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Rapid and reproducible two-dimensional capillary electrophoresis analysis of Barrett's esophagus tissues

James R. Kraly¹, Megan R. Jones¹, David G. Gomez¹, Jane A. Dickerson¹, Melissa M. Harwood¹, Michael Eggertson^{1,7}, Thomas G. Paulson^{2,3}, Carissa A. Sanchez³, Robert Odze⁶, Ziding Feng³, Brian J. Reid^{2,3,4,5}, and Norman J. Dovichi¹

¹ Department of Chemistry, University of Washington, Seattle WA 98195-1700, USA

⁴ Department of Medicine, University of Washington, Seattle WA 98195-1700, USA

⁵ Department of Genome Sciences, University of Washington, Seattle WA 98195-1700, USA

² Divisions of Human Biology, PO Box 19024, Seattle, WA 98109

³ Divisions of Public Health Sciences, Fred Hutchinson Cancer Research Center, PO Box 19024, Seattle, WA 98109

⁶ Department of Pathology, Harvard Medical School and Brigham and Women's Hospital, Boston, Ma 02115

Abstract

We have constructed a high-speed two-dimensional capillary electrophoresis system with a compact and high sensitivity fluorescence detector. This instrument is used for the rapid and reproducible separations of Barrett's esophagus tissue homogenates. Proteins and biogenic amines are labeled with the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde. Labeled biomolecules are separated sequentially in two capillaries. The first capillary employs capillary sieving electrophoresis using a replaceable sieving matrix. Fractions are successively transferred to a second capillary where they undergo additional separation by micellar electrokinetic capillary chromatography. The comprehensive two-dimensional separation requires 60 minutes. Within-day migration time reproducibility is better than 1% in both dimensions for the 50 most intense features. Between-day migration time precision is 1.3% for CSE and better than 0.6% for MECC. Biopsies were obtained from the squamous epithelium in the proximal tubular esophagus, Barrett's epithelium from the distal esophagus, and fundus region of the stomach from each of three Barrett's esophagus patients with informed consent. We identified eighteen features from the homogenate profiles as biogenic amines and amino acids. For each of the patients, Barrett's biopsies had more than 5 times the levels of phenylalanine and alanine as compared to squamous tissues. The patient with high-grade dysplasia shows the highest concentrations for 13 of the amino acids across all tissue types. Concentrations of glycine are 40 times higher in squamous biopsies compared to Barrett's and fundal biopsies from the patient with high-grade dysplasia. These results suggest that two-dimensional capillary electrophoresis may be of value for the rapid characterization of endoscopic and surgical biopsies.

Introduction

Capillary electrophoresis with laser induced fluorescence is a sensitive technique well suited for the separation and detection of small amounts of biological materials^{1, 2}. Several recent reports demonstrate the capabilities of CE-LIF for the rapid analysis of biomolecules. Aspinwall reported photolytic optical injection of fluorogenic labels for online CE-LIF

⁷Present address: University of Calgary, Department of Biochemistry and Molecular Biology, Calgary, AB T2N 4N1 CANADA

separations of amino acids and proteins³. Bowser has developed an online microdialysis-CE-LIF system for to analyze small molecules sampled *in vivo* or from tissue homogenates^{4–6}. Kennedy developed a CE-LIF assay for the detection of adenylyl cyclase activity using fluorescent substrate⁷. Sweedler has thoroughly investigated the role of D-Aspartate in neurochemical processes using CE-LIF to analyze large neurons^{8–9}. Allbritton has characterized kinase activity in single cells¹⁰.

We have developed a two-dimensional capillary electrophoresis (2D-CE) system with an ultrasensitive laser-induced fluorescence detector for the generation of fingerprints from samples as small as single mammalian cells^{11, 12}. We employ the fluorogenic reagent 3-(2-furoyl)quinoline-2-carboxaldehyde (FQ)¹³ to fluorescently label primary amines while generating low background signals and minimizing peak broadening due to incomplete labeling^{14–15}. The first generation 2D-CE instruments employed relatively low electric fields for the separation, which required 3–5 hours¹². These long separations were particularly susceptible to drift due to coating instability, temperature changes, and buffer evaporation.

In this paper, we report several modifications to our original system. Use of narrow inner diameter capillaries facilitates application of higher electric fields for the separation, which dramatically decreases the analysis time; a comprehensive two-dimensional separation of a complex mammalian cellular homogenate now is complete in one hour. Furthermore, the incorporation of dynamic coatings and passive temperature control help to stabilize electroosmotic flow and current. As a result, separation performance and component mobility is highly reproducible; the within-day migration time precision is better than 1% in both separation dimensions for the 50 most intense features generated in a homogenate prepared from a complex biological sample.

We apply this rapid, reproducible, and sensitive technique to characterize biopsies sampled from patients with Barrett's esophagus. Chronic gastroesophageal reflux can damage the normal squamous epithelium of the esophagus and lead to the development of specialized intestinal epithelium, known as Barrett's esophagus^{16, 17}. Barrett's esophagus patients have a 30 to 40 fold increased risk of progressing to esophageal adenocarcinoma, which is a malignancy with rapidly increasing incidence in western countries and has abysmal mortality rates^{18–20}. Surgical removal of the esophagus essentially eliminates cancer risk; however, esophagectomy has a 5–20% mortality rate, depending on the surgical volume of the institution, which makes it a poor option for cancer prevention²¹. Prognostic indicators need to be developed to identify those patients most likely to progress to cancer, so that they can receive more intensive surveillance for early detection of cancer.

To supplement the pathological and molecular profiling of Barrett's esophagus patients, we employ 2D-CE to monitor expression differences associated with tissue types and at different points in neoplastic progression. Squamous, Barrett's, and fundal biopsies are obtained from each of three patients during endoscopic surveillance. Tissues are fixed in 70% ethanol within 15 seconds of sampling and are homogenized within 15 minutes. Samples are fluorescently labeled and subjected to 2D-CE analysis. Spots are classified as proteins and biogenic amines based on enzymatic treatment. Co-migration has been used to identify 18 of these components.

EXPERIMENTAL SECTION

Materials and Chemicals

CHES, Tris, dextran (513 kDa), sodium hydroxide, sodium dodecyl sulfate (SDS), trypsin, amino acids, biogenic amines, and standard proteins were purchased from Sigma-Aldrich (St. Louis, MO, USA). Water was from a Barnstead Nanopure water supply (Boston, MA, USA). FQ and potassium cyanide were from Molecular Probes (Eugene, OR, USA). Fused-silica

capillaries were from Polymicro Technologies (Phoenix, AZ, USA). The dynamic coating reagent, UltraTrol LN, was purchased from Target Discovery (Palo Alto, CA, USA). Drummond glass capillaries were from Drummond Scientific Company (Broomall, PA, USA).

Stock solutions high purity of amino acid, biogenic amine, and protein standards were prepared daily at 2–10 mg/mL in ddH₂O. Standards were diluted to 5–100 μ M in 1% SDS prior to fluorescent labeling.

Cell Culture and Preparation of Cellular Homogenate

An immortalized cell line of premalignant epithelial esophageal cells (CP94251) was a kind gift from Professor Peter Rabinovitch of the University of Washington. Cells were cultured with modified MDCB 153 media.²² Cells were harvested when approximately 70% confluent. After brief exposure to 0.25% trypsin and 3.5 mM EDTA, cells were collected in PBS and rinsed three times.

Roughly 4×10^6 cells were lysed and homogenized in 500 μ L of 1% SDS using a pulsed high power probe sonicator equipped with a cooled water bath (Branson Ultrasonics, Danbury, CT, USA). Typically, cells were homogenized after 5 minutes of pulsed sonication with an 80% duty cycle and 80% relative intensity. Cellular homogenates were stored at 80 °C in 20 μ L aliquots.

To prepare a tryptic digest, homogenates were exposed to trypsin at 33 μ g/mL and incubated at 37 °C overnight to digest the protein content. The digest was reconstituted in 1% SDS and stored at –80 °C in 20 μ L aliquots.

Endoscopic Biopsies

Endoscopies and biopsies on patients in the Seattle Barrett's Esophagus Study were performed with a large-channel endoscope and "jumbo" biopsy forceps using a turn-and-suction technique²³. Tissues were fixed in 70% ethanol within 15 seconds of sampling and homogenized within 15 minutes using a PowerGen 125 homogenizer (Fisher) in 400- μ L of a 1% SDS solution. Fifty aliquots were prepared from each sample and archived at –80 °C.

The Seattle Barrett's Esophagus Study was approved by the Human Subjects Division of the University of Washington in 1983 and renewed annually thereafter with reciprocity from the IRB of the Fred Hutchinson Cancer Research Center (FHCRC) from 1993 to 2001. Since 2001, the study has been approved annually by the IRB of the FHCRC with reciprocity from the Human Subject Division of the University of Washington.

Fluorescent Labeling

Primary amines (proteins and biogenic amines) were labeled with the fluorogenic reagent FQ to produce a fluorescent product. Optimization of FQ reactivity has been previously reported.^{14, 24} Frozen aliquots of homogenate or standards were heated to 95° C for 4 minutes to thermally denature macromolecules. Next, 5 μ L of the heated sample was mixed with 5 μ L of 5 mM KCN in a tube with 100 nmol of lyophilized FQ. This mixture was heated at 65° C for 5 minutes. The sample was diluted with 90 μ L of 100 mM CHES, 100 mM Tris, 3.5 mM SDS, pH 8.7 and stored on ice until analysis.

Capillary Electrophoresis Instrument

Earlier versions of the sheath-flow cuvette-based instrument have been described^{25–28}. We have made a few modifications to the instrument. A 15 mW laser beam at 473 nm (Lasermate) was focused through the cuvette using a 6.3X objective (Melles Griot). Fluorescence was collected with an M-PLAN 60x, 0.7 NA microscope objective (Universe Kogaku), and filtered

with a 580LP long-pass filter (Omega). Transmitted light was passed through an adjustable iris to a GRIN lens that was coupled to a 50- μ m diameter, 50-cm long fused-silica fiber optic. The iris controlled the probe volume of the detector and was adjusted to optimize the signal-to-noise ratio. Light was detected by an avalanche photodiode single-photon counting module (EG&G Canada); the photocounts were acquired at 50 Hz with a National Instruments DAQ Card (PCI-6035E) programmed with LabVIEW running on a PC. The resulting electropherograms were processed and visualized with Matlab 7.0 running on either a PC or Macintosh computer.

The instrument was operated in either one-dimensional or two-dimensional modes. In one-dimensional electrophoresis, a 20-cm long, 30- μ m inner diameter, 140- μ m outer diameter fused silica capillary was placed between the injector and sheath-flow cuvette. The distal end of the capillary was held ~30- μ m from the focused laser beam inside the cuvette. For capillary sieving electrophoresis (CSE), the separation buffer was 5% dextran (513 kDa), 100 mM CHES, 100 mM Tris, and 3.5 mM SDS, pH 8.7. The sheath flow buffer for CSE was 100 mM CHES, 100 mM Tris, and 3.5 mM SDS, pH 8.7. Sample was electrokinetically injected with 2 kV for 1 second. The running voltage was 20 kV applied with negative polarity. For micellar electrokinetic chromatography (MEKC), the separation and sheath flow buffers were 100 mM CHES, 100 mM Tris, and 15 mM SDS, pH 8.7. Sample was electrokinetically injected at 0.25 kV for 1 sec.

In two-dimensional electrophoresis, the two capillaries were aligned at an interface, described below. The injection box, interface support, and sheath flow waste reservoir were carefully adjusted to equal heights to avoid siphoning. The distal end of the second capillary was held ~30 μ m from the focused laser beam inside the sheath flow cuvette.

Unfortunately, the temperature of our laboratory fluctuated by ~3 °C over a ten-minute period. These temperature fluctuations produced changes in the viscosity of the running buffer that degrade run-to-run migration time precision. We encased the optical breadboard in a tent made from construction paper and thin plastic. This tent helped stabilize the capillaries' temperature, which stabilized the current and improved the reproducibility of the separation.

2D Capillary Interface Design

In two-dimensional electrophoresis, power was independently supplied across the two capillaries. The capillaries were held in an interface that aligned the capillaries to be coaxial within a few micrometers and with their tips separated by ~40-micrometers. The interface was manufactured using macroscopic machining technology. A 0.94-mm diameter mill bit was used to machine a 0.94-mm deep, flat-bottomed channel in a 3 cm $\times$ 3 cm Plexiglas plate. A second channel was milled to a 1.17-mm depth at right angles to the first with a 1.17-mm diameter end-mill.

Next, a nested set of capillaries was used to center the two separation capillaries within the 0.94-mm channel. First, two Drummond microcaps (Cat no. 1-000-0040) were fixed with UV-curing glue into the 0.94-mm channels. These tubes were 0.86-mm OD and 0.40-mm ID; the outer diameter of the microcaps was slightly smaller than the width of the channel, the bottom and one side of the channel were used as alignment features to ensure that the two microcaps were co-axial. Two pieces of fused-silica capillaries having 365 μ m OD and 158 μ m ID were next inserted into the 0.40 mm ID microcaps. The capillary sleeves were aligned to only slightly protrude from the microcaps in the center of the interface. A drop of epoxy was placed at each junction of the alignment capillary and microcap. Two pretreated 20 cm, 140 μ m OD and 30 μ m ID separation capillaries were inserted into the alignment capillaries so that they were approximately 40 μ m apart in the center of the interface. Each outer junction of separation capillary and capillary sleeve was sealed with a drop of epoxy. Before insertion into the

interface, the polyimide coating was removed from the ends of the cleaved separation capillaries using a gentle flame. A microscope was used to observe capillary alignment within the interface. Figure 1 shows a photograph of the 2D-CE interface and the separation capillaries.

Portions of 5 μ L Drummond pipets (Cat no. 2-000-001) were fixed with UV glue into the 1.17-mm square channels; the dimensions of these tubes were neither crucial to this application nor specified by the manufacturer, but the tubes fit snugly within the 1.17-mm channels. These tubes were connected to flexible Nalgene tubing and used to rinse the interface and replenish separation media.

A square piece of a microscope slide was fit over the interface. UV curing epoxy was applied under the slide and to each channel. A hand held UV lamp was used to cure the epoxy and seal the interface.

Preparation of 2D-CE System

The pipets orthogonal to the separation capillaries were used to rinse and treat the interface and capillaries. Tubing connected one pipet to a valve and a disposable 10 mL syringe. The second pipet was connected by a 10 cm piece of tubing to waste during rinses, but was placed in a 1.6 mL tube that contained 1.5 mL MEKC buffer and the 2nd electrode during electrophoresis. The injection end of the first capillary was inserted into the injection apparatus, and the distal end of the second capillary was inserted in to the sheath flow cuvette.

Before separations, a series of solutions was flushed through the valve and interface to waste with a disposable 10 mL syringe. After each solution filled the interface, the waste tubing was clamped, and solution forced through both separation capillaries by applying gentle pressure to the syringe. Observing solution elution in the sheath flow cuvette provided evidence of capillary cleaning and treatment. The tip of the 1st capillary was rinsed periodically as eluent droplets form. Air bubbles and particles were easily flushed from the interface and capillaries using gentle pressure from the syringe.

Roughly 25 minutes are required to prepare the system for subsequent runs. The interface and both separation capillaries were treated with 0.5 M sodium hydroxide, ddH₂O, the dynamic coating reagent UltraTrol LN, and MEKC buffer, which was 100 mM CHES, 100 mM Tris, and 15 mM SDS, pH 8.7. Each solution was purged for only 2–3 minutes. The first dimension CSE buffer, 5% Dextran (513 kDa), 100 mM CHES, 100 mM Tris, 3.5 mM SDS, pH 8.7 was purged through the first capillary for 4 minutes using the N₂ gas purge of the injection apparatus²⁸. While filling the first capillary with the CSE matrix, a syringe was used to manually pump the 2nd dimension MEKC buffer into the interface, which flushed the matrix exiting the first capillary out of the interface and to waste. Before sample injection, the valve associated with the syringe was closed and the 10 cm tubing was placed in the 2nd electrode reservoir.

2D-electrophoresis

Figure 2 presents the voltage program applied to each electrode during the experiment. Sample was electrokinetically injected onto the first capillary, followed by a preliminary electrophoresis separation in the first capillary, so that the fastest moving components approached the end of the first capillary. Successive transfers and MEKC cycles were performed to comprehensively analyze the contents of the first capillary. A fraction was transferred within the interface from the first to second capillary by applying a net 10 kV for 1 second to transfer a fraction. Sample was separated in the second dimension by applying 20 kV to both electrodes for 12 seconds. The 0 kV net potential held the contents of the first capillary stationary, while the second capillary experienced a 20 kV net potential (1000 V/cm)

to separate the transferred fractions by MEKC. Transfer and MEKC cycles were programmed to repeat until all components from the first dimension had been analyzed, typically 250 fractions were transferred and analyzed.

RESULTS AND DISCUSSION

Calibration Curve and Limit of Detection

We incorporated several changes to our fluorescence detector. Most importantly, we now use an avalanche photodiode photodetector instead of a photomultiplier tube. This solid-state device has four-fold higher quantum yield in the red portion of the spectrum, which results in improved detection limits. A more compact and efficient 473 nm solid-state laser has replaced the 488 nm argon ion laser. By decreasing the excitation wavelength, the Raman interference is shifted to the blue, which allows the use of an emission filter that better matches the spectrum of the FQ-labeled analytes. Figure 3 shows the peak generated by the injection of 20 pL of a 3 nM solution of FQ labeled carbonic anhydrase. The data were first treated with a five-point median filter and then convoluted with a 31-point Gaussian filter with a 0.14-s standard deviation. Noise was estimated by the standard deviation of the smoothed baseline from 10 to 60 seconds. The resulting peak had a signal-to-noise ratio of 525, which corresponds to an instrumental detection limit (3σ) of 330 ymol or 200 copies of labeled protein.

1-D Capillary electrophoresis separations of Barrett's esophagus homogenates

Figure 4A shows the one-dimensional CSE separation of BE cellular homogenate. The profile consists of groups of intense and well-resolved peaks bordering a broad band of poorly resolved components. Proteins separate according to their molecular weight during the CSE separation²⁹. A set of standard proteins was used to generate a calibration curve between migration time and molecular weight in the CSE separation (data not shown). Figure 4B shows the MEKC separation of the BE cellular homogenate. The majority of fluorescent components migrate in the first third of the separation profile. In both cases, the separation efficiency is quite high. Separation efficiency ranged from 100,000 to 500,000 plates for well resolved components ($500,000 - 2.5 \times 10^6$ plates/meter).

Total analysis time and separation window time have been dramatically decreased from our previous reports. CSE separations of BE have a separation window of approximately 100 seconds and a total analysis time of 183 seconds. MEKC separations of BE have a separation window of approximately 84 seconds and a total analysis time of 136 seconds. There are several factors that led to faster separations while maintaining peak capacity and separation efficiency. Field strength was increased by decreasing capillary length and increasing applied voltage. High-field separations using 50 μ m ID capillaries produced band broadening, most likely a result of Joule heating. Narrower capillaries more efficiently dissipate heat because of the increased surface to volume ratio.³⁰ Field strengths in excess of 1000 V/cm were applied to 30 μ m i.d. capillaries without noticeable band broadening.

For a one-dimensional separation, peak capacity is defined as the duration of the separation window divided by 4 times the average peak standard deviation. The average peak width is 1.2 s for CSE separations and 0.8 s for MEKC separations, resulting in peak capacities of 83 and 105, respectively.

Components were tentatively classified by their diffusion constant, which was estimated by performing one-dimensional electrophoresis and measuring peak width after stopping the electropherogram for 0–30 minutes. The results, not shown, reveal a clear discrimination between low molecular weight components with diffusion coefficients near $3 \times 10^{-6} \text{ cm}^2 \text{ s}^{-1}$ and high molecular weight components with diffusion coefficients near $3 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$. These

components react with FQ, and therefore contain primary amines. Obvious target compounds are biogenic amines and amino acids for the low molecular weight components and proteins for the high molecular weight components.

Proteins complex with SDS, which results in a narrow range of size-to-charge ratio, and leads to molecular-weight based separation in sieving electrophoresis. FQ-labeled amino acids and biogenic amines have very different migration behavior than proteins in CSE. Figure 4C presents the separation of FQ labeled amino acids by CSE. Indeed, the migration times suggest a very high molecular for these compounds, and demonstrates that the separation mechanism for low molecular weight components is different than that of proteins in CSE.

2D-CE of BE Cellular Homogenate

The 2D-CE separation of a BE homogenate required about 50 minutes. Our earlier 2D experiments required 3–5 hours.^{11, 12, 31} Figure 5A shows the landscape image generated by plotting the data as a surface whose amplitude is proportional to fluorescence intensity. Figure 5B highlights some of the lower amplitude components. By overexposing the gel image, lower amplitude components are more easily visualized, Figure 5C. The dynamic range of the image is about 4×10^3 .

A Gaussian surface was fit to spots as described previously¹². The average peak width (expressed as the standard deviation) of the 50 most intense components was 1.6 ± 1.1 transfers in the CSE dimension and 0.19 ± 0.10 s in the MECC dimension. The migration time in the two dimensions was uncorrelated ($r = 0.19$) and the spot capacity was 600 for this separation.

Reproducibility of 2D-CE Analysis

To determine the within-day precision of electrophoresis, four replicate separations were performed on a single labeled BE cellular homogenate, Figure 6. A Gaussian surface was fit to the 50 most intense components¹². The average relative standard deviation (RSD) in CSE mobility was 0.4%. Calculation of the MECC dimension migration time is problematic. In a one-dimensional MECC separation, the first components reach the detector 50 seconds after injection. To speed the separation, we employed a 13 s period between sample injections onto the second dimension, so that the components from several successive injections are present in the MECC column at the same time. Unfortunately, we do not know how many cycles a component spent on the second column before reaching the detector. We do know that the fastest migrating components reach the detector at least 50 seconds after injection, which is the lower limit to migration time; most components have longer migration time. If we divide the standard deviation in migration time by 50 seconds, we have an upper limit of 0.6% RSD in the MECC migration time and mobility.

Component amplitude was less reproducible. The RSD in peak height is dominated by a few outliers; the median RSD in peak height was 14%. There are at least two sources of run-to-run amplitude variation. First, non-linear regression fits of a Gaussian surface to a spot will be sensitive to the presence of neighboring features, which can distort estimates of background signal and spot width and amplitude; minor changes in resolution and peak overlap will introduce noise in peak amplitude estimates. Second, FQ-labeled compounds are not particularly stable and undergo decomposition throughout the day.

To determine the between-day precision, three aliquots of the same biopsy were thawed, labeled, and analyzed on successive days. The average RSD in CSE mobility was 1.3%, the upper limit of the RSD in the MECC dimension was 0.6%, and the median RSD in peak height was 25%.

Classification and Identification of 2D-CE Components

To classify components in the 2D-CE separation, a tissue homogenate was treated with trypsin to digest proteins and separated, Figure 7. The amplitude of many of the broad and intense spots decreased dramatically in the digested sample's profile, which classifies those components as proteins.

We rely on spiking experiments to identify components in the 2D-CE separation profiles³². For this work, biogenic amines and amino acids standards were analyzed both alone and spiked into a homogenate. We have identified 18 components, Figure 8. The other amino acids did not co-migrate with components in these samples.

Biopsy of Esophageal Epithelium

Biopsies were obtained under informed consent from a set of patients enrolled in the Seattle Barrett's Esophagus Cohort. Patients are monitored by endoscopic surveillance every 6–12 months as determined by their degree of dysplasia. Flow cytometry is used to measure DNA content of biopsies and has shown that increased aneuploidy and tetraploidy are risk factors for progression to cancer^{33–35}. Biopsies are also subjected to molecular analysis of p16 and p53 tumor suppressor genes³⁶. Each patient selected for 2D-CE biopsy analysis was characterized as having loss of heterozygosity (LOH) at chromosome 9p but not at chromosome 17p; that is, the Barrett's tissue has lost one allele on the short arm of chromosome 9 that contains the p16 tumor suppressor gene. LOH on chromosome 9p has been shown to be an early event in the progression to esophageal adenocarcinoma. Both copies of the short arm of chromosome 17 are intact in these patients; this region contains the p53 tumor suppressor gene, the loss of which puts patients at a much higher risk of developing cancer.

Figure 9 is a photograph taken during an endoscopy of a person with Barrett's esophagus. Normal squamous epithelium is whitish pink and found nearer the throat (A). The darker red lining (B) is the metaplastic columnar epithelium characteristic of Barrett's esophagus. Fundus (C) is a normal gastric tissue located near the junction of the stomach to the esophagus. Biopsies are roughly 4-mm long, 2-mm wide, and 1-mm thick. The location of the biopsy site is recorded as the distance from the incisors.

Two biopsies were excised within a 1-cm region of squamous and fundal tissues. The samples were homogenized, labeled, and separated in parallel, Figure 10. Common features from adjacent electropherograms were analyzed to calculate 2D-CE mobility and spot area. For both tissue types, relative standard deviation in 2D-CE mobility was better than 1% for all common features. For the adjacent fundal biopsies A and B, 70% of the components in the 2D-CE profiles have less than 20% deviation in spot volume, and only 5% differed by 50% or more. Analysis of adjacent squamous biopsies A and B determine that 69% of the components have less than 20% deviation in spot volume, and only 5% of the components differed 50% or more. These results validate the reproducibility of sampling, tissue processing, and electrophoresis.

Figure 11 shows the 2D-CE profiles of squamous, Barrett's, and fundal endoscopic biopsies from three patients. The data are presented as overexposed gel images to help visualize the low amplitude components. We have identified a set of features that may distinguish the tissue type and relative disease state of the patient. Figure 12 shows highlighted regions of interest from the nine 2D-CE profiles of biopsy homogenates. 2D-CE component labeling is the same as in Figure 8.

The majority of the amino acids have similar expression across both tissue types and patients. A few (phenylalanine, glycine, and alanine) show strikingly different results. For each of the three patients, Barrett's tissues had more than 5 times the levels of phenylalanine and alanine as compared to squamous or normal esophageal epithelium. These differences are most

pronounced for Patient 2, who has high-grade dysplasia. Across each tissue type, the patient with high-grade dysplasia shows the highest concentrations for 13 of the 18 amino acids identified. The most obvious example is glycine, whose concentration is more than 40 times higher in squamous tissues as compared to Barrett's and fundal biopsies from that patient.

CONCLUSIONS

Capillary electrophoresis has been remarkably successful in nucleic acid analysis, supplanting alternative technologies for sequencing and most genotyping applications. It has been much less successful in the analysis of other biological components. This lack of success can be attributed to several factors, including the requirement of chemical derivatization of low concentration mixtures, the resolution of highly complex samples, and the need for highly reproducible separations. These issues are addressed in this paper.

Other issues remain. Identification of components is required for many, but not all, applications. Identification by spiking is tedious; there is a need for on-line identification of components, presumably by mass spectrometry. Unfortunately, mass spectrometry is much less sensitive than fluorescence, particularly for intact proteins. Most protein separations appear to require the use of surfactants to minimize interaction with capillary walls; these surfactants can interfere with mass spectrometry.

Speed of analysis remains an issue. The current instruments rely on sequential analysis of samples. There is a need for parallel analysis of many samples, by analogy with capillary array instruments developed for DNA sequencing³⁷.

Finally, resolution of proteins reported in this paper is unsatisfactory. CSE has proven to be an efficient separation method for proteins, but MECC, at least under the conditions employed in this paper, generates poor resolution. Alternative separation methods, such as isoelectric focusing, are problematic. Multiple labeling of primary amines leads to a complex mixture of reaction products^{38–39}, which leads to complex IEF separations. This phenomenon is eliminated in MECC and CSE by use of FQ as a labeling reagent and the addition of anionic surfactant to the separation medium, but that surfactant is incompatible with IEF⁴⁰.

Presuming that issues of component identification, sample throughput, and improved resolution of proteins are solved, two-dimensional capillary electrophoresis has the potential for use in high-throughput characterization of biopsies and other clinical samples. For example, an automated 96 capillary instrument that operates with a one-hour separation period can process a thousand samples per ten-hour day, which may allow routine screening of biopsies and tumors for prognostic markers. Of course, this technology may ultimately be applied to the characterization of single cells, where cell-to-cell heterogeneity provides additional information to classify patients^{29,31}.

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Figure 1. 2D-CE interface. Two 140 μm OD $\times$ 30 μm ID separation capillaries are inserted into two 365 μm OD $\times$ 148 μm ID capillary sleeves and aligned coaxial to each other approximately 40 μm apart.

Figure 2.

Voltage timing diagram for 2D-CE experiments. All voltages are applied in negative polarity and the sheath-flow cuvette is held at ground potential. The voltage applied to each capillary is determined by the net potentials between electrode 1, electrode 2, and the detector (ground).

Figure 3. Injection of 3 nM FQ-labeled carbonic anhydrase separated by MEKC. The 3σ limit of detection is 330 yoctomol (10^{-24} mol).

Figure 4.

One-dimensional capillary electrophoresis separations. A) CSE separation of BE cellular homogenate. B) MEKC separation of BE cellular homogenate. C) CSE separation of amino acid standards, peak 4 – glutamic acid, 5 - lysine, 6 - phenylalanine, 7 – isoleucine, 9 – arginine, 10 - methionine, 12 – alanine, 17 - glutamine, 18 - histidine. All separations at -1000 V/cm.

Figure 5.

Two-dimensional electrophoresis separation of BE cellular homogenate **A)** Landscape plot. Fluorescence intensity is plotted on the z-axis. Component intensity spans 4 orders of magnitude. **B)** Expanding the data 10X illustrates the high sensitivity of laser induced fluorescence detection. **C)** The same data viewed as a gel image by overexposing the image to show the low amplitude components.

Figure 6.

Four 2D-CE landscape images from replicate experiments showing the separation of BE cellular homogenate. The within-day migration time reproducibility is better than 1% for the 50 most intense components.

Figure 7.

A) 2D-CE separation of BE cellular homogenate B) Separation of BE cellular homogenate treated with trypsin enzyme. Regions are highlighted to show the disappearance of the protein content with the mixture

Figure 8.

Identified biogenic amines in 2D-CE separations of BE homogenate. 1 - spermidine, 2 - putresceine, 3 - cadaverine, 4 - glutamic acid, 5 - lysine, 6 - phenylalanine, 7 - isoleucine, 8 - valine, 9 - arginine, 10 - methionine, 11 - glycine, 12 - alanine, 13 - serine, 14 - threonine, 15 - tryptophan, 16 - asparagine, 17 - glutamine, 18 - histidine.

Figure 9. Endoscopic view of Barrett's esophagus. A) squamous and B) Barrett's. The fundus is in the stomach beyond C.

Figure 10.

Gel images showing 2D-CE separations of adjacent biopsies of squamous and fundal biopsies from a 64 year old female patient with high grade dysplasia. The position of each biopsy is noted as the distance from the incisors.

Figure 11.

Gel images showing 2D-CE separations of Barrett's, squamous, and fundus tissue homogenates from each of three patients. The position of each biopsy is noted as the distance from the incisors.

Figure 12.
Landscape images of the biogenic amine region from Figure 11. Spot labeling from Figure 8.