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Radial Capillary Array Electrophoresis Microplate and Scanner for High-Performance Nucleic Acid Analysis

Yining Shi,[†] Peter C. Simpson,[†,§] James R. Scherer,[†] David Wexler,[†,||] Christine Skibola,[‡] Martyn T. Smith,[‡] and Richard A. Mathies*,[†]

Chemistry Department and School of Public Health, University of California, Berkeley, California 94720

The design, fabrication, and operation of a radial capillary array electrophoresis microplate and scanner for high-throughput DNA analysis is presented. The microplate consists of a central common anode reservoir coupled to 96 separate microfabricated separation channels connected to sample injectors on the perimeter of the 10-cm-diameter wafer. Detection is accomplished by a laser-excited rotary confocal scanner with four color detection channels. Loading of 96 samples in parallel is achieved using a pressurized capillary array system. High-quality separations of 96 pB*R*322 restriction digest samples are achieved in <120 s with the microplate system. The practical utility and multicolor detection capability is demonstrated by analyzing 96 methylenetetrahydrofolate reductase (MTHFR) alleles in parallel using a noncovalent 2-color staining method. This work establishes the feasibility of performing high-throughput genotyping separations with capillary array electrophoresis microplates.

As the Human Genome Project enters its large-scale production stage and more and more genes are being mapped and sequenced, the demand for high-throughput, high-performance and cost-effective nucleic acid analysis technologies is increasing.[1] Traditionally, automated slab gel electrophoresis has been used for DNA sequencing, gene mapping, and disease diagnosis.[2] More recently capillary electrophoresis (CE) has been recognized as a powerful method for DNA fragment sizing and sequencing[3−6] because it provides rapid, high-resolution, and sensitive separations.[7−9] To address the throughput challenges, capillary-array electrophoresis (CAE) systems have been developed and applied to DNA fragment sizing and genomic sequencing.[10−16] However, as the number of capillaries in an array increases, it becomes more difficult to manufacture and manipulate the array; furthermore, electrokinetic sample injection in CE is inefficient, sensitive to the presence of salt and template, and biased against longer fragments.[17−19] Even more advanced approaches to high-throughput nucleic acid analysis are needed.

In 1992, Manz and co-workers[20] introduced the use of photolithographic microfabrication techniques to produce CE separation channels. Since then microfabricated CE devices have been used to perform separations of fluorescent dyes,[21,22] fluorescently labeled amino acids,[23−25] DNA restriction fragments,[26,27] PCR

* Corresponding author: (tel.) 510-642-4192; (fax) 510-642-3599; (e-mail) Rich@zinc.cchem.berkeley.edu.

[†] Chemistry Department.

[‡] School of Public Health.

[§] Present address: Kiva Genetics Inc., 2375 Garcia Ave., Mountain View, CA 94043.

[||] Present address: GeneTrace Inc., 1401 Harbor Bay Parkway, Alameda, CA 94502.

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products,[26,28] short oligonucleotides,[29] short tandem repeats,[30] and DNA sequencing fragments.[17,31,32] Furthermore, integrated microdevices have been developed that can perform PCR amplification followed by amplicon sizing[33] and DNA restriction digestion followed by size-based separation.[27] However, to make these individual analysis devices practically useful and cost-effective for high-throughput nucleic acid analyses, the microfabrication of high-density array structures that can perform parallel analyses of multiple samples is essential.

Over the past few years, we have developed several generations of CAE microplates that can analyze multiple samples in parallel.[26,34−36] In our 48-channel/96-sample CAE microplate design, 96 DNA samples can be injected, separated, and detected in less than 8 min using a laser-excited galvoscanner for detection.[36] Because of the rectilinear channel configuration of this 48-channel/96-sample microplate, there were some design and operation limitations. These limitations included the risk of cross contamination between samples due to the serial analysis of two different samples on the same channel,[28] the reduced sample analysis rates resulting from serial injection, the temporally irregular and inefficient reciprocating galvoscanner sampling, and the turns in the separation channels that can reduce resolution.

We present here a 96-channel radial CAE microplate design together with a novel four-color rotary confocal fluorescence detection system that addresses the limitations of our earlier CAE design. The radial CAE microplate (see Figure 1) has a common anode reservoir in the center of a circular 10-cm-diameter wafer and an array of 96 channels extending outward toward injector units at the perimeter of the wafer. The rotary confocal detection system consists of a rotating objective head coupled to a four-color confocal detection unit.[7] To facilitate rapid parallel loading of 96 samples into the radial CAE microplates, we have developed a 96-capillary array loader. The sensitivity and analytical performance of this system are evaluated using fluorescent dyes and restriction digest standards.

To illustrate the practical utility of the radial CAE microplate analysis system for large-scale genetic analyses, we have examined the C677T polymorphism in the methylenetetrahydrofolate reductase (MTHFR) gene that encodes a protein critical in the regulation of folate and methionine metabolism.[37] MTHFR catalyzes the irreversible reduction of 5,10-methylenetetrahydrofolate

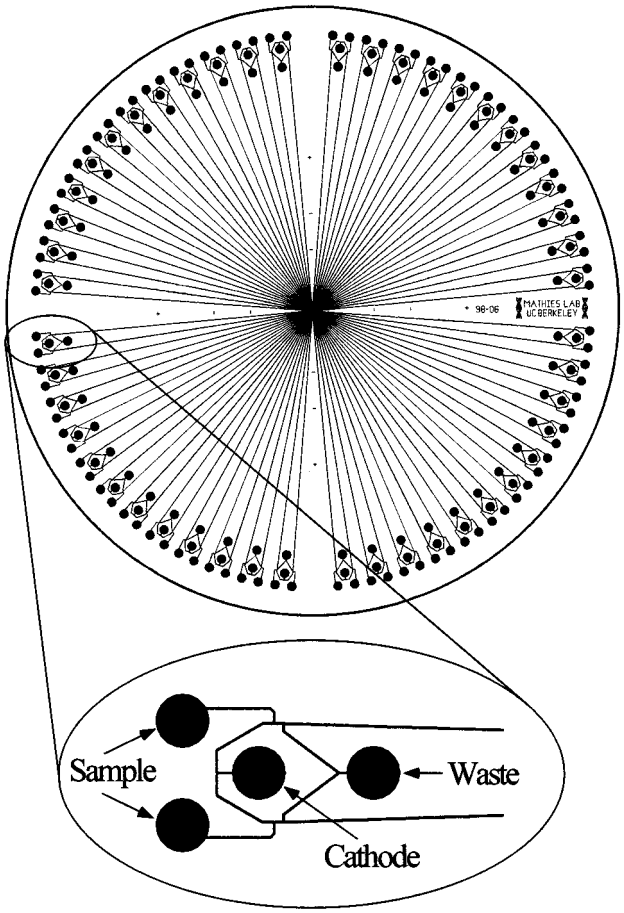

Figure 1. Mask pattern for the 96-channel radial capillary array electrophoresis microplate. Separation channels with 200-μm twin-T injectors were masked to 10-μm width and then etched to form 110-μm-wide by ~50-μm-deep channels. The diameter of the reservoir holes is 1.2 mm. The distance from the injector to the detection point is 33 mm. The substrate is 10 cm in diameter.

which is the predominant circulatory form of folate and a carbon donor for the remethylation of homocysteine to methionine.[38] This C-to-T (alanine to valine) base pair substitution has been linked to an increased risk of neural tube defects, hyperhomocysteinemia, and occlusive vascular disease.[39−43] Previous studies have shown that as many as 15% of individuals are homozygous for the 677T variant, which results in significantly reduced levels of enzyme activity.[44,45] We show here that 96 MTHFR alleles can be sized against an internal standard in less than 100 s on CAE microplates

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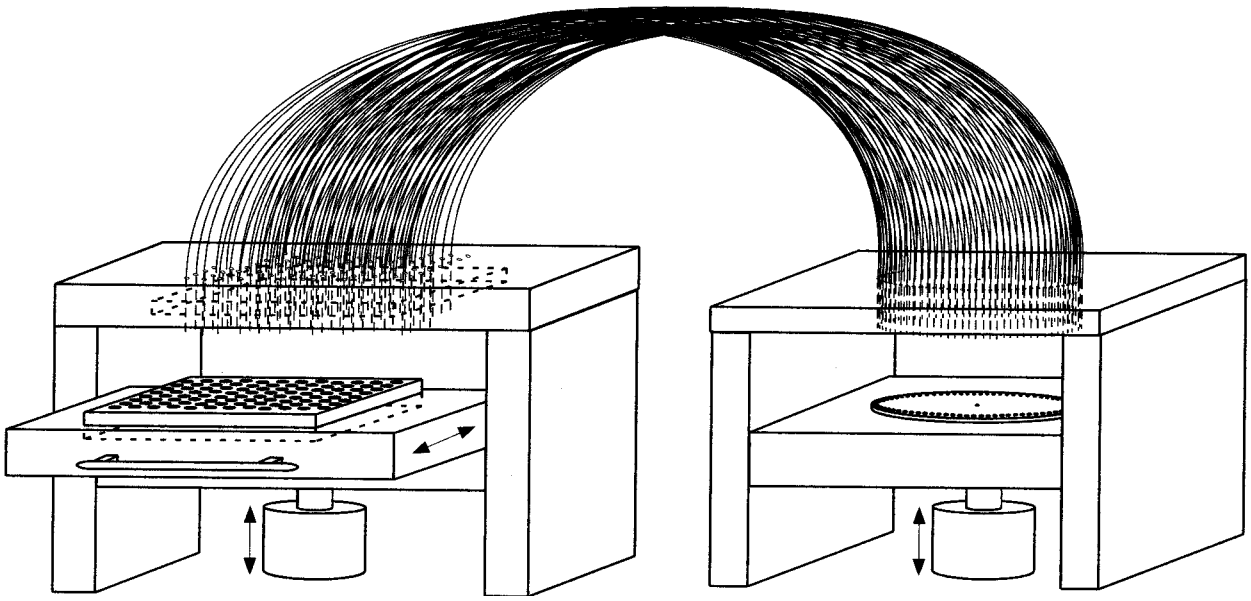

Figure 2. Schematic of the 96-sample capillary array loader. Pressurization of the microtiter dish chamber to 21 kPa (~3 psi) is used to transfer 96 samples (transferred volume ~1 μL) to the sample reservoirs of the radial microplate.

using a two-color, noncovalent bisintercalation dye labeling method.

EXPERIMENTAL SECTION

Microfabrication and Microplate Design. Capillary array electrophoresis microplates were fabricated at the U. C. Berkeley Microfabrication Laboratory as described previously.[35,36] The mask design used to fabricate the radially symmetric channel structure on 10-cm-diameter Borofloat substrates is presented in Figure 1. Channels were masked to 10-μm width and then isotropically etched to form 110-μm wide by ~50-μm deep channels. The distance from the 200-μm twin-T injector to the detection point is 33 mm. The channels extend radially out from a 2-mm-diameter central anode reservoir and are grouped into four 24-channel quadrants. The repeated unit consists of two channels with independent 1.2-mm-diameter sample reservoirs and common cathode and waste reservoirs. All reservoirs were formed by diamond drilling the substrate before bonding. Grouping the anode, cathode, and waste reservoirs reduces the number of reservoirs per plate to 193. This design was chosen over previous rectilinear designs[34,36] because the radial design is easier to lay out, and it does not contain turns that can reduce resolution.[46] Furthermore, the radial design facilitates the use of a rotary confocal scanner that has improved data-acquisition characteristics compared with reciprocal scanners (see below).

Capillary Array Loader. It was necessary to develop a loading system that can transfer samples from the rectilinear format of microtiter plates to the radial microplate. A schematic of our capillary-array loading device is presented in Figure 2. Capillaries, aligned to the microtiter plate reservoirs with a manifold, extend to a second manifold aligned with the sample wells in the radial microplate. Each of the 96 quartz capillaries (72-μm i.d., 200-μm o.d., Polymicro, Phoenix, AZ) has a length of 46 cm with a total internal volume of 1.77 μL. The tips of the capillaries extend into the wells of the microtiter dish and into the reservoirs of the CAE microplate. After introducing the microtiter dish and the microplate into their holders, a pneumatic system drives the microtiter dish and the microplate upward, putting the respective reservoirs in contact with the capillary tips. An O-ring seals the chamber holding the microtiter dish, and pressure is used to drive the samples through the capillary-array system. Depending on the pressure used (12.4−32.7 kPa or 1.80−4.75 psi) the system can deliver sample at rates from 2.1 to 5.4 μL/min with a relative standard deviation of 3−4% of the collected volumes ($N = 24$). These delivery rates were determined by weighing the amount of water delivered over six capillaries at four different pressures (1.8, 2.75, 3.6, and 4.75 psi). This loading system is similar in concept to a device described earlier by Panussis et al. for loading DNA sequencing samples on slab gels.[47] The loader can be rinsed and regenerated by flushing the capillaries with water for ~2 min followed by pressurizing the chamber with air to displace the remaining water.

Sample Preparation. DNA samples were prepared using the polymerase chain reaction (PCR) followed by digestion with *Hin*fI to detect the C677T allelic variation in the MTHFR gene. Blood was collected by venipuncture, and DNA was isolated using proteinase K treatment, followed by phenol/chloroform extraction and ethanol precipitation. The genotyping protocol was adapted from Frosst et al.[44] for the analysis of the C-to-T (alanine to valine) transversion at position 677 of the MTHFR gene. Briefly, 0.5−2.0 μg of human genomic DNA was amplified with the forward primer 5′-TGAAGGAGAAGGTGTCTG-CGGGA-3′ and the reverse primer 5′-AGGACGGTGCGGTGAGAGTG-3′. The thermal cycling conditions were a 2-min denaturation period at 94 °C and 40 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. This was followed

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by a 7-min extension at 72 °C. The 50-μL PCR reaction mixture contained 10 mM Tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg/mL gelatin, 200 μM each dNTP, 50 ng of each primer, and 2.5 unit Taq DNA polymerase (Sigma Chemical Co., St. Louis, MO). *Hinf*I digestion buffer (2.5 μL) and 10 units of *Hinf*I enzyme (Boehrniger Mannheim, IN) were added to 25 μL of the PCR product. The mixture was incubated at 37 °C for at least 2 h. Following restriction digestion, the samples were desalted for 30 min using 0.10-μm pore dialysis membranes (Millipore, Bedford, MA) floating on deionized water in a beaker placed in an ice bath.

DNA-dye complexes were prepared using methods described in the literature.[48,49] Restriction digests of pB*R*322 *Msp*I (New England Biolabs, Beverly, MA) were diluted to 10 ng/μL in 1/100 X TAE buffer (0.4 mM Tris, 0.4 mM acetate, 0.01 mM EDTA, pH 8.2) for use as a stock solution. Samples for one-color pB*R*322 *Msp*I fragment sizing were formed by mixing 10 μL of the 10 ng/μL stock with 90 μL of 63 nM thiazole orange homodimer (TOTO) solutions at a ratio of 1 dye/25 bp DNA. For two-color MTHFR genotyping, 10 μL of 10 ng/μL pB*R*322 stock was pipetted into 90 μL of 63 nM thiazole orange thiazole indolenine heterodimer (butyl TOTIN) and the resulting DNA-TOTIN complexes used as a standard sizing ladder. The MTHFR PCR product was prelabeled with TOTO by adding 1 μL of the dialyzed PCR mixture (1:4 dilution) into 9 μL of 63 nM TOTO solution. All preformed DNA-dye complexes were incubated in the dark for 30 min at room temperature before electrophoresis. Samples for two-color genotyping were made by mixing the butyl TOTIN-prelabeled pB*R*322 *Msp*I ladder with the TOTO-prelabeled PCR product (1:1 v/v) in a 96-well microtiter dish prior to loading.

Electrophoresis. The separation buffer contained 1.0% (w/v) hydroxyethylcellulose (HEC) in 1 X TPA-TAPS (80 mM tetrapentylammonium 3-{tris(hydroxymethyl)methylamino}-1-propanesulfate, 1 mM EDTA, pH 8.4).[48] The use of this TPA buffer minimizes exchange of the noncovalent dye labels. The channels of the CAE microplates were coated with a thin layer of polyacrylamide using the Hjerten protocol.[50] The channels were filled with HEC buffer by pumping the HEC into the anode reservoir using a syringe. The HEC in the sample reservoirs was then removed manually by aspiration, and the 96 samples were loaded simultaneously using the loading system described above.

The loaded microplates were placed onto the microplate holder on the rotary scanner, and electrical contact to the four types of reservoirs was achieved in-parallel using a circular electrode array plate. The circular electrode array was made by placing an array of stainless steel wires through a 6-mm-thick Plexiglas ring and wiring all common wires in-parallel to the four high-voltage power supplies (Stanford Research Systems, Series PS300, Sunnyvale, CA). A computer program written in LabView (National Instruments, Austin, TX) was used to automatically time and switch the appropriate voltages. Injection was performed for 50 s by applying +100 V to the anode, sample, and cathode reservoirs and +150 V to the waste reservoirs. Separation was carried out immediately following injection by applying +1100 V (~200 V/cm) to the anode reservoir, +100 V to the cathode reservoir, and +270 V to the sample and waste reservoirs.

Radial Microplate Scanner. To detect fluorescently labeled DNA fragments that are separated on the 96-channel radial microplate, a laser-excited confocal fluorescence scanner with a circular scanning path was constructed (Figure 3). The radial design has the fundamental advantage that high scan rates can be achieved with high positional accuracy and speed uniformity. In overview, the exciting laser beam is reflected by a dichroic beam splitter and mirror into the bottom of a vertical rotating hollow shaft. At the top of the shaft, the beam is displaced 1 cm with a rhomb prism after which it is focused into the channels by a microscope objective. Fluorescence from the sample is collected by the microscope objective at full aperture and passed along the reverse optical path to a four-color confocal fluorescence detector.[7]

The hollow shaft of the scanner is supported by two high-precision bearings b_1 and b_2 that are anchored to a support structure (not shown). The shaft is coupled by means of a timing belt B to a solid shaft that is supported by similar bearings b_3 and b_4 and connected to a microstepping motor (Zeta 6104 indexer and 57-83 motor, Compumotor, Rohnert Park, CA) driven at 50 000 pulses/rev. A scan head assembly is connected to the upper end of the hollow shaft. The assembly contains a rhomboid prism (Precision Optical, Costa Mesa, CA, 1-cm-square aperture, parallel beam displacement of 1 cm) with antireflection-coated entrance and exit faces and a microscope objective (20 X, NA 0.5, Rolyn Optical, Covina, CA). The radial microplate is supported on a holder with three 80 pitch thread micrometer screws that rest in kinematic grooves in a supporting platform. The screws allow us to match the microplate plane with that of the objective focal plane. One end of a fiber optic (200-μm diameter, Edmund Scientific, Barrington, NJ), housed in a stainless steel hypodermic needle, is positioned between channels of the microplate in the scan path as an optical trigger. The other end of the fiber is coupled to a photodiode that starts data acquisition when the focused beam hits the fiber optic.

A 488-nm beam from an Ar^+ laser (2017, Spectra Physics, Mountain View, CA) is aligned to pass through an aperture which can be moved along a rail r_1 between positions a_1 and a_2 along the *y* direction and spaced apart by ~60 cm. A 45° dichroic beam splitter (505DRLP, Omega Optical, Brattleboro, VT) deflects the laser beam to mirror m_2. Translations of the dichroic mount t_{1y} and mirror mount t_{2x} position the beam on the center of the bottom end of the rotating hollow shaft. Orthogonal adjustments on m_2 center the beam at the upper end of the shaft, thereby ensuring that the beam is centered on the aperture of the objective. In the alignment process, an aluminized glass plate is placed in the microplate holder and adjusted so that the laser beam returns on itself through aperture a_1. The return beam passes through the dichroic and is centered on the first and last apertures, a_3 and a_4, of the four-color detector by adjusting mirrors m_3 and m_4. The four-color detection system consists of four sequential confocal PMT modular units and is capable of measuring fluorescence from four spectral regions as described earlier.[7]

The detection sensitivity was determined by flowing fluorescein solutions through the microchannels. The extrapolated limit of detection in 1 X TAE buffer (pH 8.2) was determined to be 10 pM (S/N = 2) at a laser power of 70 mW. The photophysical parameters of this scanner may be estimated using a formalism previously introduced for optimizing fluorescence detection.[51] The

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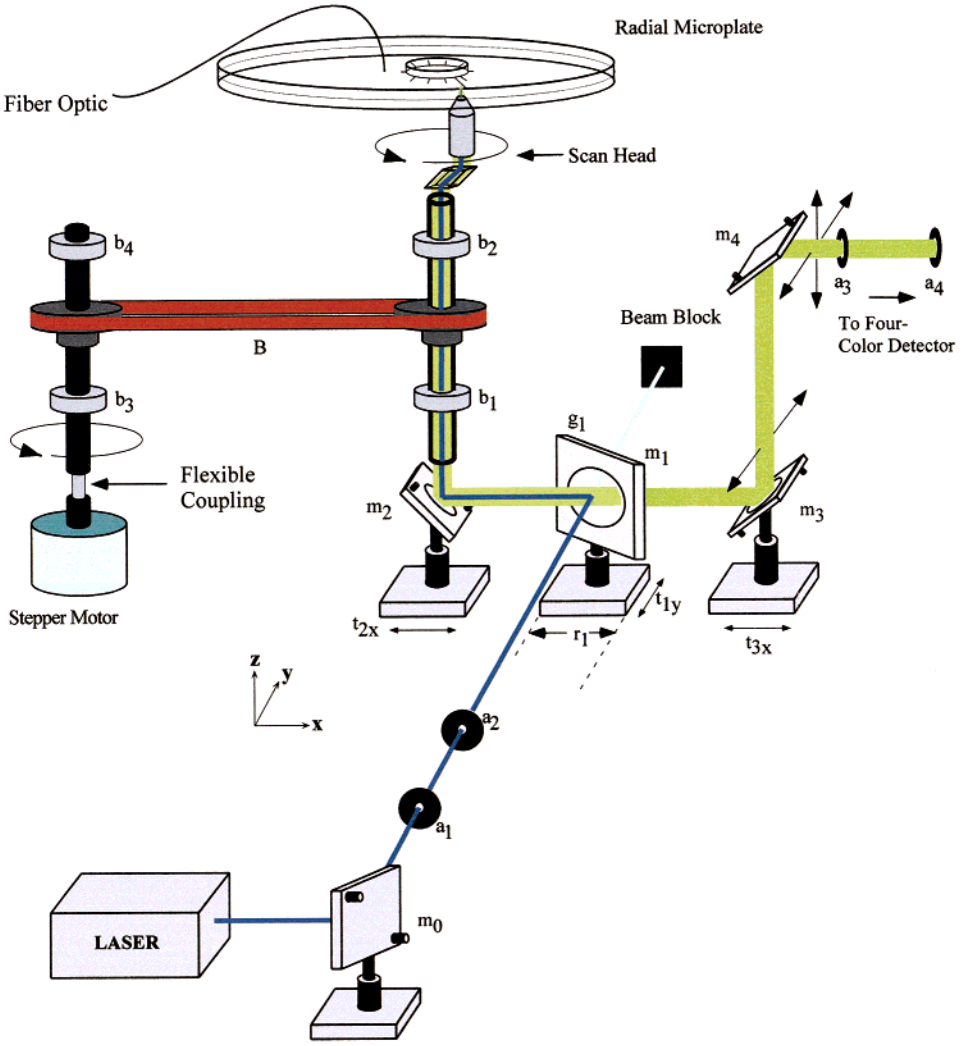

Figure 3. Schematic of the rotary confocal-fluorescence scanner used to detect separations on the radial CAE microplate. This scanner, which utilizes four independent analog-to-digital converters to simultaneously acquire four-color data, collected 2880 data points/revolution/color at 86.80 μs intervals (4 Hz rotation rate) giving a pixel spacing of 21.8 μm.

beam waist inside the microchannel is estimated to be ~3 μm in diameter. The depth of field is ~±4.5 μm, so the effective illumination volume is a cylinder ~5 μm in diameter and ~9 μm high. The transit time t_t of the beam moving at ~25 cm/s (4 Hz scan rate) across this volume is ~20 μs. The cross-sectional area of the illumination cylinder is ~20 μm^2, and the illumination intensity I is ~8.6 × 10^{23} photons/cm^2 s. The rate of photon absorption for fluorescein is given by $k_a = \sigma_a I = 2.6 \times 10^8$ photons/s, where σ_a is the absorption cross section (3.04 × 10^{-16} cm^2). Taking the rate of fluorescence decay k_f to be 2.3 × 10^8 /s and the quantum yield for photodestruction Φ_{pd} to be 2.7 × 10^{-5}, the photodestruction lifetime $\tau_d = 1/k_d = 1/k_f\Phi_{pd} = 164$ μs, where k_d is the rate of photodestruction. The dimensionless transit time $\tau = \tau_t/\tau_d \approx 0.12$ and the dimensionless illuminated intensity $\kappa = k_a/k_f \approx 1$. Since the optimum operating parameters occur when κ and τ are both ~1 this indicates that the parameters are near optimum for the scanner.[51] To maximize the fluorescence collection we use 150-μm detection confocal pinholes. The four-color detector has an image magnification of 12 so it collects fluorescence from a 12-μm-diameter cylindrical volume that completely includes the exciting laser beam and the whole channel depth. For the fragment sizing experiments reported here, the velocity of an average 180-bp fragment is ~470 μm/s. In the time of one revolution (0.25 s) this fragment band moves ~115 μm while the laser spot only samples ~5 μm of the band. Although only 4% of the molecules in the band volume are irradiated, the band shape is well sampled with 4−5 data points at the 4 Hz scan rate. Sampling of as much as 20% of the molecules in such a band would be achieved at 20 Hz scan rates with a corresponding reduction in the τ value.

Data Acquisition. The velocity of the stepper motor and data collection rate must be set to values that are consistent with the electrophoretic fragment velocity. The data acquisition board (2400a, Microstar Laboratories, Bellevue, WA) has four independent 12-bit ADCs with adjustable sampling times in increments of 0.05 μs. In this work, only one channel of the four-color detection system was used to acquire data for DNA fragment sizing, while two channels were used for MTHFR genotyping. The electrophoretic runs described here were obtained by scanning

(51) Mathies, R. A.; Peck, K.; Stryer, L. *Anal. Chem.* **1990**, *62*, 1786−1791.

at 4 Hz which allows us to collect 2880 data points/revolution/color giving a data rate of approximately 11.52 kHz/color. The distance moved by the laser beam in one sample time is 21.8 μm which gives ~5 data points across a 110-μm-wide channel. The signals from the photomultipliers were filtered with a 5.76 kHz low-pass filter (model SR640, Stanford Research Systems, CA) prior to digitization with the 12-bit Microstar ADC. The data were logged on a PC as a 16-bit TIFF image, and electropherograms were generated in IPLAB (Signal Analytics, Vienna, VA) by averaging data points across each channel. Software to permit the automatic reduction of these image files on the scanner is in development. Although a 4 Hz sampling rate was used for this work, the scanner is capable of collecting data at any rate up to 20 Hz.

RESULTS AND DISCUSSION

Figure 4 presents the image and electropherograms of a separation of 96 pBR322 *Msp*I samples labeled with TOTO using the 96-channel radial CAE microplate and the rotary confocal scanner. The horizontal direction indicates the migration time, while the vertical direction indicates the channel number. The image consists of four groups of channel images corresponding to the four quadrants of grouped channels. All 96 samples were loaded onto the radial microplate with our capillary array loader in ~20 s and successfully injected. The microchannel separations were detected without any cross-talk between adjacent channels because of the excellent spatial resolution of the confocal detection system. The 17 fragments that make up the pBR322 *Msp*I-TOTO complex samples were all resolved on the expanded 16-bit TIFF image and on the electropherograms in these 120-s separations. Baseline resolution of the 180- and 190-bp fragments was achieved, the resolution of the 190- and 201-bp fragments was 0.8 ± 0.14 (N = 96), and the resolution for the 238- and 242-bp fragments was 0.68 ± 0.12 (N = 96).

The signal intensities of identical fragments separated on the microplate were relatively consistent from channel to channel; the relative standard deviation for the least intense 67-bp peak was 35.2%, while that for the most intense 527-bp peak was 24.7% (N = 96). This channel-to-channel intensity variance most likely arises from the nonuniform sample amounts injected as a result of inhomogeneities of the HEC matrix in the injector wells. The injection, separation, and detection of all 96 samples were complete in 170 s, corresponding to a separation analysis rate of 1.8 s/sample. The migration times for a given DNA fragment depended upon the channel; for example, the migration times exhibited a relative standard deviation of 3.9% for the 180-bp fragment and 8.0% for the 622-bp fragment (N = 96). We believe that this relatively large variation in migration time from channel to channel is due to nonuniform positioning of the array electrodes in the relatively small (1.2-mm-diameter by 1.0-mm-deep) injector reservoirs, as well as the aforementioned inhomogeneities of the HEC matrix. However, the 4−8% channel-to-channel variation in migration time for individual channels is not a limitation when performing multicolor genotyping analyses or DNA sequencing since, as is the case in conventional CAE-based DNA analyses,[14] each channel is internally referenced to a migration standard.

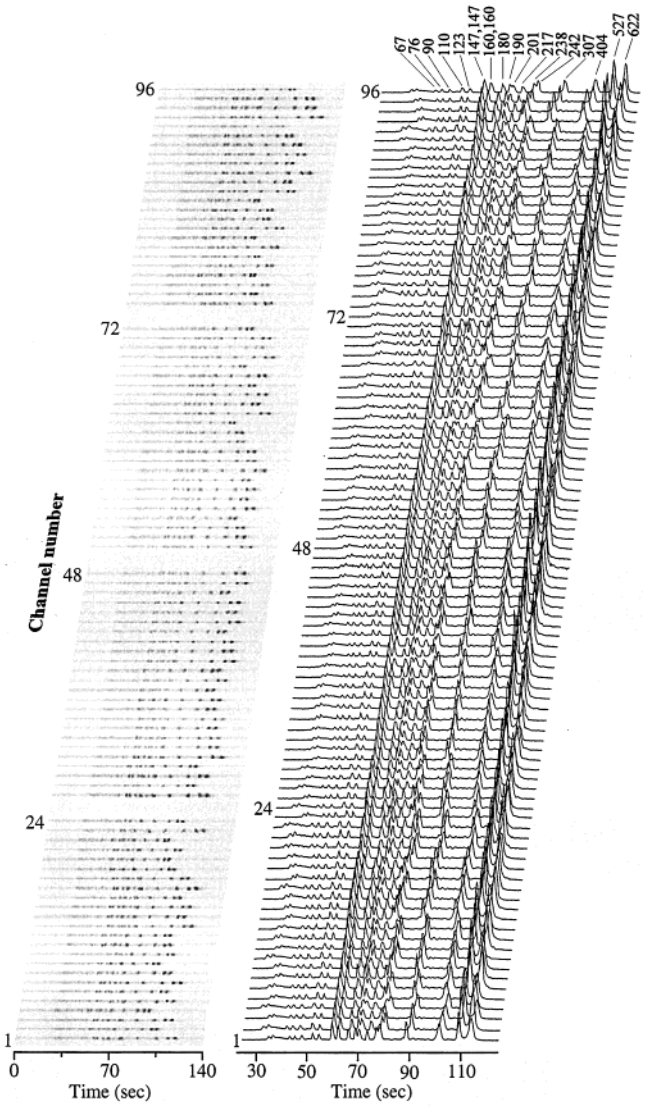

Figure 4. Image and electropherograms of a separation of 96 pB*R*322 *Msp*I-TOTO complex samples with the 96-channel CAE radial microplate and scanner. The separation buffer contained 80 mM TPA-TAPS, 1 mM H_2EDTA, pH 8.4, 1.0% (w/v) HEC. The 96 samples (TOTO/DNA = 1:25, DNA concentration = 1 ng/μL) were loaded simultaneously using the 96-capillary loading assembly prior to injection. Injection electric field = 100 V/cm. Separation electric field = 200 V/cm. Fluorescence was detected from 510 to 540 nm. The numbers at the top indicate the fragment sizes in base pairs.

To demonstrate the capabilities of the 96-channel radial microplates and the rotary scanner for rapid, high-throughput multiplex detection, we performed an analysis of methylenetetrahydrofolate reductase (MTHFR) allelic variation. PCR amplicons of the MTHFR gene were digested with *Hinf*I to assay for the C-to-T substitution at nucleotide 677. In this assay, the 677C allele gives a product migrating at 198 bp while the 677T variation introduces a *Hinf*I restriction site, reducing this fragment size to 175 bp. Figure 5 presents electropherograms of 96 MTHFR samples genotyped using the 96-channel radial CAE microplate. The red signal (>645 nm) comes from the pBR322 *Msp*I standard ladder that was prelabeled with TOTIN, and the green signal (510−540 nm) comes from the MTHFR samples prelabeled with TOTO. The 96 pBR322-TOTIN and MTHFR-TOTO complex samples were mixed (1:1 v/v) and loaded simultaneously on the

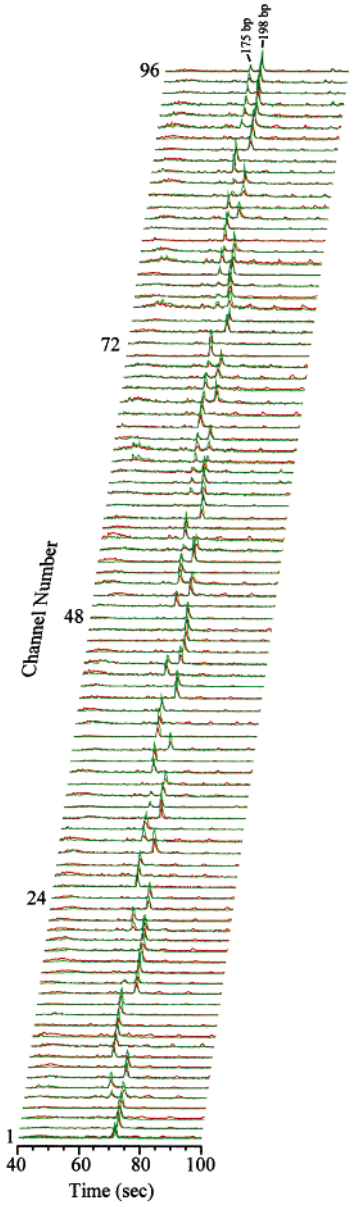

Figure 5. Electropherograms of 96 MTHFR samples genotyped using the 96-channel radial CAE microplate with two-color fluorescent labeling and detection. The red signal (>645 nm) is from the pB*R*322 *Msp*I standard ladder and the green signal (510−540 nm) is from the MTHFR samples. The pB*R*322 *Msp*I (1 ng/μL) standard was prelabeled with butyl TOTIN (1dye:25 bp), and the MTHFR DNA (1:4 dilution) was prelabeled with TOTO. The 96 pB*R*322-TOTIN and MTHFR-TOTO complex samples were mixed (1:1 v/v) and loaded simultaneously on the microplate using the loading array prior to injection. Other conditions are as in Figure 4.

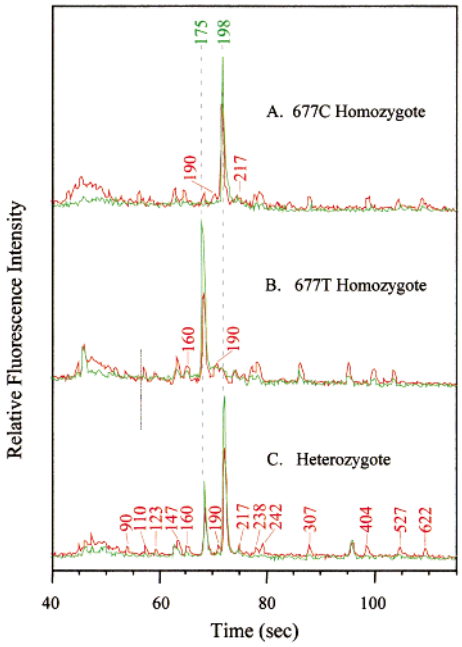

Figure 6. Representative electropherograms of three MTHFR samples from Figure 5 illustrating the different genotypes. (A) Genotype of a homozygous 677C allele (ala/ala). (B) Genotype of a homozygous 677T allele (val/val). (C) Heterozygous genotype (ala/val).

microplate using the 96-capillary loading array prior to injection. The green MTHFR peaks were easily identified in all 96 electropherograms on the basis of their characteristic green-to-red fluorescence ratio (1.8 ± 0.3, N = 129). The resolution of the MTHFR separations is more evident when selected electropherograms are expanded as shown in Figure 6. The green 198-bp MTHFR peak in electropherogram A overlapped with the 201-bp standard peak and migrated between the 190- and 217-bp standard peaks. This band was thus assigned as the 198-bp homozygous MTHFR 677C genotype. The green 175-bp peak in electropherogram B overlapped with the 180-bp standard peak and migrated between the 160- and 190-bp standard peaks. This band was thus typed as the 175-bp homozygous MTHFR 677T genotype. In electropherogram C, green MTHFR bands are seen at both 198 and 175 bp indicating that this sample is from a heterozygote.

These MTHFR fragments were sized using local linear regression on the basis of the linear correlation of the fragment size and the migration time over the fragment size range 90−242 bp. With this linear regression method, the green MTHFR peaks in Figure 6 were determined to be 199 bp for electropherogram A; 178 bp for electropherogram B; and 178 and 198 bp for electropherogram C. Sizing determinations using linear regression analysis for the 96 MTHFR samples analyzed in Figure 5 gave: 199 ± 1 for the 677C samples; 177 ± 1 for the 677T samples; 177 ± 1 and 199 ± 1 for the heterozygotes. The results performed with the microplates agree with conventional agarose slab gel electrophoresis experiments in all cases. In the slab gel experiments, the digestion products were visualized after electrophoresis on a 4% agarose gel with ethidium bromide.

In summary we have presented the design, construction, and evaluation of a radial 96-channel CAE microplate and rotary confocal fluorescence scanner for rapid, high-throughput, and high-sensitivity nucleic acid analysis. Using a one-color detection mode, fragment sizing of 96 pBR322 *Msp*I samples was achieved in <2 min. Two-color multiplex sizing of 96 MTHFR samples was also completed in <100 s. This separation sample throughput of 0.56−0.64 samples/s (including 50 s for injection) is an order of magnitude better than the throughput of our 12-channel design[34] and five times better than that obtained with our 48-channel design.[36] Compared with our previous 12-channel and 48-channel designs, the 96-channel radial microplate has several advantages which contribute to the improved performance for nucleic acid analysis. First, the symmetric radial layout allows us to use a rotary confocal scanning detection system capable of interrogating the

microplate from below with a rotating objective. This inverted format facilitates access to the microplate to introduce samples, reagents, electrodes, etc. A major advantage of this design, in comparison with our previous reciprocal mechanical and galvo scanning systems,[34,36] is that measurements can be made on each channel of the microplate at a regular time interval and with a very high sampling rate. Indeed, measurements can be made on as many channels as can be packed into the 6.28-cm circumference. Since 96 channels only occupy ~15% of the total beam path, there is ample space for more channel lanes with no change in the data acquisition rates of the detection system. For example, our experience indicates that a channel spacing of 163 μm (100-μm channel + 63-μm glass separator) is practical, which would allow us, in principle, to detect as many as 384 channels. The fabrication and operation of such a chip will require a design with a practical reservoir layout. Second, although it is feasible to use multiplex injection methods to increase the total number of samples analyzed per microplate,[36] the radial design, using one sample reservoir connected to each separation channel, minimizes the possibility of cross contamination between samples. Cross contamination between multiple-injected PCR samples was observed in a CE microdevice with a single separation channel layout.[28] Third, the radial design also has the aforementioned advantage of a simple and symmetric layout as well as the elimination of turns that can reduce separation performance. Finally, compared with the 0.33 NA galvo scanning system we used earlier, the radial scanner has improved light-gathering capability because of its NA 0.5 objective.

The radial microplate design incorporates additional improvements that make this system more practically useful for high-throughput operation. (1) The 96 different samples prepared in a conventional 96-well microtiter dish can be simultaneously loaded into the radial microplates in under 20 s with the capillary-array loader. Compared with manual loading, the parallel loader minimizes and equalizes evaporation and gives more uniform sample concentrations. (2) The 192 reservoirs in the injector units are electrically addressed using a single circular electrode array plate. (3) The common anode facilitates cleaning, regeneration, and matrix-filling of all 96 channels in parallel. With these procedures, the microplates were found to be reusable for as many as ~100 runs without noticeable deterioration in separation performance.

Recently, Ulvik et al.[52] reported genotyping analyses of MTHFR polymorphisms using multiple-injection capillary electrophoresis and one-color laser-induced fluorescence detection. With a single capillary, they showed that as many as 10 samples can be serially injected and separated in ~16 min, corresponding to a separation throughput of 0.01 sample/s. In comparison, our two-color results show a >50-fold improvement in separation throughput to 0.5−0.6 sample/s. With appropriate engineering, it should be possible to operate our system with a separation throughput of 96 samples in under 30 min which translates to more than 4600 analyses per day. Even higher throughputs are possible by employing size and color multiplexing.

The loader, microplate, and scanner presented here comprise a microfabricated DNA analysis system that is capable of performing ultrahigh-throughput genotyping. In the future, the evaluation of the phenotypic effects that are linked to the MTHFR C677T polymorphism and other polymorphisms important in health care and pharmaceutical development will require large-scale epidemiological studies to determine allelic frequencies in various populations. Our analysis of 96 samples in less than 90 s per microplate demonstrates the power of microfabricated CAE devices for practical, large-scale, and high-performance nucleic acid based epidemiological studies.

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