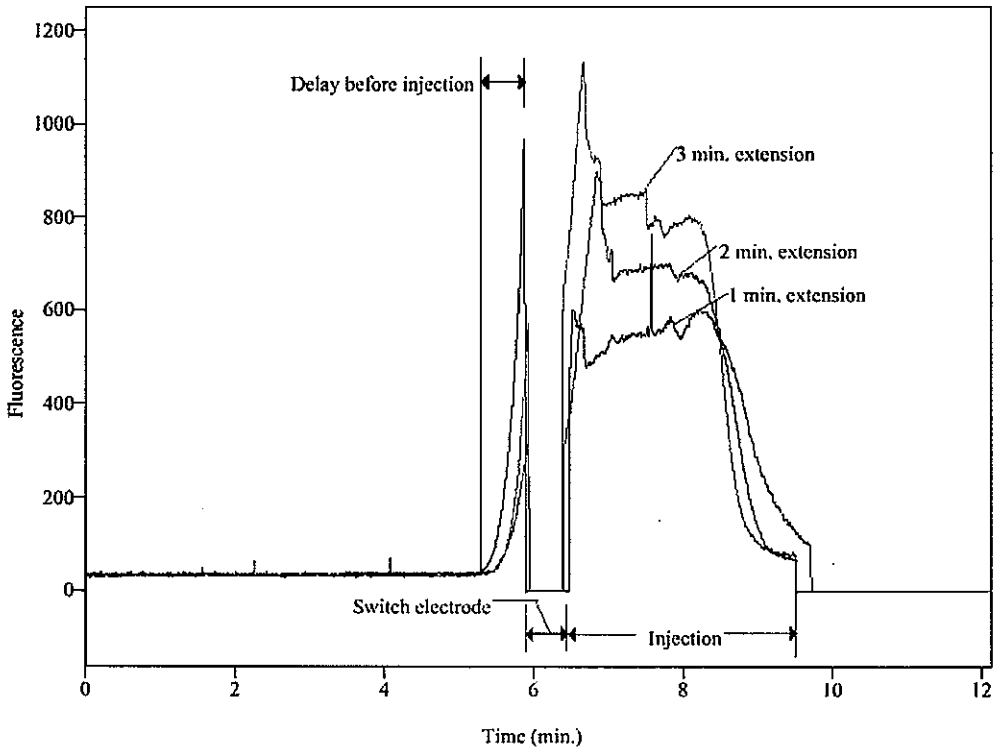

1200
1000
800
600
400
200
0
Fluorescence
Delay before injection
3 min. extension
2 min. extension
1 min. extension
Switch electrode
Injection
0
2
4
6
8
10
12
Time (min.)

FIGURE 4

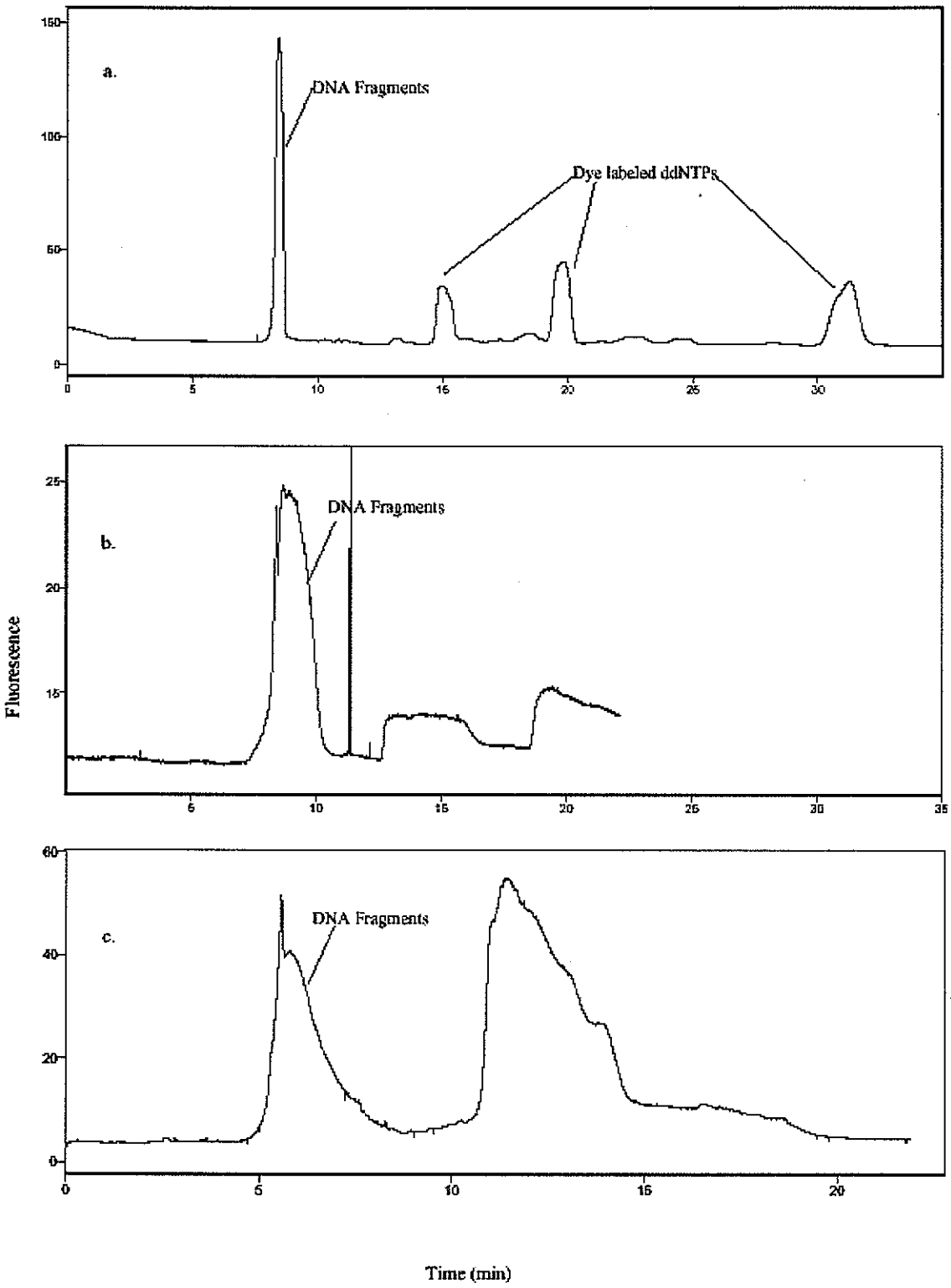

a.
DNA Fragments
Dye labeled ddNTPs
b.
DNA Fragments
c.
DNA Fragments
Fluorescence
Time (min)

FIGURE 5

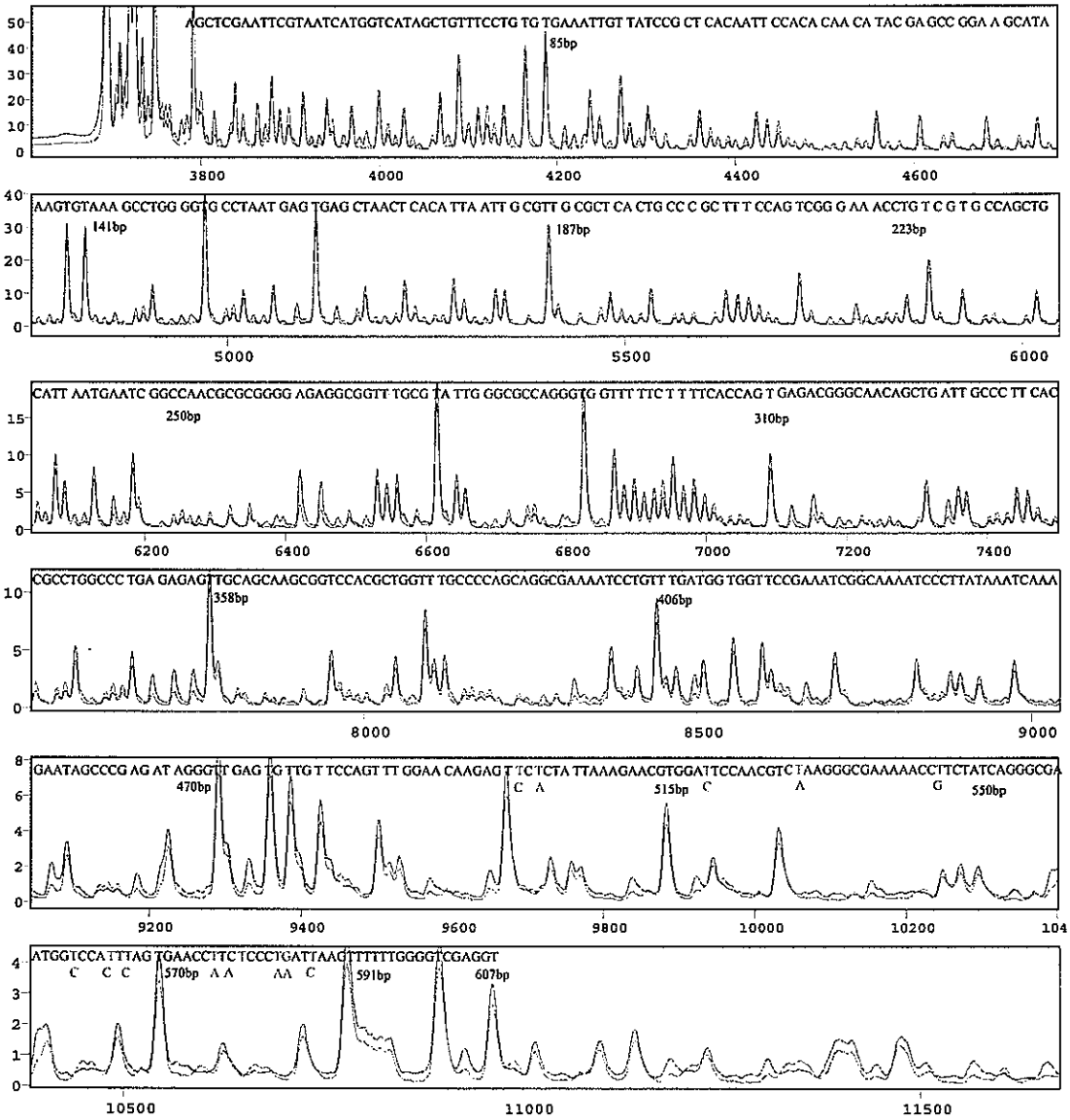

85bp
141bp
187bp
223bp
250bp
310bp
358bp
406bp
470bp
515bp
550bp
570bp
591bp
607bp

FIGURE 6

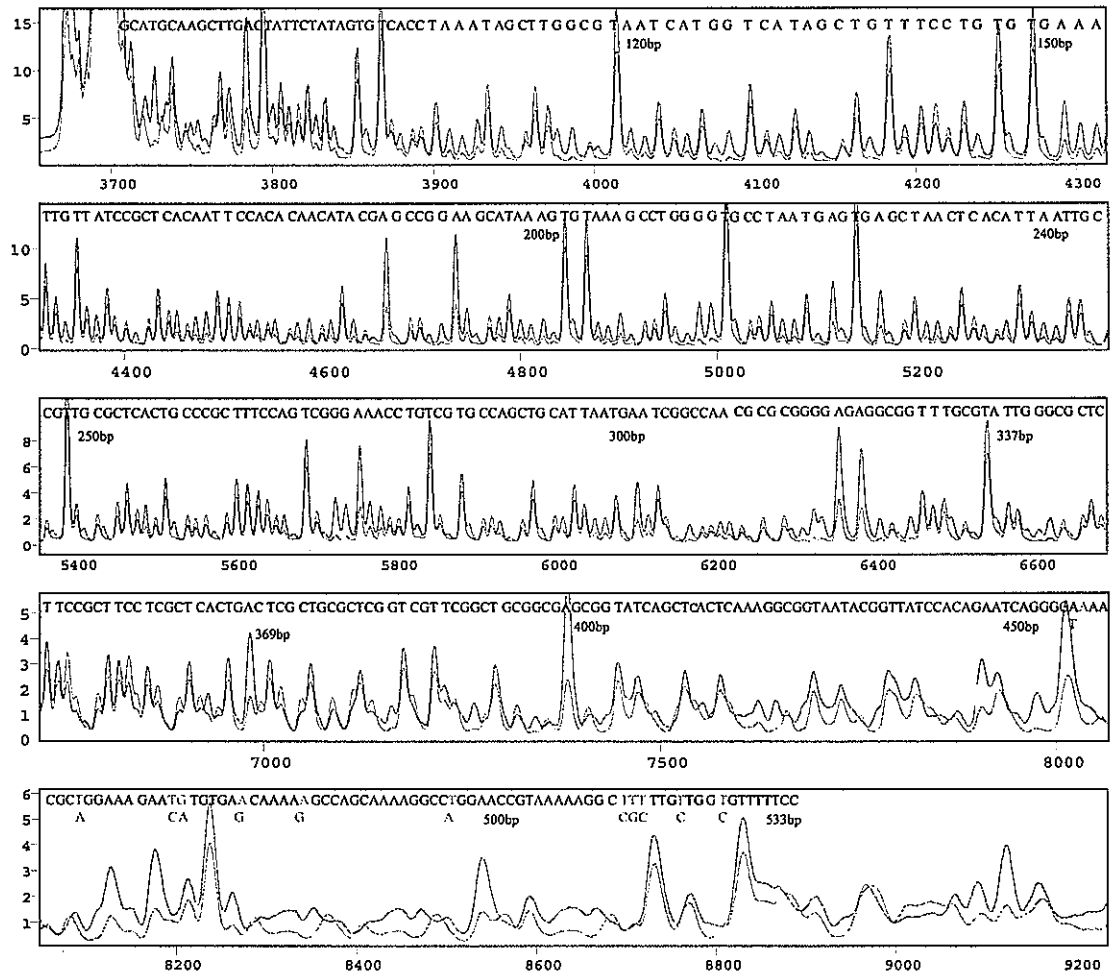

GCATGCAAGCTTGACTATTCTATAGTG TCACCTAAATAGCTTGGCG TAAT CAT GG T CA TAG C T GT T TCC TG TG TG A A A
120bp
150bp
3700
3800
3900
4000
4100
4200
4300
TTG TT ATCCGCT CACAAT T CCACA CAACATA CGA G CCG G AA GCATAA AG TG TAAA G CCT GG G G TG CC T AA T G AG TG A GC T AA CT C ACA T T A ATTG C
200bp
240bp
4400
4600
4800
5000
5200
CGTTG CGCTCACTG CCCGC TTTCCAG TCGGG AAACC TGTCG TG CCAGCTG CAT TAATGAA TCGGCCAA CG CG CGGGG AGAGGCGG T T TGCGTA TTG GGCG CTC
250bp
300bp
337bp
5400
5600
5800
6000
6200
6400
6600
T TCCGCT TCC TCGCT CACTGAC TCG CTGCGCTCG GT CGT TCGGCT GCGGCGAGCGG TATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGAAAA
369bp
400bp
450bp
7000
7500
8000
CGCTGGAAA GAATG TGTGAA CAAAAA GCCAGCAAAAGGCCTGGAACCGTAAAAAGG C TTT TTGTTG G TGTTTTTCC
A
CA
G
G
A
500bp
CGC
C
C
533bp
8200
8400
8600
8800
9000
9200

FIGURE 7

CHAPTER 6. GENERAL CONCLUSIONS

Massively multiplexed capillary electrophoresis with laser induced fluorescence detection is a proven high-throughput analysis technique for DNA sequencing. But continuous improvements in performance and reduction in cost are imminent to make it a true state-of-art technique.

The work in this dissertation has described the development of a novel approach to laser-beam step-scanning fluorescence excitation and single-element detection for multiplexed capillary electrophoresis. Uniform separation efficiency and good signal to noise ratio were obtained. The detection limits achievable for this approach were similar to those of single-capillary fluorescence detection. Advantages include the efficient utilization of light due to the high duty-cycle of step scan, good detection performance due to the reduction of stray light, ruggedness due to the small mass of the galvanometer mirror, low cost due to the simplicity of components and flexibility due to the independent paths for excitation and emission. With slight modification of the setup, two-dimensional laser scanning excitation was easily achieved to accommodate different excitation wavelengths for multicolor excitation. Successful high throughput CGE DNA sequencing were demonstrated.

A simple but reliable microthermocycler which is capable of cycle-sequencing amplification of both ss- and ds-DNA was developed. The reaction volume for DNA sequencing sample preparation was successfully dropped to nano-liter regime, which was comparable to the subsequent separation analysis. Integrated close system was constructed including online reaction, purification and gel separation, which was ready for multi-capillary high throughput operation.

For the first time, we also performed parallel CZE and MEKC on the same 96-capillary array instrument originally developed for DNA analysis. Without the use of the sieving matrix and multiple color detection, the experiment is in fact much simplified. In this way, the throughput of CE was improved 100 fold. The use of two internal standards greatly improved the reproducibility of multiplexed CE and made it reliable enough for most industrial and pharmaceutical applications.

For the future efforts, several other interesting applications can be envisioned based on the multiplexed capillary electrophoresis and nano-liter integrated sample preparation system such as genotyping, peptide mapping, and combinatorial screening. One area that is of great interest is single-cell analysis with the ultimate goal to screen cell populations for disease markers. If the proper injection scheme could be achieved, such as by coupling a capillary array with flow cytometry, this concept may eventually be suitable for clinical diagnosis.

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this thesis.