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Multistage Isoelectric Focusing in a Polymeric Microfluidic Chip

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This paper reports a protocol that improves the resolving power of isoelectric focusing (IEF) in a polymeric microfluidic chip. This method couples several stages of IEF in series by first focusing proteins in a straight channel using broad-range ampholytes and then refocusing segments of the first channel into secondary channels that branch from the first one at T-junctions. Experiments demonstrate that several fluorescent proteins that had focused within a segment of the straight channel in the first stage were refocused at significantly higher resolution due to the shallower pH gradient and higher electrical field gradient. Two variants of green fluorescent protein from the second-stage IEF fractionation were further separated in a third stage. Three stages of IEF were completed in less than 25 min at electric field strengths ranging from 50 to 214 V/cm.

The conventional method of protein separation in proteomics is two-dimensional polyacrylamide gel electrophoresis (2D-PAGE),[1] where in the first dimension, proteins are resolved by polyacrylamide gel isoelectric focusing (IEF PAGE) and, in the second dimension, by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). However, 2D-PAGE is labor-intensive, and the whole process, including stain/destain, usually takes two or more days to complete.[2] Microchip electrophoresis is a promising alternative to PAGE gels since it has the potential to provide rapid protein analysis,[3] straightforward integration with other microfluidic unit operations, on-line detection,[4] smaller sample sizes, and lower fabrication costs.[5] However, the application of 2D electrophoresis on a microchip is much more challenging than 2D-PAGE due to the difficulty of coupling the two orthogonal separation techniques.

Several research groups have explored integration of zone electrophoresis and IEF on microfluidic chips. Chen et al.[6] described a method for carrying out 2D gel electrophoresis in a microfluidic system where integration of IEF and SDS electrophoresis was made possible by the ability to assemble and disassemble multiple poly(dimethylsiloxane) (PDMS) layers during a run. This system, however, required complicated sample/gel loading procedures as well as the alignment, bonding, and peeling of six PDMS layers. Herr et al.[7] performed 2D electrophoresis in a plastic microfluidic chip containing an IEF channel that intersected a zone electrophoresis channel. In this system, proteins focused in the IEF channel are mobilized to the intersection by electroosmotic flow (EOF), and then, after the IEF field is turned off, zone electrophoresis is applied as the second dimension of separation. Each band focused in the IEF channel was processed in this sequence and the IEF bands far from the intersection were refocused repeatedly before they were subjected to zone electrophoresis.

Li et al.[8] integrated liquid-phase IEF with SDS−PAGE in a microfluidic chip. This chip, which consists of a single IEF channel networked with an array of orthogonal channels, provides a means to perform parallel SDS−PAGE on each of the IEF-focused proteins without rearranging the chip. More recently, Wang et al.[9] developed a microfluidic chip that is able to select a defined pI range out of an IEF channel for further zone electrophoresis using two sets of pneumatically actuated PDMS valves.[10] A segment of channel shared by IEF and zone electrophoresis is used to transfer the focused proteins from IEF to zone electrophoresis by opening the valve on the zone electrophoresis channel and closing the valve on the IEF channel.

All of the examples above represent different schemes for coupling two orthogonal separation techniques in an effort to achieve maximum resolution with minimum loss of sample. However, IEF performance in these examples is low when compared with results reported for ampholyte-based, liquid-phase IEF separation of myoglobins with a resolving power of 0.02 pH unit.[11,12] In this paper, we developed a method for improving IEF

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resolving power by implementing an IEF staging technique, which first focuses proteins in a broad pH range and then refocuses segments of that broad pH range in narrow pH ranges.

IEF staging is a fairly routine purification strategy at preparative scales. The multicompartment electrolyzer with isoelectric membranes developed by Righetti and co-workers[13−15] for preparing narrow pH cuts from wide pH range carrier ampholytes is prototypical of staged IEF apparatuses. Narrow pH cuts can be further fractionated in the multicompartment electrolyzer with corresponding isoelectric membranes to produce ultra-narrow pH cuts. The IsoPrime multicompartment electrolyzer (Amersham Biosciences, Piscataway, NJ) and the Rotofor system (Bio-Rad Laboratories, Hercules, CA) are preparative protein purification devices that fractionate proteins in free solution by liquid-phase IEF and can reprocess the fractions that may contain other contaminating proteins as well as the protein of interest. Staged IEF using the IsoPrime multicompartment electrolyzer and the Rotofor system for maximum resolution of protein separations have been reported elsewhere.[16−18] To our knowledge, IEF staging has not yet been applied in microscale separations.

In this work, the concept of "staging," in which a single dimension of separation is reapplied to a sample to improve resolution, is introduced for microchip electrofocusing. The term "multistage fractionation", in which a single protocol is reapplied to a sample to improve resolution, is used to distinguish this work from "multidimensional fractionation," in which two or more orthogonal separation techniques are sequentially applied to a sample. Microchip IEF is staged by first focusing proteins in a straight channel using broad-range ampholytes and then refocusing segments of that first channel in secondary channels that branch out from the first one. The following experiments demonstrate that staging IEF can significantly increase the resolving power of IEF.

EXPERIMENTAL SECTION

Materials and Reagents. Recombinant green fluorescent protein (GFP variants 1 and 2, pI = 6.0 and 6.31, MW ∼28 000) was obtained from Upstate Biotechnology (Lake Placid, NY). Allophycocyanin (APC, pI = 4.38, MW ∼104 000) and r-phycoerythrin (PE, pI = 4.78, MW ∼240 000) were purchased from Molecular Probes (Eugene, OR). pIs of PE, APC, and two major GFP variants were determined by IEF PAGE with IEF pI markers (Sigma, St. Louis, MO). Methylcellulose (MC, viscosity of 2% aqueous solution at 25°C: 400 cP), Pharmalyte pH 8−10.5 ampholine were purchased from Pharmacia Biotech (Uppsala, Sweden). Pharmalyte pH 3−10, 3−4, 4.2−4.9, 4.5−5.4, and 6.7−7.7 carrier ampholytes and NaOH were acquired from Sigma. H_3PO_4 was obtained from J.T. Baker, Inc. (Phillipsburg, NJ). All MC concentrations used in this paper are given in weight/weight units, and all ampholyte concentrations are in volume/volume units.

Fabrication of PDMS Microchips. Fabrication of PDMS microchips was performed using the procedure reported in our previous work.[19] Briefly, a positive pattern of the desired channel structure is formed on a glass substrate using a positive photolithography technique.[20,21] PDMS prepolymer and curing agent (Sylgard 184, Dow Corning Inc., Midland, MI) were uniformly mixed at a ratio of 10:1, respectively, and degassed for 2 h at 0.001 Torr. The liquid elastomer is cast onto a positive pattern formed on the glass substrate and cured in a hot kiln for 6 h at 80 °C. At the end of the curing process, the elastic polymeric material is carefully peeled from the glass substrate to become the bottom layer of the microchip. The open channel on this bottom layer is irreversibly sealed with a flat surface of another layer of PDMS substrate containing holes as reservoirs after both surfaces have been plasma-oxidized.[22] This microchip (Figure 1) consists of a straight channel and two secondary channels that branch out from the straight channel (Figure 1A). All channels are 300 μm wide and 10 μm deep (Figure 1B). Built-in positioning scales (Figure 1B) that run along channels in 1-mm increments are used to identify protein band positions.

PDMS Microchannel Conditioning. Our previous work[19] demonstrates that EOF is greatly reduced in PDMS by applying a dynamic coat of MC to the channel walls. To achieve this, the following conditioning procedure was used before running IEF separations. First, all channels were flushed for 1 min at 10 psi with 1 M NaOH to obtain uniformly deprotonated surface silanol groups. The flush solution was allowed to stay in the channel for 10 min and was then pushed out of the channel using 5 psi nitrogen gas. To coat the channel walls, 0.4% MC was introduced into the channel and allowed to remain for 10 min. After this solution was removed using nitrogen gas, all channels were carefully pressure-filled with a mixture of 4% ampholyte, 0.4% MC. and protein sample (total protein concentration, 0.12−0.2 μg/μL). Excess sample solution was removed from the reservoirs using a micropipet, and electrode solutions were loaded into the reservoirs. The 2.5% MC was added to the electrode solutions to suppress pH gradient drift as well as to discourage the intrusion of the electrode solutions into the separation channel.

Multistage IEF Operation. Platinum wire electrodes were placed in the two reservoirs at opposite ends of the straight channel, and first-stage focusing was carried out at constant voltage using an XHR 600-1 power supply (Xantrex Technology Inc., Vancouver, Canada) while the branch channel reservoirs were left electrically floating. After first-stage focusing had finished, the electrodes were quickly removed from the straight channel reservoirs and placed in the branch reservoirs to perform the second stage of focusing.

An important aspect of multistage IEF is the use of anodic and cathodic reservoir solutions to define a narrow pH range in a channel network initially filled with broad-range ampholytes. Initially, all of the networked channels are filled with 4% broad-

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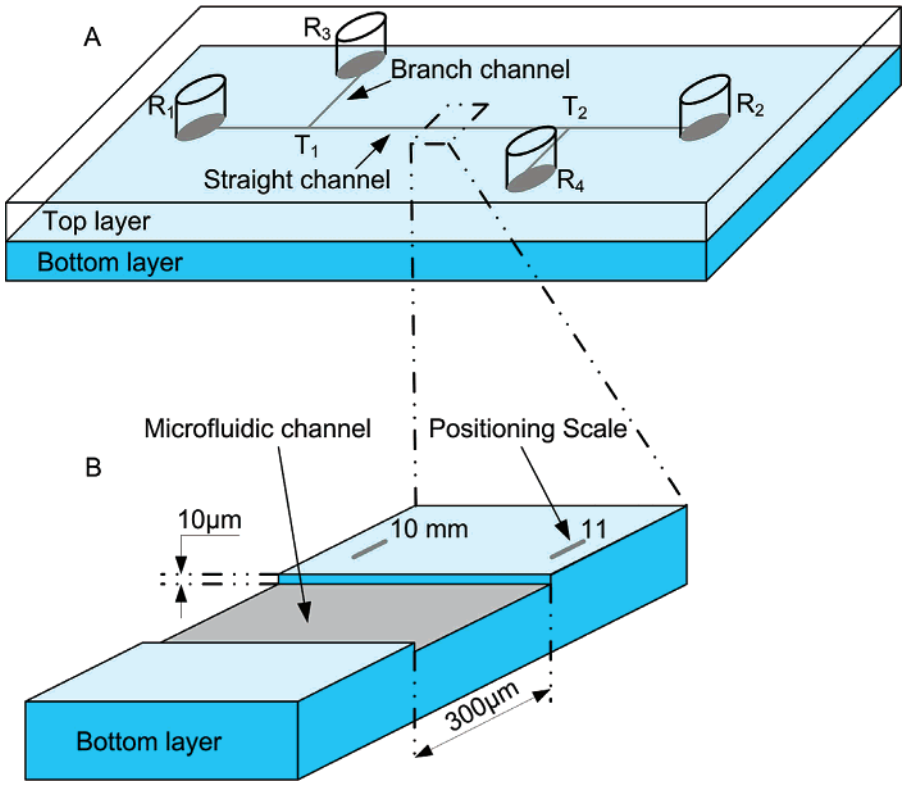

Figure 1. (A) Schematic of a PDMS microchip typically used for two-stage IEF. The length of the straight channel and branch channels are 20 and 5 mm, respectively. The chip for three-stage IEF has two additional branch channels which are not shown in this figure. R_1-R_4 are reservoirs, and T_1-T_2 are T-junctions. (B) Schematic of the microchannel with depth and width of 10 and 300 μm, respectively. A millimeter positioning scale is etched into the surface of the bottom layer.

range ampholytes (pH 3−10), 4% MC, and protein. Then, to run the first stage of electrofocusing, strong acid (50 mM H_3PO_4) and strong base (50 mM NaOH) were used as electrode solutions in the straight channel reservoirs to define a pH range of 3−10. In the second and third stages, narrow-range ampholyte solutions were diluted in 2.5% MC solution and then used as reservoir solutions to define narrower pH ranges. For example, commercially available ampholyte solutions with pH ranges of 3−4 and 6.7−7.7 can be used as anodic and cathodic solutions, respectively, to define a pH range of ~3.5 to ~7.2. Those ampholytes in the separation media that have pI values out of this narrower pH range will presumably migrate out of the separation channels and into the reservoirs during second- and third-stage IEF.

Imaging. The loaded chip was mounted underneath the objective lens of a Leica DMLB fluorescence microscope equipped with a CCD camera (SPOT RT color, Diagnostic Instruments, Inc., Sterling Heights, MI), and the channel was checked for the presence of fluorescent proteins. The fluorescent proteins were excited with a mercury lamp (Osram HBO 100 W/2) using filter cubes (DMLB 513804 and DMLB 513808, Leica Microsystems, Inc.). The images were collected through 4× and 10× objectives, and the positions of the protein bands were obtained according to a channel scale fabricated into the PDMS (Figure 1B). Electropherograms were constructed from microscope still image pixel intensities using ImageJ (http://rsb.info.nih.gov/ij) to average the intensities across the channel width after subtracting the background signal intensity from the images. In cases where the protein bands were not in the same field of view, spatial electropherograms were obtained by aligning images together based on the built-in scale. Peak resolutions were obtained by using spatial moments to estimate the peak positions and widths from the electropherograms.[23]

RESULTS AND DISCUSSION

Optimization of IEF in a single PDMS microchannel was investigated in our previous work.[19] High-viscosity polymer (MC) solutions placed in the reservoirs was used to reduce the intrusion of electrode solutions into the separation channel. Dynamic coatings of MC (0.4% in separation media) were employed on PDMS surfaces to minimize both EOF and wall−protein interactions. At an electric field strength of 50 V/cm, the overall time to reach steady state was increased by ~20% in 0.4% MC separation media when compared to the separation media without MC. As can be seen from eq 1[11], the 0.4% MC used in the separation media helps to increase resolution, most likely by decreasing the diffusion coefficient

$$\Delta(\mathrm{pI}) = 3\sqrt{\left|\frac{D}{E}\left(\frac{\mathrm{d(pH)}}{\mathrm{d}x}\Big/\frac{\mathrm{d}\mu}{\mathrm{d(pH)}}\right)\right|} \qquad (1)$$

where Δ(pI) is the pI difference between resolved baseline adjacent proteins, D is the diffusion coefficient of the protein, d(pH)/dx is the pH gradient, E is the applied electric field strength

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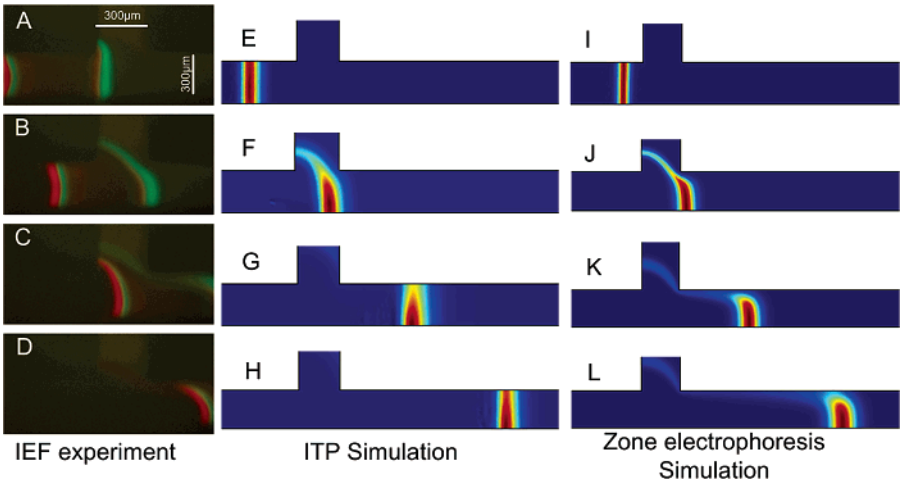

Figure 2. Band deformation at a T-junction. Time-series photos A−D were captured at junction T_1 as shown in Figure 1A at 87, 95, 107, and 123 s, respectively, after the initiation of IEF in the straight channel. Separation media: 0.4% MC, 4% Pharmalyte 3−10, PE 0.02 μg/μL, APC 0.06 μg/μL, GFP 0.04 μg/μL. Cathode solution: 50 mM NaOH containing 2.5% MC. Anode solution: 50 mM H_3PO_4 containing 2.5% MC. Voltage 100 V. Simulation results E−H and I−L are a comparison of behavior of zone electrophoresis and ITP both during and after bands pass the T-junction.

and $d\mu/d(pH)$ is the derivative of the electrophoretic mobility with respect to the pH at the protein's pI.

Equation 1 also shows that high resolution is favored by high field strengths. However, both pH gradient drift toward the cathode and bending of protein bands were observed in our previous experiments[19] as the electric field was increased to 100 V/cm in a single 2-cm channel with broad-range ampholytes. Under optimal conditions, high resolution can be achieved within 6 min in single microchannel separations using broad-range ampholytes (pH 3−10) at relatively low electric field strengths (50 V/cm).

However, in multistage IEF separations, the presence of T-junctions can lead to transient band deformation and temporarily reduced resolution. In the next section, we will first discuss band deformation at T-junctions during IEF, then demonstrate two-stage IEF and three-stage IEF and, finally, present a comparison of integrated multistage IEF with single-channel IEF.

Band Deformation at a T-Junction. In the early part of first-stage focusing, the sharpened front of a moving wave of protein (Figure 2A) approaches a T-junction (T_1 in Figure 1A). The upper part of the forward edge is stretched up and sharply twisted as it is drawn ~150 μm (roughly half the channel width) into the junction channel while the lower part of the band continues to move to the right, stretching and dispersing as it crosses the T (Figure 2B). The rear bands then catch up with the top of the front band and execute the same maneuver, dramatically lowering the resolution of the two bands (Figure 2C). After the bands have left the vicinity of the junction, they eventually refocus at slightly lower resolution as compared with the same protocol without a T-junction (Figure 2A). Figure 2D shows that a small amount of protein was left in the junction channel because it diffused deep into the junction channel and out of the returning electrical field.

The primary source of dispersion at a T-junction is the deformation of electric field lines as current passes by the open channel. This phenomenon has recently been described by Lin et al.[24] for linear zone electrophoresis, but to our knowledge, it has not been treated for nonlinear electrophoretic techniques such as IEF and ITP. One-dimensional IEF and ITP simulations are treated in detail in the works reported by Mosher et al.[25,26] and Baygents et al.,[27] and two-dimensional simulations of linear electrophoresis (zone electrophoresis) have been exhaustively investigated,[28−30] but no two-dimensional simulations of nonlinear electrophoresis (IEF and ITP) have yet been reported. Since ampholyte-based IEF simulations require computations of hundreds of components,[31] band dispersion and bending near the T-junction was instead studied using a model of ITP, which requires only about a half-dozen components to mimic dispersive behavior similar to IEF.

Although the ultimate source of dispersion at a T-junction in both linear and nonlinear electrophoresis is deformation of the field lines, the bands behave differently in linear and nonlinear systems both while and after they pass the T-junction. In particular, nonlinear systems such as IEF and ITP eventually refocus dispersed bands as shown in Figure 2E−H while linear systems such as zone electrophoresis lack the ability to resharpen the bands as shown in Figure 2I−L. The simulations suggest that multidimensional models can be developed by extending the one-dimensional models to be used in chip design to estimate dispersion and refocusing behavior in nonlinear electrophoresis at intersections. We hope to address these issues in greater detail for ITP, IEF, and other nonlinear electrofocusing techniques[32−34] soon.

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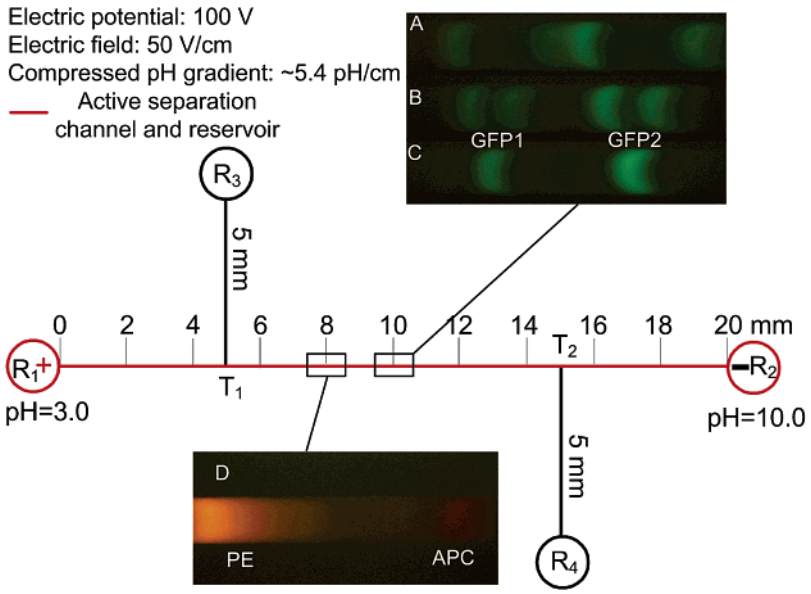

Figure 3. First-stage IEF separation in the straight channel. Photos A−C represent the final focusing stages of GFP. Photo D shows final position of PE and APC. Photos A−D at 480, 490, 580, and 610 s, respectively. Separation media: 0.4% MC, 4% Pharmalyte 3−10, PE 0.02 μg/μL, APC 0.06 μg/μL, GFP 0.04 μg/μL. Anode solution (R_1): 50 mM H_3PO_4 containing 2.5% MC. Cathode solution (R_2): 50 mM NaOH containing 2.5% MC.

Two-Stage IEF. The microfluidic chip employed to perform two-stage IEF has one 2-cm-long straight channel and two 5-mm-long branched channels (Figure 3). Initially, all of the channels were filled with a uniform separation medium, which is a mixture of protein sample (total mass ~10.8 ng) 4% ampholytes (pH 3−10), and 0.4% MC. The first stage of IEF was performed in the straight channel at an electric field strength of 50 V/cm applied between reservoirs R_1 and R_2 while reservoirs R_3 and R_4 were left electrically floating (Figure 3).

Time-series photos (Figure 3A−C) show the final focusing stages of the GFP bands. The final positions of the focused protein bands at this stage, shown in Figure 3C and D, were located between 7 and 11 mm from the anode and remained stationary. However, due to compression,[19] a 3−10 pH gradient spanned ~13 mm in this 2-cm-long channel yielding a nominal pH gradient of ~5.4 pH/cm rather than the ideal gradient, 3.5 pH/cm. Figure 4A shows that the four variants of GFP were resolved into two bright bands and two dim bands. The electropherogram in Figure 4A shows two peaks corresponding to the two bright GFP variants. The other two bands were too dim to show up in this electropherogram.

After first-stage focusing had finished, an electric field strength of 100 V/cm was applied between branch channel reservoirs R_3 and R_4 while R_1 and R_2 were left electrically floating. In an effort to refocus the protein bands in segment T_1−T_2, a narrow pH interval of ~3.5 to ~7.2 was established between the branch reservoirs R_3 and R_4 by using pH 3−4 and pH 6.7−7.7 ampholyte solutions as anodic and cathodic solutions, respectively. Ampholytes and proteins whose pIs were out of this pH interval were assumed to migrate into the anodic or cathodic reservoir shortly after the initiation of second-stage focusing.

The resolution of the two bright GFP bands at the end of the first stage (Figure 4a) was ~2.33 at an electric field strength of 50 V/cm. Immediately after initiation of the second stage, the GFP bands (Figure 4B) became tighter compared to those in Figure 4A due to their quick response to a doubling of the electric field strength from 50 to 100 V/cm. Figure 4b shows that the resolution of two bright GFP bands right after the initiation of the second stage at an electric field strength of 100 V/cm was ~2.50, which is lower than the 3.30 (Table 1) anticipated by eq 1. The discrepancy between measured and anticipated resolution arises because the GFP bands were further bent, a phenomenon that accompanied application of the higher electric field strength and is possibly due to temperature gradients[35,36] or electrohydrodynamic instabilities.[37,38]

It took a relatively long time for ampholytes to migrate to their pI positions and establish a shallower a pH gradient so the distance between two bright GFP bands (Figure 4A and B) did not increase until ~1 min after of initiation of the second stage. Figure 4C−F and c−f shows that the two bright GFP bands separated gradually

Table 1. Comparison of Measured and Expected Resolutions of Two GFP Variants during Two-Stage IEF

	end of first stage	right after doubling of electric field strength at the beginning of second stage	end of second stage
electric field strength, V/cm	50	100	100
pH gradient, pH/cm	5.4	5.4	1.85
resolution of GFP1 and 2	2.33	2.50[a]	4.68[a]
		3.30[b]	5.64[b]

[a] Experimentally measured resolutions during the second stage. [b] Resolutions were calculated based on eq 1.

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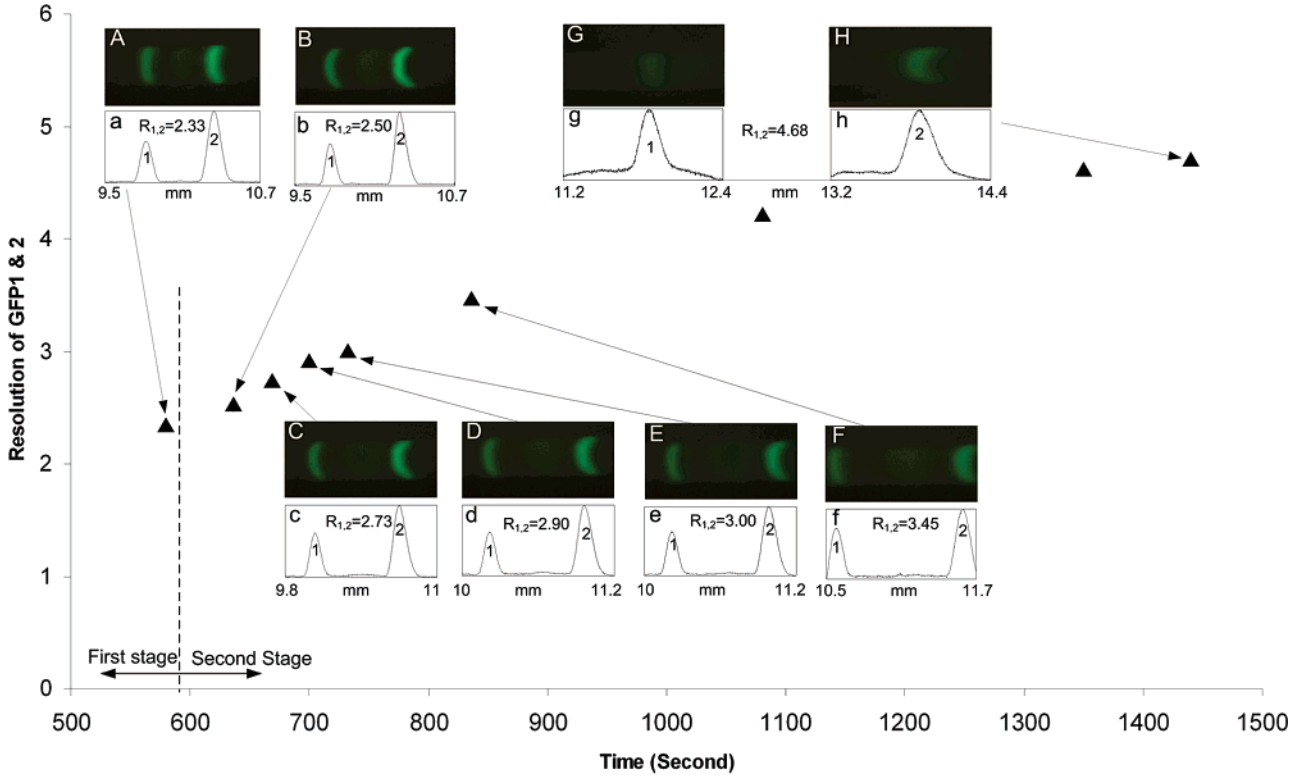

Figure 4. Refocusing of GFP in a second stage after the first stage shown in Figure 3. Graphs a–h are the electropherograms of the corresponding photos A–H. Numbers 1 and 2 stand for the two bright variants of GFP. Photos G and H represent GFP variants 1 and 2, respectively, since they were too far apart to be captured in a single photo. $R_{1,2}$ is the resolution of GFP variants 1 and 2. Anode solution (R_3): 4% 3–4 ampholytes containing 2.5% MC. Cathode solution (R_4): 4% 6.7–7.7 ampholytes containing 2.5% MC. Voltage 200 V.

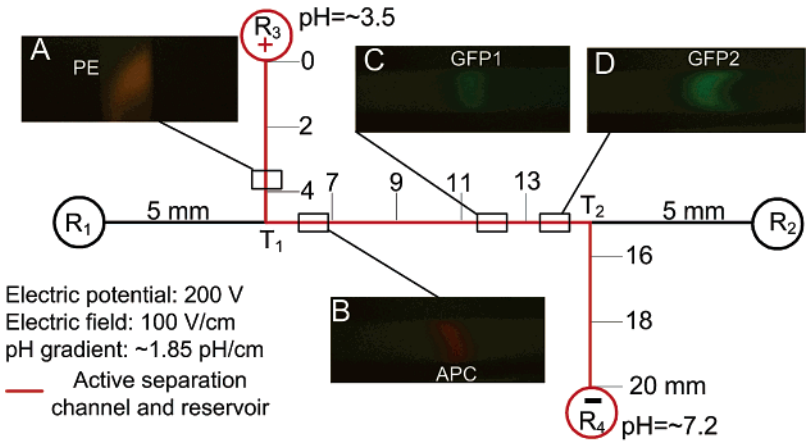

Figure 5. Final positions of refocused proteins at the end of second-stage IEF. Experimental conditions were shown in Figure 4. Photos A–D were taken at 780, 840, 850, and 870 s, respectively, after the initiation of the second-stage IEF. Resolution of GFP1 and GFP2 is 4.68, which is 2-fold greater that at the end of the first stage.

and broadened as they migrated toward their new positions with the band broadening largely due to the shallower pH gradient established in this stage. According to the pIs of these proteins and the positions in which they were focused (Figure 5), the pH gradient was estimated to be ~1.8 pH/cm. At the end of the second stage, the distance between these two bands (Figure 4G and H) was ~2.0 mm and the resolution was ~4.68, which is roughly 2-fold greater than that at the end of the first stage. Table 1 shows that the resolution of 4.68 is only 17% less than the theoretical resolution of 5.64 predicted from eq 1 using twice the electric field strength and one-third of the pH gradient in the first stage. Thus, the resolving power of IEF in this microchip was significantly increased by integration of second-stage IEF.

Three-Stage IEF. The two-stage IEF described above supports the hypothesis that a segment of the pH gradient in the first-stage channel can be successfully refocused into secondary channels. In an effort to further test this concept, we implemented a third stage on the microfluidic chip. The procedure used in the first two stages of three-stage IEF is virtually identical to that of the two-stage IEF described above. The third stage is designed to further expand a portion of the segment of the pH gradient that was expanded in the second stage.

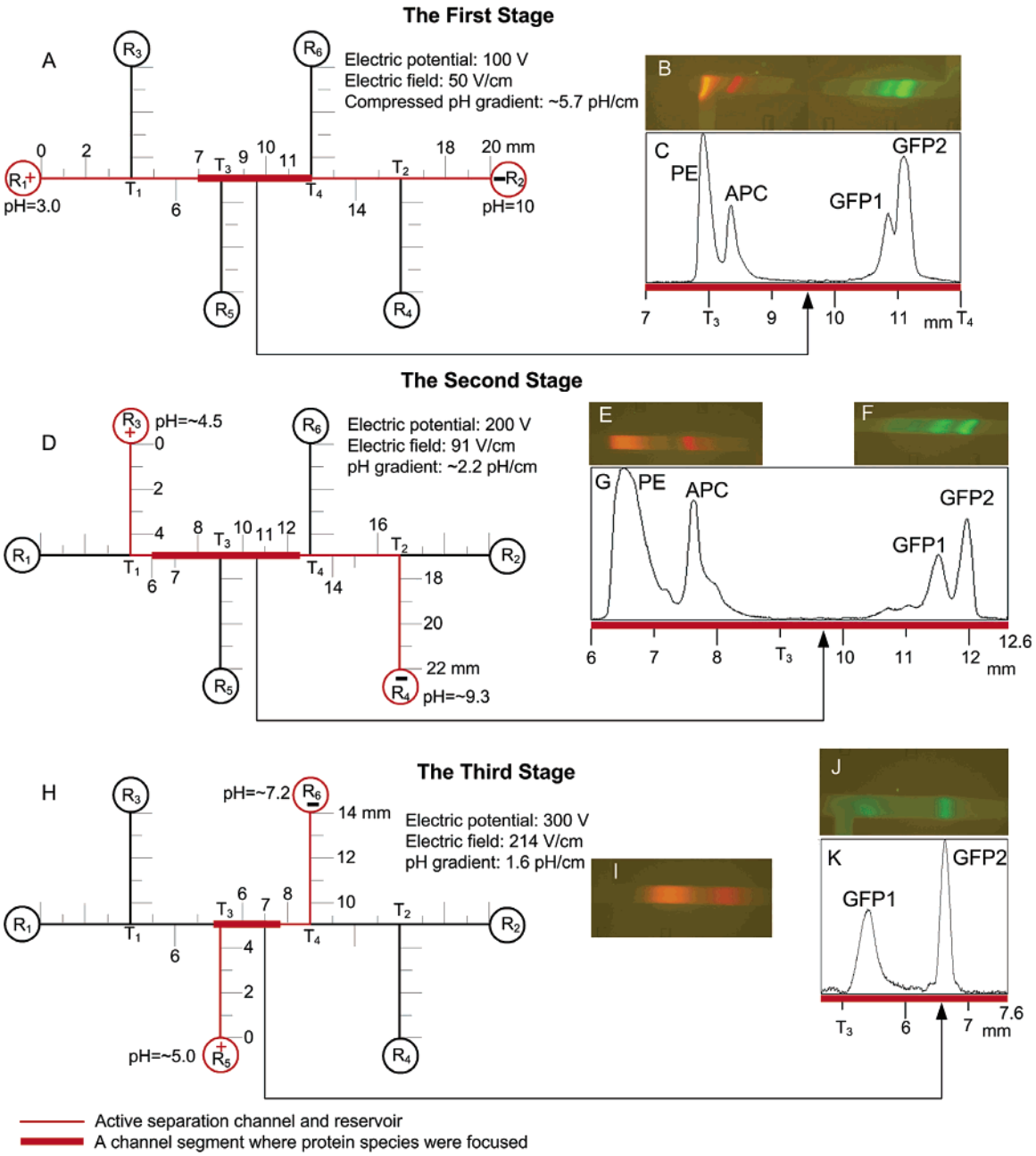

Figure 6. Three-stage IEF separation. (A, D, H) The layout of IEF at each stage. (B−C, E−G, I−K) Photos and electropherograms of separation results at each stage. Separation media: 0.4% MC, 4% Pharmalyte 3−10, PE 0.04 μg/μL, APC 0.06 μg/μL, GFP 0.08 μg/μL. Electrode solutions: R_1, 50 mM H_3PO_4; R_2, 50 mM NaOH; R_3, 4% 4.2−4.9 ampholytes; R_4, 4% 8−10.5 ampholytes; R_5, 4% 4.5−5.4 ampholytes; R_6, 4% 6.7−7.7 ampholytes. All electrode solution contains 2.5% MC. The applied electric field and pH gradient at each stage is indicated in the figure.

When an electric field strength of 50 V/cm was applied between R_1 and R_2 (Figure 6A), a compressed pH gradient of ~5.7 pH/cm was established in the straight channel. Panels B and C in Figure 6 indicate that PE, APC, and GFP bands were located in the segment T_3−T_4. It can be clearly seen from Figure 6B that PE and APC were completely resolved and that the two GFP variants were grouped tightly. Since Figure 6C was obtained by averaging the intensities transversely across the channel while the protein bands are tilted, the PE and APC peaks overlap.

The second stage was used, in part, to further separate PE and APC and, in part, to migrate them out of the segment, T_3−T_4. In this stage, pH 4.2−4.9 and pH 8−10.5 ampholyte solutions were employed as anodic and cathode solutions, respectively, and a pH gradient of ~2.2 pH/cm was established between R_3 and R_4 (Figure 6D) after an electric field strength of 91 V/cm was applied. After PE and APC migrated out of segment T_3−T_4 and before GFP reached T_3, this stage was stopped. Panels E, F, and G in Figure 6 show the positions of the protein bands at the end of the second stage. The reader should note that, at this moment, IEF has not yet achieved steady state and that all of the protein bands were still moving toward their pI positions. Compared with Figure 6C, the electropherogram in Figure 6G indicates that the protein bands were further resolved and the distance between APC and GFP1 was increased by 25%.

The third stage was designed to further separate the two GFP variants that were kept in segment T_3−T_4 at the end of the second

Table 2. Comparison of Positions of Protein Bands Obtained from Staged IEF, Single-Channel IEF and Theoretical Calculation Based on pIs with the Same pH Range and Electric Field

		staged IEF	single channel IEF	calcd based on pI
pH Range 3.5−7.2;[a] Electric Potential 200 V; Channel Length 20 mm				
distances of protein bands from anode (mm)	PE (pI = 4.38)	4.3 ± 0.5[c]	4.7 ± 0.2	4.7
	APC (pI = 4.78)	6.5 ± 0.4[c]	6.7 ± 0.3	6.9
	GFP1 (pI = 6.00)	12.0 ± 0.6[c]	12.2 ± 0.3	13.5
	GFP2 (pI = 6.31)	13.6 ± 0.6[c]	13.9 ± 0.1	15.2
distance between GFP1 and 2 (mm)		1.7 ± 0.3[c]	1.7 ± 0.2	1.7
resolution of GFP1 and 2		3.9 ± 0.7[c]	3.9 ± 0.3	
pH Range 5.0−7.2;[b] Electric Potential 300 V; Channel Length 14 mm				
distances of protein bands from anode (mm)	GFP1 (pI = 6.00)	5.2 ± 0.7[d]	5.4 ± 0.3	6.4
	GFP2 (pI = 6.31)	6.6 ± 0.7[d]	6.6 ± 0.3	8.3
distance between GFP1 and 2 (mm)		1.4 ± 0.2[d]	1.2 ± 0.1	1.9
resolution of GFP1 and 2		2.1 ± 0.4[d]	1.9 ± 0.1	

[a] 4% ampholytes pH 3−4 and 6.7−7.7 were used as anolyte and catholyte, respectively. [b] 4% ampholytes pH 4.5−5.4 and 6.7−7.7 were used as anolyte and catholyte, respectively. [c] Results were obtained from the second stage of two-stage IEF. [d] Results were obtained from third stage of three-stage IEF. Each data point represents the average of three measurements and the standard error.

stage. In the third stage, pH 4.5−5.4 and pH 6.7−7.7 ampholyte solutions were employed as anodic and cathodic solutions, respectively. After an electric field strength of 214 V/cm was applied, a shallower pH gradient of ~1.6 pH/cm was established between R_3 and R_4 (Figure 6H). Panels J and K in Figure 6 show that two GFP variants were separated by ~1 mm while the PE and APC bands in Figure 6I have broadened, most likely due to diffusion since they were outside of the electric field (Figure 6H).

This microfluidic chip (Figure 6) could also be used to process two different second-stage IEF separations. In this case, R_3 and R_5 could be used as anodic and cathodic reservoirs to expand the segment T_1−T_3 while the segment T_4−T_2 could be expanded between reservoir R_6 and R_4. These two separations can be performed serially or in parallel if a mechanical valve[10,39] is implemented between T_3 and T_4.

Comparison of Integrated Multistage IEF with Single-Channel IEF. An alternative way to improve resolution of microchip IEF is by performing single-channel IEF in narrow pH ranges and at higher electric fields. To compare this with the second stage of two-stage IEF and the third stage of three-stage IEF in the same separation media, the electrode solutions and electric fields were chosen to set up pH ranges of 3.5−7.2 and 5−7.2 in a single channel, respectively. Table 2 shows that both cases give similar results in the pH ranges of 3.5−7.2 and 5.0−7.2. When compared to theoretical calculations based on protein pIs (Table 2), the pH gradient in both cases is shifted ~1.5 mm toward the anode. This shift is most likely due to drift of the pH gradient,[40,41] a major drawback inherent in ampholyte-based liquid-phase IEF at high voltages.

Ideally, staging allows the initial fractionation over a broad range, pH 3−10, in the straight channel to be distributed across a multichannel array of seven secondary stages, each secondary stage covering a pH unit. By contrast, to accomplish the same thing in a single-channel IEF chip would require seven parallel channels to cover pH 3−10 with a unit pH in each channel. The advantages of performing narrow-range IEF in a single channel are elimination of dispersion arising from the T-junction and all seven parallel channels can be run at the same time, which reduces analysis time. However, if valves[10,39] are implemented in a multistage IEF chip, the secondary stages can be run in parallel, instead of serially, and dispersion arising from the T-junctions could be reduced. A major advantage of integrated multistage IEF over parallel single-channel IEF is the compact design of the former, especially when detection, integration of second-dimensional separation, and integration to mass spectrometers are considered.

Multistage IEF provides tertiary stages when the resolving power provided by a secondary stage is not high enough to separate out the protein of interest. However, for single-channel IEF, when a narrow pH range is not capable of separating out the target protein, it is difficult to further resolve this prefocused target protein out of contaminating proteins.

CONCLUSIONS

Multistage IEF of an ampholyte/protein/polymer mixture has been demonstrated in a microfluidic chip. Sequentially shallower pH gradients were successfully established in the second and third IEF stages, allowing the target proteins to refocus at 2× higher resolution in the later stages, thus further resolving GFP variants beyond the first stage.

The final positions of the protein species in the later focusing stages are determined by their pIs in the newly established pH gradients. The use of narrow pH range amphoytes as anode and cathode solutions to set the pH values of two end points allows ready control of pH range inside the channel. The directions that protein bands are going to mobilize are determined by their current positions and the prospective positions in the newly established pH gradient, which is eventually defined by the pH values of the anode and cathode solutions. These experiments have demonstrated that controlled mobilization of the protein species enables selective separation of target proteins.

These experiments also showed that band deformation at a T-junction is primarily due to the deformation of electric field lines as current passes through the T-junction. Two-dimensional simula-

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tion of nonlinear electrophoresis showed excellent agreement with the IEF experiment of band deformation at a T-junction and also illustrated the difference in behavior between linear and nonlinear electrophoresis during and after protein bands pass a T-junction. When T-junctions are relatively close in three-stage IEF chips, the focused protein bands are skewed due to deformation of the electric field. Such deviations reduce the axial resolution, but this distortion may potentially be reduced by implementation of PDMS valves.[10,39]

Multistage IEF provides increased resolution of proteins since the serial application of IEF allows on-chip fractionation in a broad pH range followed by subfractionation in a shallower pH gradient without additional reagents. The microchip design flexibility afforded by soft lithography allows us to increase resolution beyond the 2× demonstrated here by increasing the number of primary channel segments as well as the number of secondary branches. The implementation of valves should also speed the process by allowing tandem, parallel operation of the stages rather than the serial operation illustrated here. Eventually, it is expected that the multistage unit operation will be incorporated into a larger network as a single dimension in a separation cascade that leads ultimately to mass spectrometry.

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