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PAPER IN FOREFRONT

# Capillary array isoelectric focusing with laser-induced fluorescence detection: milli-pH unit resolution and yoctomole mass detection limits in a 32-channel system

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**Abstract** We report a multiplexed capillary electrophoresis system employing an array of 32 capillaries with a micromachined sheath-flow cuvette as the detection chamber. The sample streams were simultaneously excited with a 473-nm laser beam, and the fluorescence emission was imaged on a CCD camera with a pair of doublet achromat lens. The instrument produced mass detection limits of 380±120 yoctomoles for fluorescein in zone electrophoresis. Capillary isoelectric focusing of fluorescent standards produced peaks with an average width of 0.0029± 0.0008 pH. Capillary coating stability limits the reproducibility of the analysis.

**Keywords** Capillary electrophoresis/electrophoresis · Fluorescence/luminescence · Bioanalytical methods · Spectroscopy/instrumentation

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## Introduction

Isoelectric focusing is a very powerful tool for the characterization of amphiprotic compounds. Like many other forms of electrophoresis, isoelectric focusing can be performed either on a thin slab or in a capillary. Unlike most other forms of electrophoresis, slab gel isoelectric focusing tends to produce higher resolution separations than capillary isoelectric focusing. Slab gels allow use of immobilized pH gradients, which produces outstanding resolution that approaches 0.001 pH unit [1–3]. Capillary isoelectric focusing (cIEF) usually employs solution-phase ampholytes; the best peak widths have been generated using whole-column imaging detection and are on the order of 0.02 pH units [4–6]. Other capillary isoelectric focusing methods employ a point detector near the end of the capillary; resolution of 0.004 p*I* has been achieved with UV absorbance detection [7].

The performance of isoelectric focusing improves at lower protein concentration by minimizing the disruption of the pH gradient and eliminating protein precipitation [7–8]. However, UV absorbance has limited sensitivity in capillary electrophoresis due to the short optical pathlength. Fluorescence detection of proteins usually requires use of labeling chemistry. Most labeling reactions convert cationic lysine residues to neutral or anionic products, which severely degrades electrophoretic performance [9]. Recently, Wolfbeis reported fluorogenic reagents that convert the cationic lysine residue into a cationic fluorescent product [10, 11]. We have employed these reagents for ultrasensitive fluorescence detection in capillary isoelectric focusing with excellent resolution [12, 13].

Our fluorescence system provided ultrasensitive detection. However, it was based on a single capillary. Samples

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must be analyzed sequentially, which decreases the throughput of the instrument. There is a need to improve throughput for screening applications. Unlike microfluidic devices, which require cumbersome detection systems, capillary array electrophoresis has proved to be well suited to high-throughput analysis. As an example, Mao et al. reported multiple capillary isoelectric focusing based on whole-column imaging [14]. That system used cylindrical optics to disperse a 100-mW excitation laser beam across the array of capillaries. Such geometry is inherently inefficient and produces very low excitation power per capillary. That instrument produced ~100 am mass detection limits for labeled standard proteins. In this paper, we describe a capillary array electrophoresis system that produces over six orders of magnitude superior detection limits. This instrument employed a 10-mW laser beam to excite fluorescence in a sheath-flow cuvette detector. This extremely efficient detector uses a stationary laser beam to simultaneously excite fluorescing sample migrating from each capillary.

## Experimental section

The overall instrument scheme consists of capillaries, injection block, sheath-flow cuvette, excitation source, excitation and emission optics, and a CCD detector. The basic design of the instrument is similar to others reported from this group [15–17].

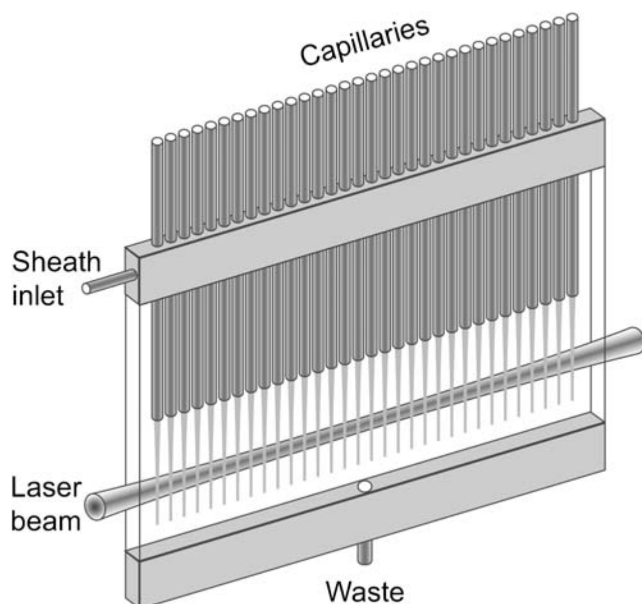


**Fig. 1** Sheath-flow cuvette. An array of 32 capillaries is held in a microfabricated sheath-flow cuvette. Sample migrates from the tips of the capillaries, forming thin filaments in the fluid-filled chamber. A single low-power laser beam simultaneously excites fluorescence from the sample streams

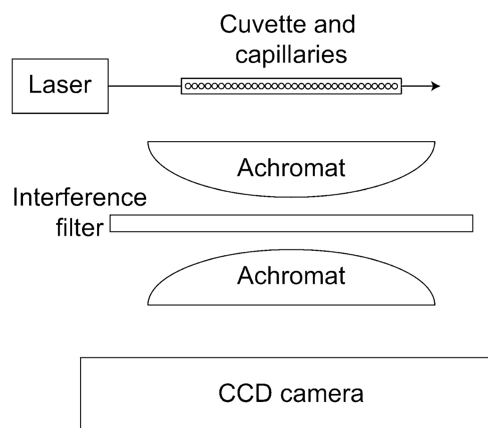


**Fig. 2** Optical design of the instrument. A beam from a solid-state laser is focused into the sheath-flow cuvette shown in Figure 1. Fluorescence is collected and collimated with an achromatic lens, filtered with an interference filter, and imaged onto a CCD camera with an identical achromatic lens

*Sheath-flow cuvette* The sheath-flow cuvette, Fig. 1, is similar to a 16-capillary version developed by this group [16]. Briefly, the cuvette consists of four parts: two identical etched Pyrex pieces that serve as the front and back of the cuvette and two spacers made from 145±3 μm thick microscope cover slips. The identical front and back pieces have a set of long grooves that act as alignment guides to equally space the capillaries. A set of V-shaped stoppers holds the capillary ends uniformly across the cuvette. The cuvette was assembled with UV-curing glue. The glue was illuminated for about 30 min with 18 W 366 nm lamp and cured at 50 °C for 12 h. A wafer-dicing saw was used to trim the ends, which were then polished with diamond paste. The cuvette was held in a home-built stainless-steel fixture.

*Excitation and detection* The optical system is shown in Fig. 2. A 10-mW laser operating at 473 nm was used for excitation of fluorescein, fluorescein-based p*I* standards, and the P503-labeled proteins. A 10-mW laser operating at 532 nm was used for excitation of the tetramethylrhodamine-labeled glycolipids standards.

The beam was focused into the cuvette from the side to simultaneously illuminate each sample stream. The laser beam was focused to a 35-μm spot size with a 1× microscope objective (Melles Griot) in the cuvette. The beam remains reasonably collimated over a distance given by twice the confocal distance ($z_c$) for a Gaussian laser beam,

$$z_c = \pi {\omega_o}^2 / \lambda \tag{1}$$

where $\omega_o$ is the beam waist spot size and $\lambda$ is wavelength. The beam is collimated over 15-mm in this case, which is larger than the length of the cuvette.

The emission channel for this instrument is essentially a monochromatic version employed in our two-dimensional

capillary array detector [17]. Fluorescence was collected at right angles to the laser beam and sample streams with a pair of 50 mm *f*/1 doublet achromat lenses (Melles Griot, USA). The surface closest to the cuvette of the combined lenses was about 50 mm from the cuvette and the back surface was about the same distance from the CCD sensor. The first lens collected and collimated the fluorescence emission while the second imaged the fluorescence onto the camera. A 590DF35 interference filter was used for P503 labeled proteins and fluorescein-based p*I* standards; the same filter was used for tetramethylrhodamine-labeled glycolipids. The filter was from Omega Optical, Brattleboro, BT, USA and was placed between the lenses to block scattered and stray light. The lenses imaged the cuvette onto a monochrome CCD camera (Photometric CoolSNAP $ES^2$) equipped with an interline-transfer progressive-scan image sensor with 1,392×1,040 imaging array of 6.45 μm× 6.45 μm pixels. Image acquisition was performed with commercial imaging software, Metavue v. 7.1 (MDS Analytical Technology, Concord, ON, Canada).

*Capillary preparation* cIEF separations of proteins were performed using a set of 32 cross-linked polyacrylamide coated capillaries, 30 cm long 50/150 μm I.D/O.D. The capillaries were coated with procedures similar to Ramsay et al. [12, 13] using a simple home-built apparatus to purge reagents through the capillaries. In order to improve the coating performance, as reported by Gao and Liu [18], a small percentage ( 0.01–0.02%) of *N*, *N*-methylene-*bis*-acrylamide (BIS) was added to the acrylamide solution for the polymerization reaction. A brief description of the capillary coating procedure is as follows: at 30 psi, eight 2-m long capillaries were simultaneously flushed with 1 M NaOH for 2 h, then rinsed with distilled water for 1 h. Next, 1% (*w*/*v*) acetic acid solution was rinsed through the capillaries for 3 h. A solution containing 1:1 ratio of 3-(trimethoxylsilyl) propyl methacrylate (MAPS) and methanol was purged at 15 psi through the capillary for 1 h. Both ends of the capillaries were placed in the MAPS/ methanol mixture overnight. On the second day, the capillaries were flushed with methanol for 1 h at 15 psi and dried with $N_2$ gas for another 1 h. After the capillaries were dried, a carefully degassed fresh solution of 4% (*w*/*v*) acrylamide and 0.02% (*w*/*v*) BIS, 0.1% (*w*/*v*) TEMED, and 0.1% (*w*/*v*) ammonium persulfate was flushed through the capillaries at 15 psi until the solution was visualized coming out the outlet end. The flow was then interrupted and the capillaries were immediately rinsed and stored in distilled water until use. Zone electrophoresis of fluorescein was performed using a set of 32 uncoated capillaries, 30 cm long 50/150 μm I.D/O.D. Zone electrophoresis of tetramethylrhodamine-labeled lipid standards was performed using a set of 32 uncoated capillaries, 30 cm long 25/150 μm I.D/O.D.

*Samples and reagents* All reagents were purchased from Sigma-Aldrich unless otherwise stated. All solutions were prepared with distilled deionized water and vacuum filtered through a 0.22-μm filter. Chromeo P503 dye was purchased from Active Motif. Uncoated fused silica capillaries were purchased from Polymicro Technologies. Fluorescein-based p*I* standards were graciously provided by the Karel Slais group (Institute of Analytical Chemistry, Academy of Sciences of the Czech Republic) [19]. Tetramethyl rhodamine-labeled lipids standards were provided by Ole Hindsgaul and Monica M. Palcic (Carlsberg Laboratory) [20, 21].

Protein samples were originally dissolved in water at a concentration of 10 mg/mL and stored at −20 °C. New aliquots were taken each day, thawed at room temperature, and labeled with Chromeo P503 dye as previously described [10, 11, 22]. Briefly, 5 μL of protein solution was added to a mixture of 15 μL of 10 mM borate buffer and 5 μL of P503 dye. The labeling reaction took about 25 min at room temperature. At the completion of the labeling reaction, indicated by change in color from blue to pink, 175 μL of 0.1% (*v*/*v*) Tween 20 in 1% (*v*/*v*) Triton X100 solution was added to quench the reaction. For all cIEF studies, lower protein concentrations were made by linear dilution in 4% ampholyte solution containing 2% Pharmalytes 3–10, 0.67% each of Pharmalytes 4–6.5, 5–8, 8–10.5 in 0.1% Tween 20/1% Triton X. Just before a run, protein samples were spiked with fluorescein-based p*I* standards (p*I* 6.5 and 5.5) which were prepared in distilled water.

*Separation procedure* For all separations, the capillaries were cut to 30 cm length and the anode end of the capillaries were placed in a multi-well format injection block connected to a CZE1000R high-voltage power supply (Spellman, Hauppauge, NY, USA). The other ends of the capillaries had the amide coating removed and were inserted into the sheath-flow cuvette until they reach the V-shaped stoppers in the cuvette. The capillaries were sealed in place and a dilute fluorescein solution was passed through the capillaries for alignment of the instrument. During injection, a gold-plated V-bottom multi-well plate was used to hold the samples; a single capillary sits in a single well. Either a similar gold-plated microtiter plate or a 70 mm Petri dish was used to hold the run buffer during separation.

For all cIEF separations, the capillaries were filled with analytes prepared in a 4% ampholyte solution by purging the solution through the capillaries for about 30 s. All cIEF focusing were performed at 20 kV with 10 mM phosphoric acid as the anodic buffer and 40 mM NaOH as the sheath-flow liquid (cathode). The sheath-flow liquid was changed to 20 mM aspartic acid during cIEF chemical mobilization at same electric field strength.

*Data acquisition and processing* Fluorescence images were acquired using Metavue v 7.1. Fluorescence image frames streamed at one binning mode and 300 ms exposure time resulted in 3.3 fps. All 32 spots were captured simultaneously on a single frame. Each frame acquired corresponds to a 0.3-s CCD exposure taken every 0.3 s. Spatially equal regions of interest were defined for the fluorescence spots generated from the sample stream. By choosing binning and exposure time, it is possible to increase the rate of image acquisition and thereby guarantee that even the fastest moving bands are not broadened due to blurring, but a major limitation to that is the computational power of the processing computer. For example, a typical 40 min cIEF experiment will produce around 24,000 image frames (about 3.0 GB disk space or 10 GB memory space) for 10 fps acquisition rate. For data extraction, the acquired image stacks were background corrected, and the integrated intensity for each region of interest corresponding to each capillary was exported as a text file. The exported data was unbundled to a set of 32 data column with simple Labview program and then treated with five-point median filter to remove spikes due to the passage of particles through the laser beam. Migration time was calculated from the number of frames acquired and the acquisition rate. Electropherograms were then plotted for each capillary. A separate Labview voltage trigger program was used to control the high-voltage source. All data acquisition and processing were carried out on PC equipped with Intel core Quad CPU at 2.33 GHz and 4 GB of RAM. Peak alignment was performed using a two-point procedure based on the fluorescein-based p*I* standards (p*I* 6.5 and 5.5) peak positions.

## Results and disccussion

*Free-solution electrophoresis* As an initial evaluation of the system, the 32-capillary system was used for the free-solution electrophoresis of a 130 pM solution of disodium fluorescein. Mass detection limits (3 s) for the instrument were 380±120 ym fluorescein injected onto the capillary.

In a second evaluation, the instrument was used for the free-solution separation of six fluorescent sphingolipid standards (Cer: ceramide, GlcCer: glycosylceramide, LacCer: Lactosylceramide, $G_{Mx}$: Monosialogangliosides, $G_{Ax}$: Asialogangliosides, x refers to the metabolic parameter). The electrophoretic migrations of the standards are well-known based on previous work from our group [20, 21]. The $G_{M1}$ and Cer peaks were identified in each electropherogram; the relative standard deviation in peak position was 3% for both components. We used the migration time for those components in a two-point alignment algorithm; Fig. 3 shows the aligned electropherograms.

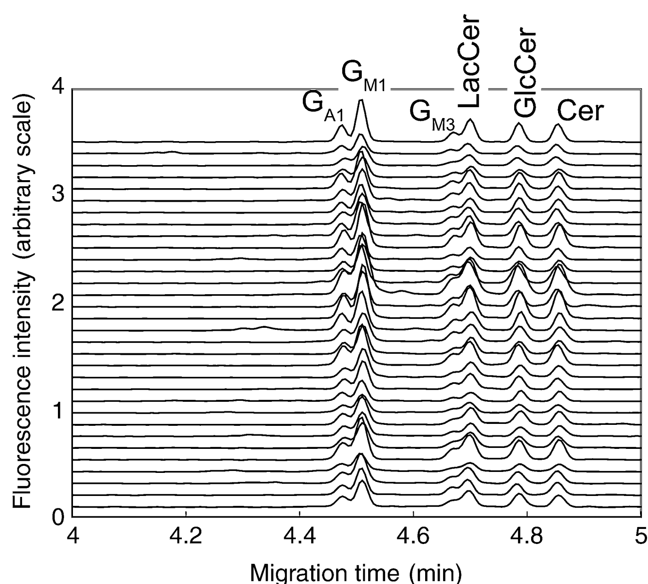


**Fig. 3** Separation of sphingolipid standards using free-solution electrophoresis in the 32 capillary instrument

The relative standard deviation in peak height between the 32 capillaries was 35% for the $G_{M1}$ peak. This variation in sensitivity likely reflects variation in overlap between the sample streams and the laser beam and capillary-to-capillary variation in injection volume. The average detection limit for the 32 capillaries was 5±2 zmol injected onto the column, which is about an order of magnitude poorer than the detection limit produced with a single capillary instrument [23].

*cIEF standard separation* Two fluorescein-based p*I* standards (p*I* 6.5 and 5.5) were analyzed by capillary isoelectric focusing. The reproducibility of the peak positions was poorer than for the free-solution electrophoresis; the relative precision for peak 1 position was 5% and for peak 2 was 8%. Capillary isoelectric focusing is particularly sensitive to electro-osmosis induced by flaws in the capillary coating, which likely accounts for the poorer capillary-to-capillary precision in peak migration time. Figure 4 presents a typical electropherogram.

A Gaussian function was fit to the peaks; the average peak width for the p*I*=6.5 standard was 0.0027±0.0003 pH and the p*I* 5.5 standard was 0.0036±0.0006 pH. The insert of Fig. 4 presents a particularly sharp peak with standard deviation of 0.0021 pH units.

*cIEF protein separation* Myoglobin (p*I* 7.2 and 6.8) and ovalbumin (p*I* 5.5) were labeled with Chromeo P503, mixed with the standards, and analyzed by cIEF. This mixture was run on 26 capillaries; the rest were blanks. The blanks showed a significant background signal due to fluorescence from impurities present within the ampholytes;

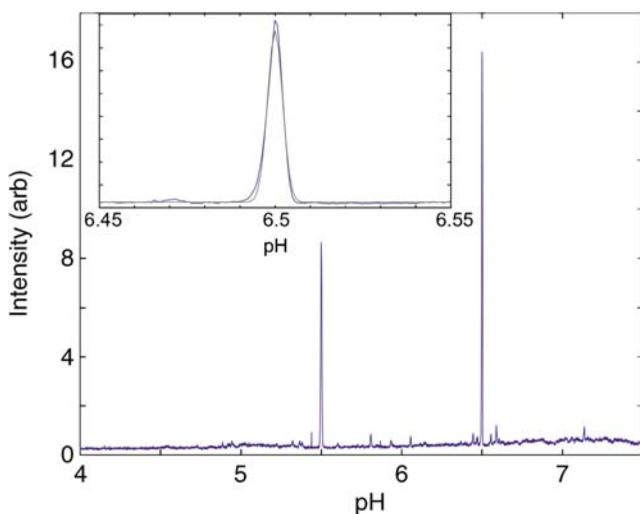


**Fig. 4** Capillary isoelectric focusing of p*I* 5.5 and 6.5 standards. The insert shows the raw data and the least-squares regression analysis with a Gaussian peak (*green*) for the 6.5 p*I* standard. The standard deviation of the Gaussian function was 0.0021 pH units

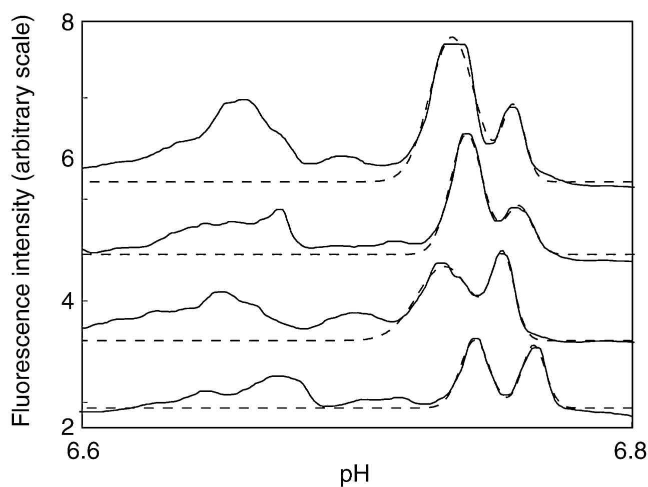


**Fig. 6** Capillary isoelectric focusing of myoglobin components. The smooth curves are the data and the dashed curves are the least-squares regression fit of a pair of Gaussian functions to the peaks. The peaks were separated by 0.020±0.001 pH unit

similar behavior has been noted elsewhere [10]. Figure 5 presents the electropherograms generated from the protein sample, after alignment with the p*I* standards and conversion to pH scale. The alignment algorithm is not perfect; a residual ~0.05 pH jitter is observed in the protein p*I*. In addition, the estimated p*I* of ovalbumin (5.2) is not consistent with the literature value. There are two possible sources of this inconsistency. Either the p*I*s of the standards are not accurate or the labeling chemistry modifies the p*I* of the proteins. We demonstrated that labeling does not change the p*I* of proteins [12], which implies that the p*I*s of the standards are not accurate.

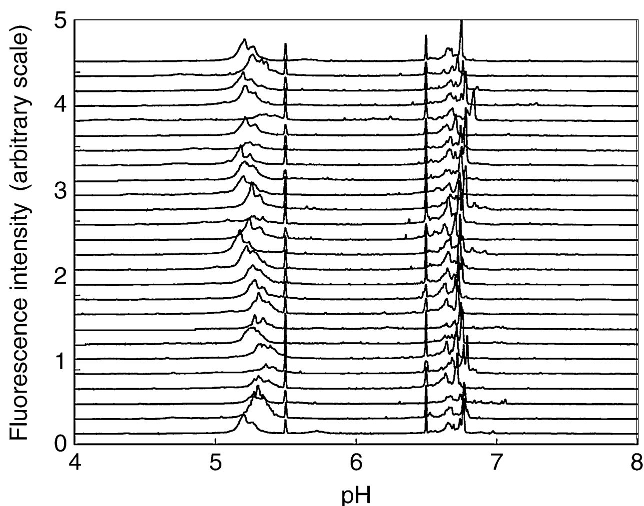


**Fig. 5** Capillary isoelectric focusing separation of myoglobin, ovalbumin, and p*I* 5.5 and 6.5 standards. The electropherograms were aligned based on the migration time of the standards, and the pH scale was based on the nominal pH of the standards

Resolution varied between capillaries, which may be attributed to non-uniform capillary surface coatings. A few capillaries produced outstanding resolution of protein isoforms. Figure 6 presents four separations of the myoglobin peak. The components of the doublet with nominal p*I* of 6.75 were separated in apparent p*I* by 0.020±0.001 pH unit.

*Detection limit* The detection limit (3 s) for the cIEF of fluorescently labeled ovalbumin was 200±100 pM, averaged across the capillaries. These values are in agreement with previous work where Chromeo P503 labeled proteins in 4% non-photobleached ampholytes with excitation at 473 nm wavelength. Future work will investigate the use of photobleached ampholytes and 532 nm excitation for Chromeo P540-labeled proteins. We have reported that the background signal drops dramatically for the longer wavelength excitation and that photobleaching reduces the fluorescence background signal from ampholytes [13]. The combination of these improvements should produce one to two orders of magnitude improved detection limit. However, there is one issue with the use of the green laser, which prevented its use in this experiment. The fluorescein-based p*I* standards are not excited at 532 nm. A two-color instrument analogous to one developed for DNA sequencing would allow the detection of fluorescence from standards and sample without interference [24].

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