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Side-entry excitation and detection of square capillary array electrophoresis for DNA sequencing

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Abstract

In high throughput DNA sequencing based on capillary electrophoresis, efficient coupling of the laser to each capillary is a challenge. Our group previously reported two multiple point irradiation schemes. The present work describes a more efficient excitation and detection method in which the laser light propagates through the capillary array without undergoing a serious reduction in power. An array of square capillaries (340 μm O.D.×75 μm I.D.) was sandwiched between two fused-silica plates with an index-matching solution in between. The light was directed into the channel across the capillary array from the side. DNA sequences of PGEM/U from 24 capillaries were obtained even with a relatively low-power laser. The excitation scheme can be scaled up to hundreds of capillaries to achieve high-speed, high-throughput DNA sequencing, genetic typing and drug screening.

Keywords: Detection, electrophoresis; Instrumentation; DNA

1. Introduction

The challenge of the Human Genome Project is to increase the sequencing efficiency by 100 times over the existing state of the art in order to complete sequencing of the human and possibly other model organisms' genomes by the early 2000 [1]. Several technologies, such as capillary gel electrophoresis (CGE) [2–10], single-molecule sequencing [11–13], sequencing by hybridization [14–17], and sequencing by mass spectrometry [18–31] emerged as a result of this initiative. Among these, CGE technology is expected to play an integral role in achieving the designated goal of the Human Genome Project.

A number of properties of CGE, such as superior heat dissipation and short running time, make CGE a good choice over conventional slab-gel methods. The sequencing chemistry, tagging chemistry and separation mechanism are analogous to those in slab-gel systems. The use of new gel matrices [32–37] (linear polymer solutions) has overcome problems originally encountered with cross-linked gels. Even though a 25-fold increase in speed [38] has been obtained for a single capillary, much effort has been focused on developing multiple capillary systems to increase sample throughput. Several types of capillary array systems were proposed. In one configuration [4–6], a scanning confocal fluorescence excitation and detection system was developed and demonstrated in a 24-capillary array system. In another configuration [7,8], a multiple sheath flow system was used for a 20-capillary array. Other schemes include a multiple point excitation system [2,3] and a side-entry excitation system [9,10]. Another interesting format is the use of a machined channel [39,40] The capillary array system is presently the most mature format for DNA sequencing.

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In a previous experiment [9], we developed a side-entry laser irradiation and detection system for multiple capillaries in which the capillaries were immersed into water to roughly match the refractive index n of the capillaries. The reduction of the laser beam intensity along the beam path due to reflection at the capillary walls and refraction of the cylindrical columns is suppressed. Scattered light incident on the detector is also reduced because detection is performed perpendicular to the direction of propagation of the laser light and because the inter-capillary refractive index, which causes specular scattering, is matched by water. The success of this excitation geometry for a 96-capillary array depends on the packing quality, the refractive index of the gel matrix, the fused-silica and the surrounding medium outside the capillaries.

Recently, Anazawa et al. [10] reported a computer simulation for the side-entry excitation and detection scheme and evaluated the transmittance as a function of the refractive index. Where the capillaries are placed in air, the laser beam passes through the cores of all capillaries because each capillary acts as a convex lens focusing the laser light repeatedly when $2<R/r<6$ (R is the O.D. of the capillary and r is the I.D. of the capillary).

However, when the capillaries are placed in air, the laser power reduction by reflection is significant. The incident laser power is reduced by 7% after each capillary. The tenth capillary will receive about 50% of the laser power of the first capillary. At the 32nd capillary, the attenuation of the laser power is 10-fold. Higher laser powers can be used to achieve good S/N for the last capillary. However, the first capillary will have a large signal and the dynamic range of the detector is then the limiting factor. Also, the array has to be packed well so that the centers of the capillaries lie along one plane.

Here, we report the results of side-entry excitation and detection for a square capillary array. The use of larger rectangular capillaries for electrophoresis was introduced in 1937 [41]. But only until recently the application of rectangular capillaries for electrophoresis received some attention [42–46]. These published works demonstrate some advantages of rectangular capillaries over cylindrical capillaries, namely that a wider capillary increases sample capacity and provides better sensitivity for pathlength dependent detection systems (absorption, fluorescence). To our knowledge, there are no reports of CGE performed in square capillaries, especially for multiple-capillary DNA sequencing. The square channel shape is also the norm for microfabricated devices [39,40].

2. Experimental

2.1. Reagents and samples

The buffer in all the experiments was 1×TBE with 3.5 *M* urea (pH~8.3). The 1×TBE buffer solution was prepared by dissolving a pre-mix (Amerosco, Solon, OH, USA) of 89 m*M* tris(hydroxymethyl)aminomethane (Tris), 89 m*M* boric acid, and 2 m*M* ethylenediaminetetraacetic acid (EDTA) in deionized water. Urea was from ICN Biomedicals (Aurora, OH, USA). Poly(ethylene oxide) (PEO) was obtained from Aldrich (Milwaukee, WI, USA). A mixture of 0.15 g of M_r 8 000 000 and 0.14 g of M_r 600 000 PEO was dissolved in a 20-ml glass bottle with 10 ml of 1×TBE with 3.5 *M* urea. The heating and stirring block was set at the highest speed for 4 h, at which time the magnetic stirring bar in the buffer can hold a vortex. The solution was kept stirring slowly until a clear and thin gel solution was obtained. PGEM/U DNA samples were obtained from the Nucleic Acid Center of Iowa State University (Ames, IA, USA).

2.2. Capillary array preparation

Capillaries for each experiment (square prototype, 75 μm I.D.×340 μm O.D., Polymicro Technologies, Phoenix, AZ, USA) were cut from the same batch of capillary. The total length of the capillary is 65 cm with 50 cm effective length. Locations for the detection windows were marked. The polyimide coating of the window region was removed with a heating coil of chromel (0.007 in. in diameter; 1 in.=2.54 cm), which was connected to a laboratory-made low-voltage high-current power supply. The capillaries were packed side-by-side and clamped down between the two flat surfaces of a plastic holder. Five-minute epoxy (ESCO Products, Oak Ridge, NJ, USA) was used to fix the capillaries. The array was transferred to the top of a 2.5-cm wide,

5.0-cm long and 2-mm thick fused-silica plate which functioned as the bottom plate. Two pieces of 2.5-cm wide, 1-cm long and 1-mm thick glass were placed above the capillary array, with the window region open. The space between the two plates and the capillary array at each side of the window were sealed with optical glue (No. 61, Edmund Scientific, Barrington, NJ, USA). After exposure to UV light (Cole-Parmer Instruments, Chicago, IL, USA, model 9815-series lamps) for several hours, the cement cured and the array became fixed between the two plates. Two small thin fused-silica plates (1 mm) were then attached to the bottom plate along the window region to provide an optical interface for laser entry and to form a shallow well to hold the index-matching liquid (R.P. Cargille Labs., Cedar Grove, NJ, USA). The window region was covered with a 2.5-cm long, 2.5-cm wide, 150-μm thick fused-silica plate. The array was fixed in a way that it could be rotated and adjusted up and down for alignment with the laser beam.

2.3. Optics

The schematic diagram of the fluorescence-based multiple square capillary electrophoresis system is shown in Fig. 1. An argon ion laser (Coherent, Santa Clara, CA, USA, Model Innova 90) operating at 514.5 nm was used for excitation. The laser beam was focused at the center of the array by a 10-cm lens (Edmund Scientific). Two 'dummy' capillaries, which were separated by about 1.5 cm and placed on each side of the array, were filled with rhodamine-6G-spiked gel matrix. These were used to adjust the coupling of the laser beam into the array. The laser power was measured by using a power meter (Melles Griot, Irvine, CA, USA, Model 13PEM001). Fluorescence from DNA bands in each capillary was monitored simultaneously with a charge-coupled device camera (CCD, AT-200. Photometrics, Tucson, AZ, USA). A wide-angle camera lens (Nikon, Japan, Model Nikkor AF, 70-mm diameter, 28-mm f.1.) was attached to the CCD to provide a large solid angle for efficient light collection. A holographic notch-plus filter at 514.5 nm (Kaiser Optical System, Ann Arbor, MI, USA) was inserted behind the wide-angle lens to eliminate the excitation laser light. An image splitter, made of a 610- or a 630-nm long pass filter (Edmund Scientific) and a quartz plate that compensates for the optical pathlength, was placed

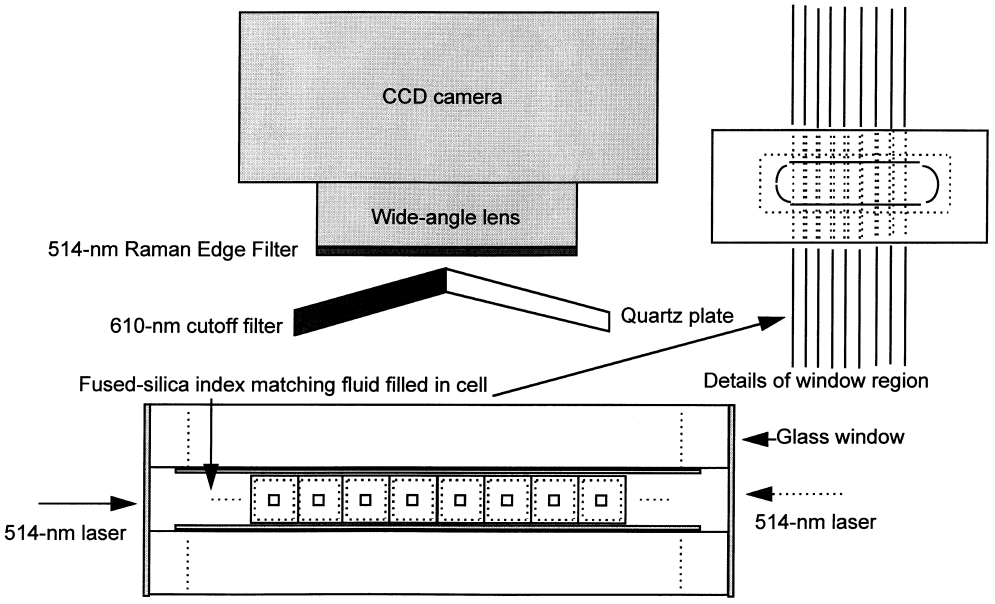


Fig. 1. Schematic diagram of the laser excitation and CCD detection system for square-capillary array DNA sequencing.

before the wide-angle lens. The fluorescence image from each capillary was thus split into two channels. The image from the 610- or the 630-nm filter was the red channel while the image from the quartz plate was the green channel.

2.4. Refractive index measurements

A Bausch & Lomb refractometer (Fisher Scientific, Pittsburgh, PA, USA) was used to measure the refractive index of the gel matrix. Refractive index varies slightly with the temperature. Thus, temperature was maintained at 25°C for the experiment using a waterbath. For refractive index measurement, the gel solution was sandwiched between two glass plates. The two glass plates were cleaned with ethanol between each measurement. Distilled water was used to calibrate the refractometer. A refractive index of 1.3335 was found, which compares well with the literature value of 1.3330 for room-temperature water [47]. A separation matrix composed of 1.5% M_r 8 000 000, 1.4% M_r 600 000 PEO in 1×TBE with 3.5 *M* urea was then tested. Two batches of each gel were tested twice; the average result of the refractive index was 1.366.

2.5. Detection of PGEM/U DNA fragments

PGEM/U DNA was used as the standard DNA sample to test the integrated system of excitation, detection, sample handling and injection. Before filling the capillaries with gel, the capillaries were flushed with methanol. Before DNA samples were injected, electrophoresis was pre-run for 10 min. DNA sequencing samples were prepared from the Sanger reaction according to the standard protocols (ABI, dyeDeoxy terminators and cycle sequencing with Taq polymerase). Each tube of dried PGEM/U sample was resuspended in 4 μl of formamide–10 m*M* EDTA (5:1, v/v) solution, heated at 95°C for 4 min to denature and put into a −10°C freezing well to cool down rapidly. The parameters such as laser power, exposure time, aperture size of the camera lens, electric field strength for injection and separation are described for each experiment below.

3. Results and discussion

3.1. Theoretical considerations

Improved excitation efficiency is expected using square capillaries for the side-entry excitation scheme (Fig. 1). The lens effect from the cylindrical capillary walls does not exist in the square capillary system. Capillaries are immersed in index matching fluid (Zeiss). This further reduces stray light arising from refraction that occurs at the interface of the two media. At the same time, laser intensity loss was minimized. The refractive index for the gel used here is 1.366. The refractive index of a fused-silica plate is 1.458. By using the side-entry excitation scheme, the reduction in laser power after each capillary is:

$$R = 2(n_1 - n_2)^2/(n_1 + n_2)^2 = 0.00212 \quad (1)$$

The transmitted light after *n* capillaries is $(0.99788)^n$. The trend of laser power reduction in this system when each capillary is filled with PEO gel is shown in Table 1. The 500th capillary still receives 34.6% of the laser power received by the first capillary. A thousand capillaries can thus be excited simultaneously in this system by coupling the laser power from both sides of the cell. Theoretically, the square capillary is therefore the ideal choice for large array DNA sequencing from the excitation and detection points of view. The same holds true for channels in microfabricated devices.

Table 1
Laser power available for the *n*th capillary in the arrangement in Fig. 1 when each capillary is filled with PEO gel (1.5% M_r 8 000 000, 1.4% M_r 600 000 PEO)

*n*th Capillary	Laser power (*P*)
1	1.000
10	0.979
50	0.899
100	0.809
200	0.654
300	0.529
400	0.428
500	0.346

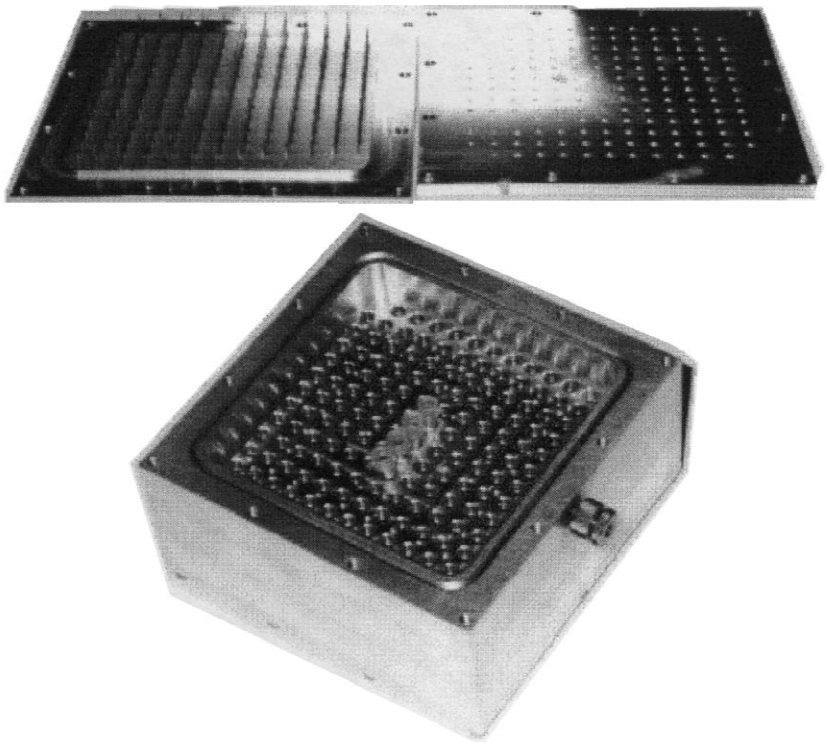


Fig. 2. Photograph of multiple capillary injection device. Top left: 96-needle plate; top right: plate for 96-well microtiter plate; and bottom: injection block including heat sink.

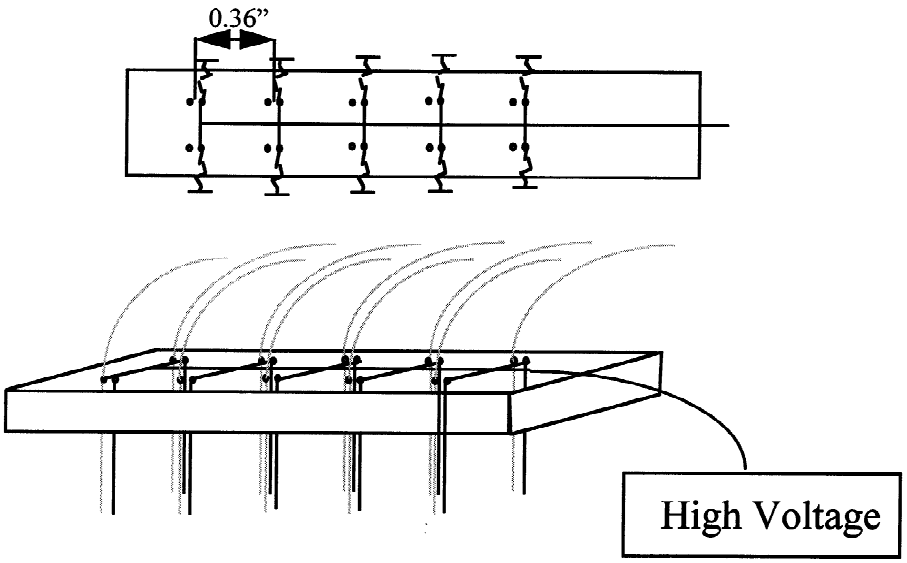


Fig. 3. External electrodes injection device for ten capillaries.

3.2. Injection

In order to demonstrate the efficiency of the excitation scheme proposed for multiple capillary DNA sequencing, we must first establish that sample injection is implemented properly. A scheme (Fig. 2) for individual injection of 100 samples was tested. The device is composed of three parts. The bottom section is an electrode plate. One-hundred needles built on an aluminum plate acted as the individual electrodes. Each needle punched through each sample vial from the bottom for injection. In the middle are the vial holders. The top plate with small holes can be used to hold the capillary array and works as the guide for inserting individual capillaries into each vial. A demonstration of four-capillary simultaneous injection using this device was first performed. The results (data not shown) show successful sample introduction into all four capillaries with uniform signal levels. After each run, the lower block can be washed or a new batch of needles can be utilized for the next experiment in order to prevent cross contamination. The combination of denaturing with hot injection (samples preheated to 85°C) can also be implemented since this device can also function as a heating block.

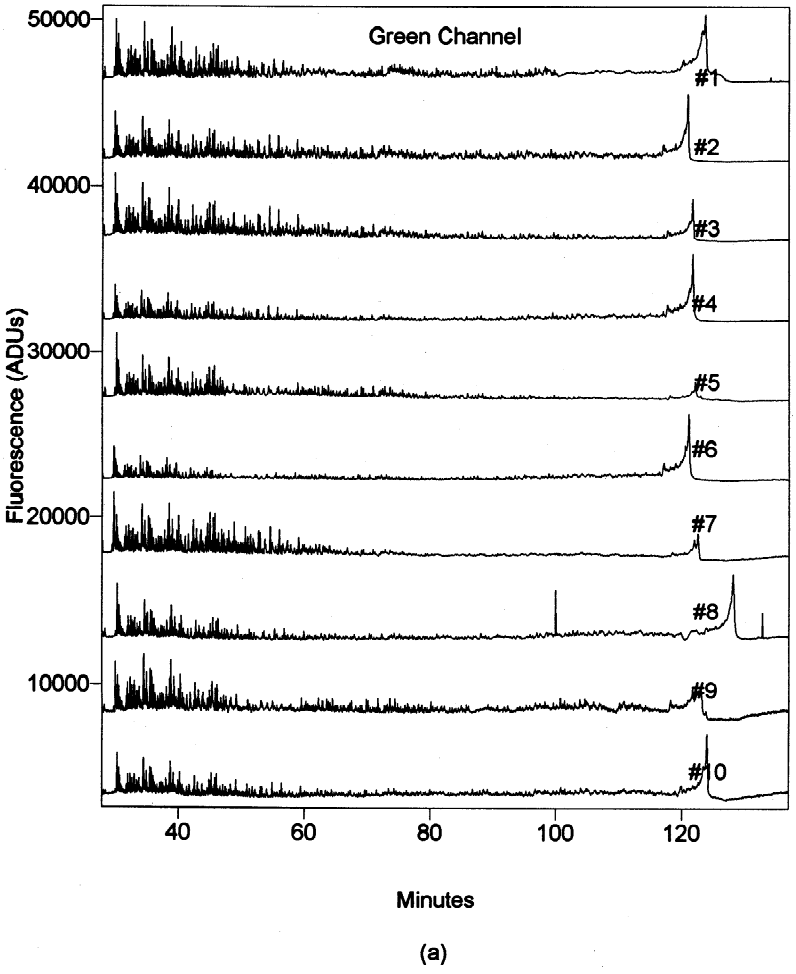
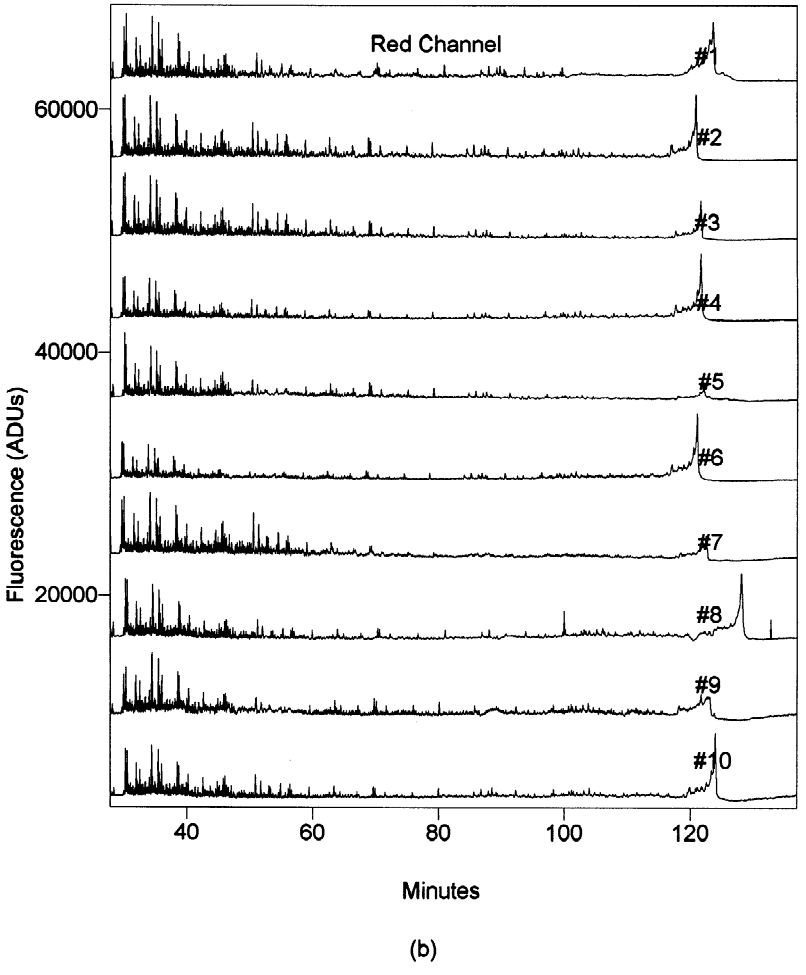


Fig. 4. DNA sequencing results for ten square capillaries. Capillary, 50 cm total length, 38 cm effective length; injection, 60 s at 80 V/cm; electrophoresis, 150 V/cm; laser power, 40 mW; exposure, 400 ms. (a) Electropherograms from the green channel and (b) electropherograms from the red channel.

In order to reduce the amount, and thus the cost, of the DNA samples used, we have chosen to greatly simplify sample injection by using ten electrodes, each for a bundle of ten capillaries, rather than 100 independent electrodes each corresponding to an individual capillary. We expect this simplification to enhance the run-to-run reproducibility, which will further aid in a confident assessment of the optical excitation scheme. The sample introduction scheme is shown in Fig. 3. Ten external electrodes have been connected to the center wire which goes to the power supply. Beside each electrode, there is another hole through which each capillary bundle will go. The spacing is designed in such a way to fit the standard 96-well microtiter plates. Instead of gluing ten capillaries inside a polyether ether ketone (PEEK) tubing for injection as a bundle, capillaries are glued onto the outside surface of a 2-cm long 1/16 in. I.D.× 1/8 in. O.D. PEEK tubing with equal spacing between capillaries. The injection electrode was inserted inside the PEEK tubing. The ten capillaries and lone electrode extended past the tubing. In this way, the PEEK tubing provides an arrangement for handling the capillary array similar to a single capillary in one sample tube. Thus, simultaneous injection of one sample into as many as ten capillaries is achieved without problems such as competition for injection, capillary action or sample depletion problems that could happen when capillaries are bundled together.



(b)

Fig. 4. (*continued*)

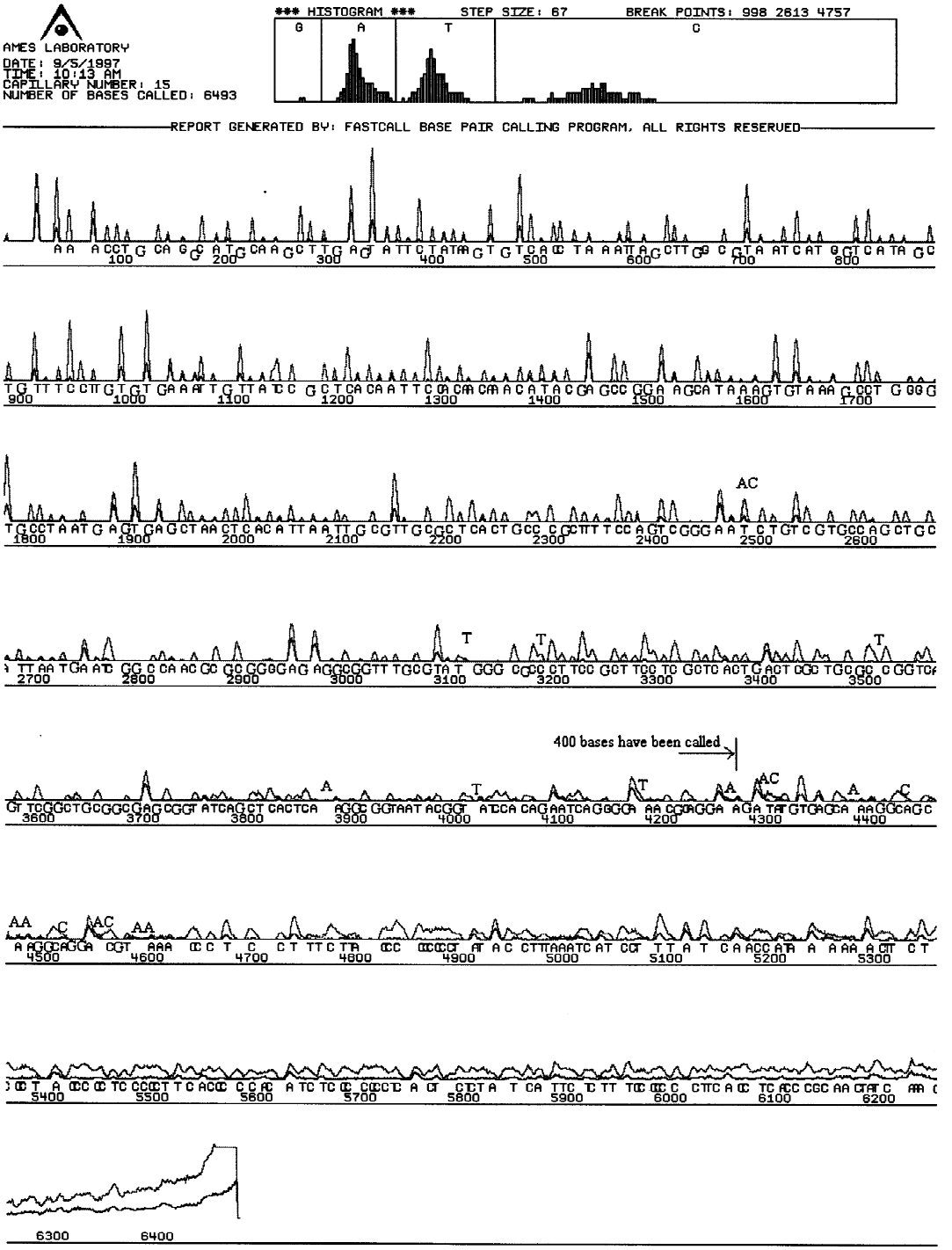


Fig. 5. Base calling on capillary 3 of the ten-capillary run of Fig. 4.

3.3. Square-capillary DNA sequencing

Theoretically, square capillaries is the ideal choice for high-throughput DNA sequencing, especially from the excitation and detection points of view. Several successful runs of DNA sequencing results were obtained using a square ten-capillary array system. The ten capillaries were divided into two

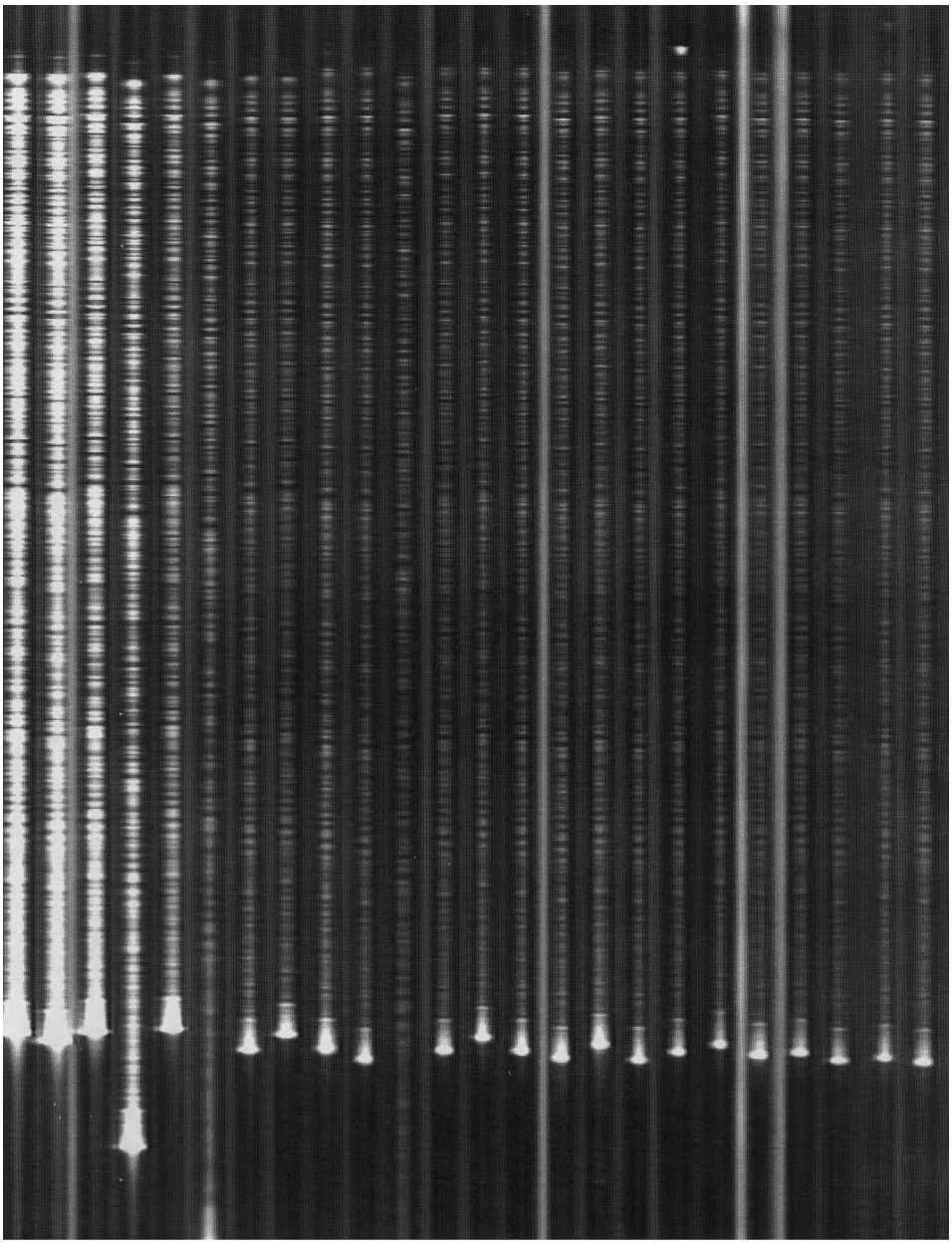


Fig. 6. Image of DNA sequencing results (red channel) for 24 square capillaries. The horizontal direction represents the array. The vertical direction represents migration time from 30 to 150 min. Capillary, 65 cm total length, 50 cm effective length; injection, 60 s at 75 V/cm; electrophoresis, 150 V/cm; laser power, 40 mW; exposure, 400 ms.

groups. Each group was injected separately. The results are shown in Fig. 4. Both S/N and resolution were excellent for this run in all ten capillaries. Base calling was performed on capillary 3 using our laboratory-developed software [48]. More than 400 bases (Fig. 5) were called with an accuracy of 98%. DNA sequencing was then performed in a square 24-capillary array. The 24 capillaries were divided into three groups, each containing eight capillaries. The results for all 24 capillaries are shown in Fig. 6. Each lane of gel image corresponds to one capillary. The first few (left) capillaries have higher intensities because the laser beam was focused on the first capillary. Still, the signal is quite uniform and even the 24th capillary has sufficient S/N for base calling. Capillaries 4, 6 and 11 exhibit longer run times (presumably due to uneven gel filling) but similar separation efficiencies. Base calling was performed using our laboratory-developed software [48]. For a typical capillary, about 300 bases were called with an accuracy of 95%. Higher accuracy and longer reads can be expected if peak deconvolution and more sophisticated software are employed.

The array size has been further scaled up to 100 square capillaries. The 100 capillaries were divided into 10 groups. Each group of 10 capillaries were bundled together at the detection end for gel filling. The results for 12 out of 100 capillaries are shown in Fig. 7. For this 100 square-capillary run, excitation efficiency is quite uniform across the array, which is the key result of the present study. Both the resolution and the S/N are not as good as those in the 10-capillary array. There are several possible reasons. Due to the fragility of the square capillary, the experiment was delayed for about 4 h during the gel filling process with the gel inside most of the capillaries. Since the column is uncoated, the electroosmotic flow is expected to increase over time at these high pH values and cause less sample to be introduced into the capillaries. It was found that for a large capillary array the signal level for simultaneous injection was lower than that of a small capillary array under the same sample introduction conditions. Therefore, longer injection times are needed to achieve similar signal levels. Automation of the sample-introduction process should alleviate these problems. Also, a new manufacturing process has recently been reported that allows square capillaries to be produced with mechanical ruggedness comparable to that of cylindrical capillaries [49].

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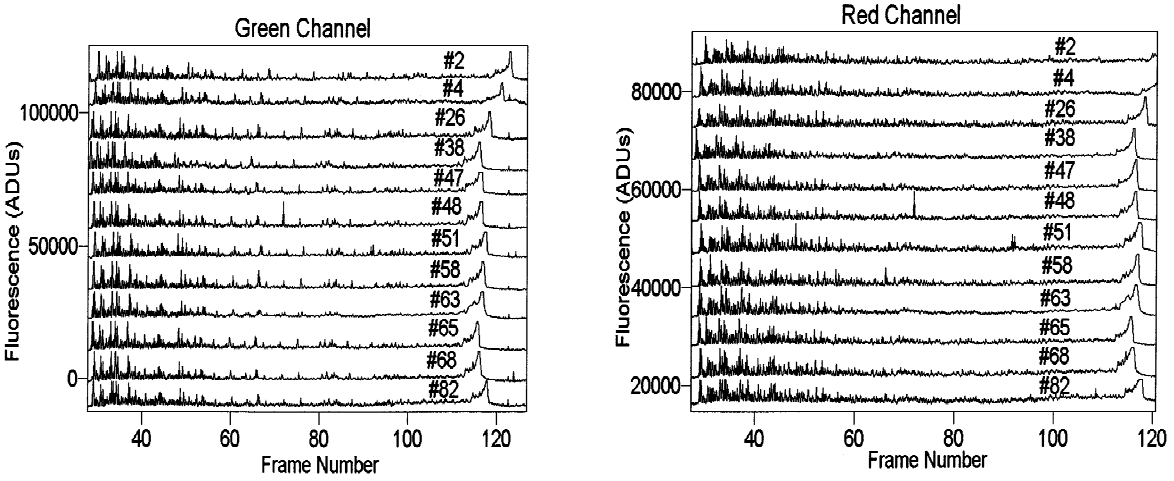

Fig. 7. Two-channel sequencing results from 100 square-capillary array. Conditions are identical to those in Fig. 6 except the exposure time is 300 ms. Twelve electropherograms each from the green (>520 nm, left) and red (>610 nm, right) channels are displayed.

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