Supporting Information to

Cascaded Free-flow Isoelectric Focusing for Improved Focusing Speed and Resolution

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Analytical Scaling Approximations for IEF

Mass transport equations are used as the starting point to formulate expressions to describe isoelectric focusing. The conservation equation for species in solution experiencing an electrophoretic force is given by:

$$\frac{\partial C_i}{\partial t} = \underline{\nabla} \cdot \left(D_i \underline{\nabla} \cdot C_i - \underline{E} z_i \mu_i C_i \right) \tag{S-1}$$

In Equation (S-1) the concentration of a species C_i is a function of position and time, electric field E, variable charge z_i, constant mobility μ_i, and its diffusion constant D_i. In order to understand the effect of geometry and physical parameters on focusing, it is helpful to non-dimensionalize the conservation equation for one-dimensional transient focusing and make two assumptions: i) a linear pH gradient is established over the total width, w, and ii) the species has a linear charge behavior in the channel ($dz_i/dx = \Delta z_i/w =$ constant). Non-dimensionalizing and expanding the derivative:[1]

$$\frac{\partial \Theta}{\partial \tau} = \frac{1}{Pe_e}\frac{\partial^2 \Theta}{\partial \eta^2} - \Theta - \frac{\partial \Theta}{\partial \eta}\left(\eta - \eta_{pI}\right) \tag{S-2}$$

The dimensionless quantities are defined as $\Theta = C_i / C_{i,0}$, $\eta = x / L$, $\tau = t / t^*$, and $Pe_e = E\mu w \Delta z / D$. The characteristic focusing time is defined as $t^* = w / E\mu\Delta z$. The location where the species focuses is defined by η_{pI}. The ratio of electrophoretic to diffusive fluxes, Pe_e, is a Peclet number similar to Peclet numbers encountered in other separation systems, typically the ratio of convective to diffusive fluxes. This number must be much less than 1 for electrophoretic forces to dominate the system.

The steady state solution takes the form:

$$\Theta = \sqrt{\frac{Pe_e}{2\pi}}\, exp\left(-\frac{\left(\eta - \eta_{pI}\right)^2}{2} Pe_e \right) \tag{S-3}$$

The Gaussian distribution of Equation (S-3) has a standard deviation of $Pe_e^{-1/2}$. By defining separation distance as 1.5 standard deviations on either side of the isoelectric point (87% of the peak area), the resolution (*Res*, the minimum difference pI divided by the pH range within the channel) becomes:

$$\frac{\Delta pI_{\min}}{\Delta pH} = \frac{3}{Pe_e^{1/2}} = 3 \cdot \sqrt{\frac{D}{E\mu w \Delta z}} = \frac{1}{Res} \tag{S-4}$$

This result is equivalent to the expressions of Vesterberg and Svensson [2] and Rilbe [3] for the minimum resolvable difference in pI. At excessively high electric fields, detrimental phenomena such as Joule heating, electroosmotic flow, and precipitation prevent ideal focusing. Therefore for any sample composition, there is an optimal electric field strength that maximizes resolution while minimizing negative effects. To examine focusing behavior in a variety of geometries, the electric field (as opposed to applied voltage) was assumed to be the same for every case. For the time scaling in Equation 2,

the time scale of focusing (t^*) increases linearly with respect to channel width, w. From Equation (4), the resolution at steady state increases with $w^{1/2}$. These dependencies show that there is a trade-off between short focusing times and resolution when the applied field is constant. This trade-off can be exploited with multiple stages to deliver high resolution separations with a minimal focusing time, analogous to shifting gears in order to maximize a vehicle's acceleration.

Considering instead the case of constant applied voltage ($V = Ew$), the time scale of focusing increases with w^2, whereas the steady state resolution is independent of distance, consistent with the findings of Das and Fan.[4] In practice, however, using a constant voltage for a range of channel widths is less feasible than maintaining a constant electric field. For example, applying the high voltages typical in cm-scale IEF (200-500V) to a channel 1 mm in width would result in field strengths of 2-5 kV/cm. Electroosmotic flow would increase by a factor of 10, and Joule heating would increase by a factor of 100. A thermally insulated 1 μL sample with conductivity similar to 2% Ampholine in deionized water (0.3 mS/cm) would be exposed to 1.3-7.5 W of electrical power, heating it at a rate of 300-1800 °C/s. Such dramatic Joule heating effects necessitate lower applied voltages for electric fields on the order of 200 V/cm, as well as active device cooling.

Simulations of BSA Focusing in Channels of Varying Widths

The numerical model discussed in the main text was also used to simulate the focusing of BSA in a channel either 1 or 3 mm long. Figure S-1 shows the simulated focusing of BSA under three different geometry configurations at identical field strengths: 1 mm, 3 mm, and 1 mm followed by focusing at 3 mm. The protein focusing follows the "double-peak approach" observed by others[5-8]: peaks form at both electrodes, move toward the pI,

and coalesce. To better quantify focusing, the simulation used low electric fields (5 V/cm for each case) for slow, low resolution focusing. The resolution (from a Gaussian fit, $R^2 > 0.999$) of the focusing is 1.6 times greater for the longer channel. Transitioning from a shorter to a longer channel reaches this higher-resolution steady state in 40% less time than the 3 mm case. These results demonstrate that the predictions for small amphoteric molecules in terms of channel geometry and residence time also extend to large proteins with complicated electrophoretic behavior.

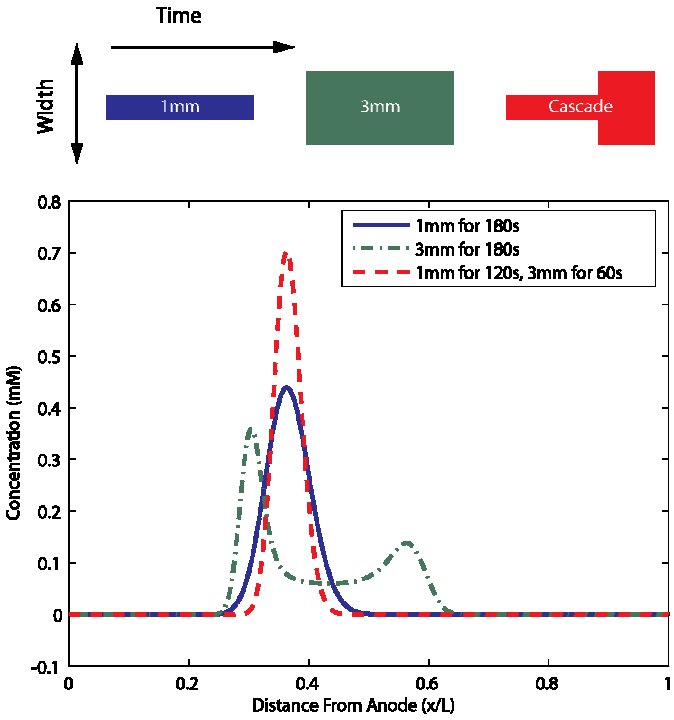

Figure S-1: Simulated focusing of a protein in channels of different widths. Solid line is steady state focusing after 3 min in a 1 mm wide channel; dash-dot line is a 3 mm channel, incomplete after 3 min. Dashed line is focusing in a 1 mm channel for 2 min, followed by a 3 mm channel for 1 minute. This more resolved steady state is reached in 40% less time.

Reduction of Albumin for Western Blotting Applications

In addition to concentrating important proteins, FF-IEF prefractionation tools have utility in reducing the detection interference caused by common contaminants. Species

present in cell culture media, such as albumin, can distort blotting results if the two proteins have similar sizes. The kinase JNK has a molecular weight near the bovine serum albumin in DMEM media, making it difficult to detect in samples rich in cell media. FF-IEF offers an orthogonal separation step to further reduce distortion. Figure S-2 shows how albumin can be separated from molecules of interest. For samples lysed in albumin-rich media, the albumin was observed to be concentrated in a few outlets (to the point of overloading the gel for #4), reducing the distortion in the rest of the pH fractions. In this case incomplete focusing at low field strengths was useful in reducing BSA and improving in the detection sensitivity of the other outlet fractions.

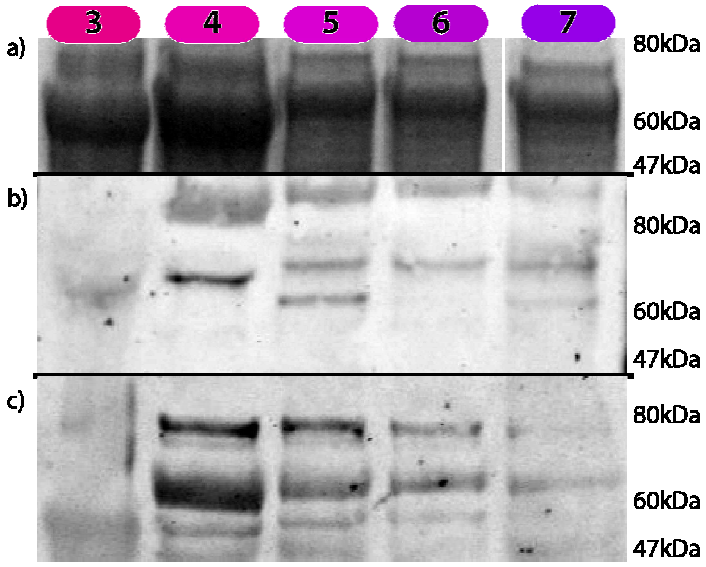

Figure S-2: Coomassie stained gel (a) and JNK western blots for (b) total JNK and (c) phospho-JNK. Whole HeLa cell lysed with 1% Triton in DMEM culture medium with 4M urea is processed at 111nL/s (18s of focusing) at ~150V/cm.

Protein Complex Focusing with a Narrow pH Range

The versatility of FF-IEF allows for many types of sample compositions, such as different pH ranges and denaturing conditions. Narrow pH ranges can be used in the device to separate proteins with similar pI, whereas milder surfactant conditions enable

focusing of protein complexes. Figure S-3 shows whole cell lysate focused in the 5-7 pH range in the presence of 4M urea. Bands were scaled to represent fold concentration over the averaged outlet sisgnal. Here, protein bands corresponding to cytochrome C, and pAKT show reasonable focusing and at least 2-fold enrichment over the lysate alone. However, ERK2 shows weaker focusing, presumably due to the multiple pIs across the 5-7 range for the various phosphorylated forms of ERK[9]. Of note is the focusing of cytochrome C to an average pI of approximately ~5.7, far from the expected value of ~9.6. This apparent shift in pI is most likely due to the focusing of a protein heterocomplex (e.g. partially disrupted mitochondria) which includes cytochrome C and is denatured by NP-40. Figure 7 in the main text shows cytochrome C focusing normally at the high end of a 3-10 pH gradient in the presence of 4M urea as well as 0.45% NP-40. This shift in cytochrome C focusing in the presence of a surfactant supports the hypothesis that it participates in a protein complex that focuses at a more neutral pI.

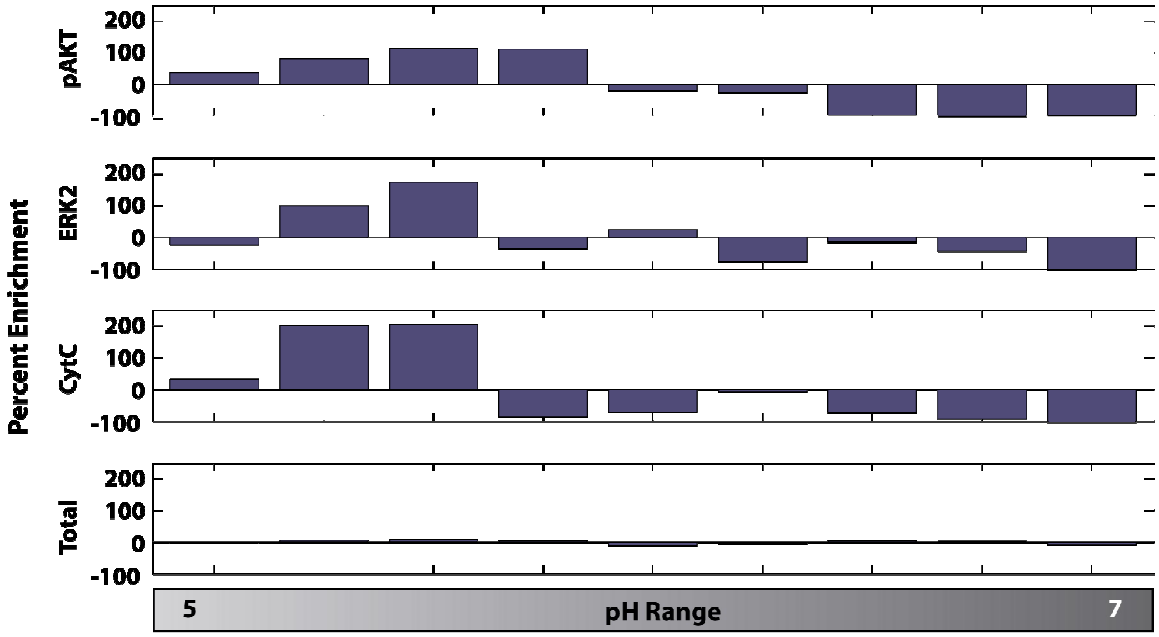

Figure S-3: Immunoblot and silver stain results for signaling proteins in HeLa cell lysate focused in a 5-7 pH gradient. Focusing of ERK2 and phospho-AKT is comparable to Figure 7 in the main text, whereas cytochrome C focuses to a pI estimated at ~5.7. These proteins are concentrated and collected from whole cell lysate at 111nL/s (14s of focusing) at ~300V/cm.

References

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