

**Development of Microfluidic Based Western Blot Technology for Fast and High-
Content Analyses**

by

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ABSTRACT

Development of Microfluidic Based Western Blot Technology for Fast and High-Content Analyses

By

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Western blotting has been a highly valued method in research for protein identification and relative quantitation in complex biological samples. Although widely adapted, Western blot assays have many limitations including labor intensive, low throughput, low information content, large sample consumption, and mediocre repeatability.

A microfluidic Western blotting system based on protein sieving on microchips followed by direct blotting onto a moving membrane is described. By flowing same separation sieving buffer by the sides of separation channel, protein bands are well defined and transferred onto membrane with minimum band broadening, which is an improvement to a previous hybrid capillary Western blotting system. Separation speed is significantly improved on a microchip due to a high electric field applied. A complete Western blot for actin is finished in about 25 min with 0.7 nM detection limit.

While speed of separation is important to some applications, in many cases resolution between closely related protein isoforms is required. 2 kDa different proteins are used to demonstrate the achievement of improved separation resolution by modifying channel dimensions and lengths. With an 8 cm long separation channel, ERK1 (44 kDa) and ERK2 (42 kDa) are baseline resolved on the membrane within 8 min.

Another advantage of using microfluidic devices for Western blotting is the potential for high throughput and high content analysis. Up to seven parallel separation channels, each with 3 sample reservoirs, are accommodated on a 3 inch by 3 inch glass chip. 21 Western blots are completed within 30 min. To demonstrate the capability of detecting multiple proteins using a small amount of cell lysate sample, 11 proteins are detected using less than 200 nL lysate sample (0.4 μ g total protein). For comparison, traditional assays would require at least 90 μ L (90 μ g total protein) to finish the same task.

Overall, microfluidic based Western blotting technology has shown promise for fast analysis, low sample consumption, and multiplexing over traditional methods.

Chapter 1 Introduction

Western Blotting Backgrounds

Western blotting, which combines protein sizing and immunoaffinity is a powerful tool to detect proteins in life scientific study. It has remained popular since it was introduced in 1979.¹ The technique is routinely implemented for basic research and also as a confirmatory test for certain diseases, like HIV, Lyme disease, and Hepatitis B.^{2,3} A schematic drawing of all steps in Western blot is illustrated in Figure 1-1. Briefly, proteins are separated according to sizes on a slab gel. All protein bands are then transferred onto a membrane for immunoassay. An antibody that is specific to the target protein is added for detection. Detailed procedures and subtleties are discussed in this chapter.

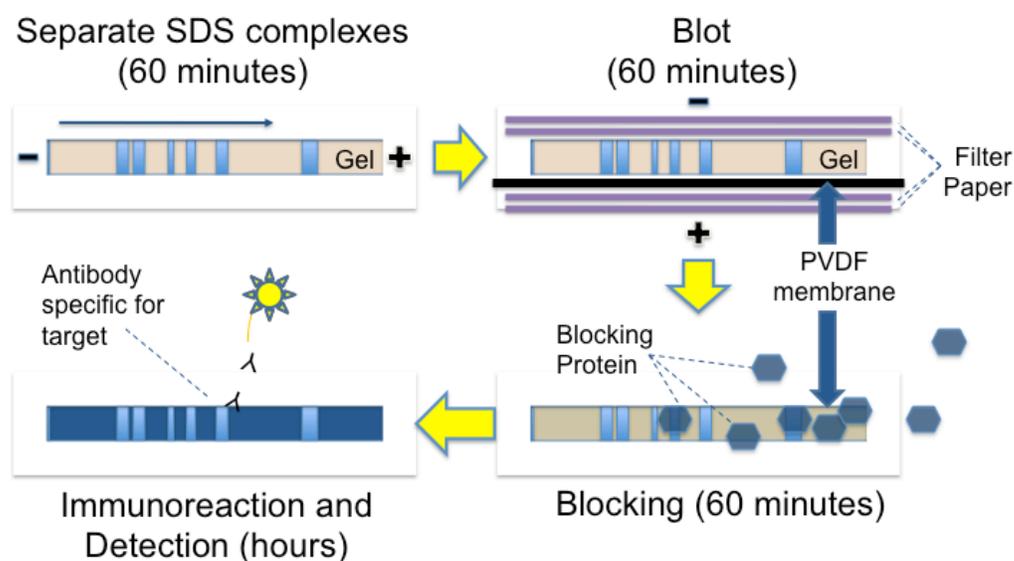


Figure 1-1. Workflow of a traditional Western blot. A full Western blot assay usually takes 6-12 hours.

In a typical Western blot, lysates are prepared from cell culture or tissues. When using serum as sample in a Western blot, it is common to dilute the sample or do albumin depletion since majority of the content is albumin. Sample is then denatured at 95 °C for 5 min in sample buffer containing glycerol, sodium dodecyl sulfate (SDS), and beta-mercaptoethanol (BME) or dithiothreitol (DTT). After the protein denaturing process, disulfide bonds are reduced, and sampled proteins become covered in the negatively charged SDS. It has been shown that the ratio of SDS to protein for most globular proteins is a constant at 1.4 mg SDS per mg protein.⁴ Due to the uniform charge per unit length of the polypeptide chain, the mobility variability among different SDS-protein complexes no longer relates to the sequence of amino acids but solely to the length of the polypeptide, which directly relates to the molecular weight of the protein.

After loading the sample onto gel, voltage is applied across the separation length, and proteins are separated using SDS-PAGE. Negatively charged SDS-proteins move toward the positively charged electrode through the polyacrylamide mesh. Smaller proteins move faster than the larger proteins and there is a linear relationship between the log value of molecular weight and the reciprocal of migration time.

Speed and resolution are related to the gel pore structure. The pore size is controlled by varying the concentration of acrylamide at a fixed concentration of crosslinking agent. The lower the acrylamide concentration of the gel, the better resolution for high molecular weight protein separation. For example, 7.5% gel gives better resolution between 150 kDa and 200 kDa than 12% gel.⁵ Gradient gel is also popular to separate a wide range of proteins. The most common gradient gel contains 4-20% acrylamide and based on the size of proteins being separated the range could be

adjusted. Cross-linked gels are of sufficient mechanical strength that they could be handled easily for follow-up studies.

While many applications use crosslinked polymer matrixes, non-crosslinked polymer matrixes have also been successful, especially for capillary-based and microdevice-based SDS-PAGE applications.^{6,7} The main reason is that the polymer solution could be replaced easily after runs and multiple runs could be obtained from the same capillary with low loss in performance. Details will be discussed in the following section.

After separation the proteins are transferred from the slab gel onto a nitrocellulose membrane or a polyvinylidene difluoride (PVDF) membrane. There are different approaches reported for transferring, such as simple diffusion, vacuum-assisted solvent flow, and electrophoretic elution. Among these methods, electroblotting is the most commonly used procedure to transfer proteins from a slab gel to a membrane. The main advantages are the speed and the completeness of transfer. In a typical wet electrophoretic transfer, membrane and gel are sandwiched between two pieces of filter paper and placed in ice-cold transfer buffer, which usually contains 10-20% methanol. When electric field is applied across the sandwiched layers, proteins move from the gel onto the membrane while maintaining separation information within the gel. Depending on the molecular weight of the target proteins, the transfer step could take between 30 min to 2 h.

The final step is the immunoassay. The membrane is first treated with blocking solution to block any non-specific binding sites. Typically 3%-5% bovine serum albumin (BSA) or non-fat dry milk in Tris-Buffered Saline (TBS) with 0.1% of detergent such as

Tween 20 or Triton X-100 is used. Blocking usually takes about 1 hour to finish. This step is necessary to reduce background in the final product of Western blots and eliminates false positives. The membrane is then probed with a specific antibody to detect a target protein. It is very common to have a two-step incubation, i.e. using a primary antibody and a secondary antibody. Because all the primary antibodies harvested from same animal hosts have the same Fc fragments, they can be recognized by same type of secondary antibodies. Therefore the cost of doing a two-step incubation is much lower than labeling individual primary antibodies for detection. The antibody incubation time also varies based on the specificity of the antibody. Usually the membrane is incubated in diluted primary antibody solution for more than 12 hours in a cold room.⁸ Then the unbound primary antibodies are washed away from the membrane surface. The membrane is then probed with secondary antibody for 1 hour at room temperature.¹⁰

After washing away the unbound probes, the Western blot is ready for detection. Commonly used methods for detecting proteins include using radioactive, enzyme-linked, or fluorescent-labeled reagents.⁹ Using radioactive detection methods make it easy to quantitate the antigen on membrane by using a scintillation counter. Chemiluminescence has been used for the detection of protein bands on X-ray film similar to radiolabeled probes. After binding of secondary antibody conjugated with an enzyme, normally horseradish peroxidase (HRP), the substrates are added. Chemiluminescence occurs when the substrate is catalyzed by HRP, and produces light as a by-product. The image of the blot is visualized by exposing the blot to film. The major drawback is the fact that quantitative results cannot be obtained. The fluorescent detection captures a digital image of the Western blot and allows for simultaneous

detection of several target proteins on the same blot by using multiple and differently colored fluorophores. Many fluorescent molecules are also stable for a long period of time, allowing blots to be stored for re-imaging at a later date. It is considered to be one of the best methods for quantification but is less sensitive than chemiluminescent detection.

Although reliable, robust, and widely adapted, Western blotting has some well-known drawbacks. It usually requires 8–20 h to complete, including gel preparation, sample treatment, separation, transfer, multiple rounds of incubations and washings. Most of these steps are performed manually, which makes the technique labor intensive and can decrease reproducibility. Analysis of large proteins is also hindered by difficulty of transferring them from slab gels onto membranes. Western blotting has not been miniaturized, which wastes materials and reduces sensitivity. The current protocol is also not suitable for sample limited applications because usually 10-20 µg total protein is needed for one assay. Another problem is the difficulty of determining multiple proteins in one sample. Multi-protein analysis is accomplished by removing antibody from the blotting membrane (also called “stripping”) followed by reprobing with antibodies for the next protein target.¹⁰ This requires re-performing the slowest step of the process. Besides, the antigens are very likely to be partially removed in the harsh antibody-removing environment, reducing the sensitivity for the next probing process.

Products Related to Western Blotting

Several commercial products have been released to improve different stages of Western blotting since it is a widely used technique in biochemical and clinical

applications. This is an ever-growing field with an estimated \$1 billion global market ^{11,12} including consumables and instruments costs.

Millipore has released a fast immunoassay module, called SNAP i.d. protein detection system.¹³ The instrument enables rapid blocking, antibody incubation, and washing by applying vacuum to pull solutions through the blots after separation and transfer are done. By shortening the time for immunoassay from 8-12 hours in traditional protocols to less than 30 min, the SNAP i.d. system offers high throughput analysis and optimization the immunodetection conditions. The system also enables antibody recollection to further reduce the reagents cost. Figure 1-2 illustrates the comparison between traditional immunoreaction and SNAP i.d. method. While traditional method relies on antibody diffusion into membrane, SNAP i.d. pulls the reagents through membrane and distributes antibodies evenly.¹³ In chapter 2 and chapter 4, we use this technique to achieve fast immunoassay. However, the sensitivity is not as good as using traditional immunoassay protocols. The current SNAP i.d. system is also not ideal for multiplexing analysis. In chapter 5, we discuss solutions to solve these drawbacks.

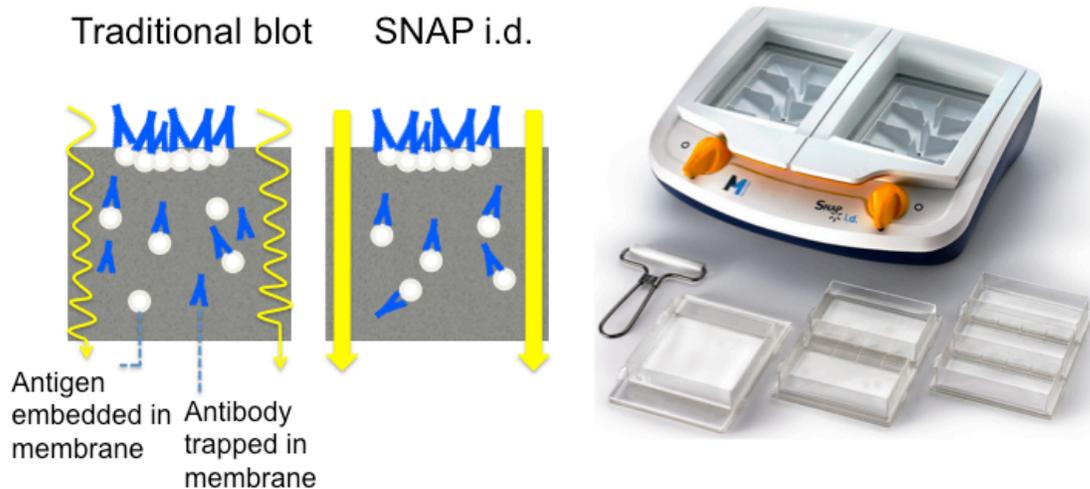


Figure 1-2. SNAP i.d. system overview. It is used to facilitate the immunoreaction step in Western blot. Compared to traditional method, vacuum is applied underneath the

membrane to evenly distribute the antibody solution thus improves efficiency and speed. Immunoreaction could be done in 30 min.

iBind released by Thermo Fisher Scientific focuses on reducing the immunoassay time. It compresses overnight incubation time to about 2.5 hours. iBind Western Systems allow all solutions to be prepared and loaded in the device at the start of the procedure, from which point all steps proceed automatically. Using a unique design, blocking reagents, antibody reagents and wash buffer sequentially flow through the membrane surface. This sequential lateral flow technology, i.e. simple capillary action, allows for timely release of antibodies onto the blots and a constant flow rate over the incubated membrane.

One group also uses microfluidic networks to apply reagents to a membrane from conventional PAGE.¹⁴ In this method, once a separation is done and transferred from the gel to a membrane, a fabricated microfluidic network with 5 microchannels is placed over the protein bands and sealed to the membrane. Multiple proteins are simultaneously probed by flowing different antibodies through the 5 channels. Comparing the antibody consumption with conventional Western blot protocols, the new antibody introduction approach significantly reduces the volume required to less than 1 μL in each microchannel. Figure 1-3 below illustrates the idea of this microfluidic based immunoblotting technique.

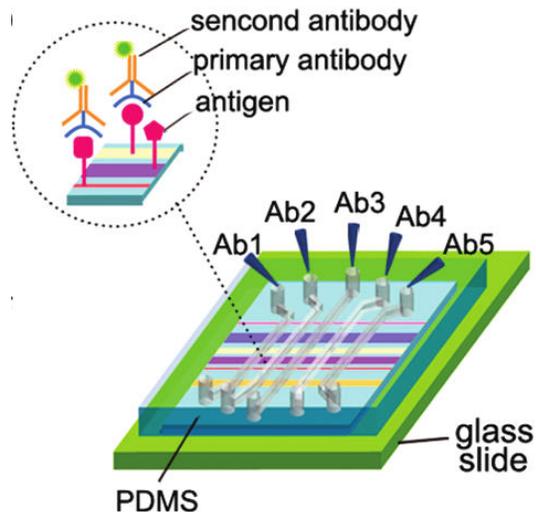


Figure 1-3. Illustration of microfluidic based immunoblotting technique. Proteins are transferred from a polyacrylamide gel to PVDF membrane by electroblotting. A polydimethylsiloxane (PDMS) microfluidic network is assembled with the blotted PVDF membrane. The microfluidic channels are oriented perpendicular to the protein bands on the membrane. 5 antibodies for specific proteins are introduced in parallel microfluidic channels.¹⁴ Reprinted with permission from Pan, W.; Chen, W.; Jiang, X. *Anal. Chem.* **2010**, *82* (10), 3974–3976. Copyright (2010) American Chemical Society.

There are products focusing on improving protein transfer speed. Thermo Fisher Scientific introduced a fast western transfer system called iBlot, which features 7 min protein transfer from gel to membrane.¹⁵ The iBlot dry blotting requires no transfer buffer and no external power supplies. The rapid transfer is possibly due to the unique design to reduce the distance between the electrodes and the integrated power supply so that the system could generate a high field and increase transfer speed. A similar product developed by Bio-rad relies on semi-dry transfer of proteins and the transfer time can be as short as 3 min.¹⁶ A gel is sandwiched between two ion reservoir stacks prewetted with transfer buffer. This allows for fast and efficient transfer possibly due to a high transfer field applied.

Another product by GE Healthcare fully integrates Western blotting.¹⁷ This analyzer uses traditional protocols of each step in Western blotting. By integrating every

step, assay variability is minimized. Reliable normalization is also achieved by multiplexing housekeeping proteins as controls.

Capillary Electrophoresis of Proteins

SDS-PAGE for proteins is the first step in Western blotting. It resolves the components of complex systems. But traditional forms of electrophoresis suffer from several disadvantages. Perhaps the most obvious one is the speed of separation. Typical SDS-PAGE takes about 1 hour due to relatively low electric field applied (100-150 V).¹⁸ The use of capillaries as an electromigration channel for separation was introduced¹⁹ and presents fast separations due to the high surface-to-volume ratio, allowing for efficient heat dissipation from high electric fields applied. For example in this work we were able to apply 460 V/cm through the microchannel and achieve 2 min separation of ladder from 11 kDa to 155 kDa.

Capillary electrophoresis (CE) performance is commonly discussed in terms of resolution and efficiency. Efficiency, or theoretical plates can be calculated using the equation below¹⁶

$$N = \frac{L^2}{\sigma^2} = \frac{V\mu}{2D}$$

where σ^2 is the variance of the zone, D is the diffusion coefficient, L is the length of separation, and V is the voltage applied across the capillary. A high voltage is usually favored to get good separation as long as it does not introduce too much variance caused by temperature.

Resolution (R_s) is a numerical index assigned to adjacent peaks to assess the ability to discriminate between them.¹⁶

$$Rs = \frac{\Delta W}{W} \left(\frac{N}{16} \right)^{1/2}$$

where $\Delta W/W$ is the relative velocity difference of the two peaks. It is noticed from the equation that higher separation efficiency improves resolution.

Another term often used to describe separation power is peak capacity, n_c . It is the maximum number of separated peaks that can be fit into the path length.²⁰

$$n_c = \frac{L}{4\sigma Rs} = 1 + \frac{N^{1/2}}{4} \ln \frac{V_{\max}}{V_{\min}}$$

where V_{\max} and V_{\min} are the largest and smallest volumes in which zones can be eluted and detected. This equation illustrates that peak capacity is proportional to the separation efficiency. A higher electric field for separation would increase the peak capacity.

A lot of work has been published using CE for small molecule separations. CE for protein separation, however, is not favored especially in uncoated bare capillaries. One early work on CE of peptides and proteins emphasized the adsorption problems.²¹ To minimize the electrophoretic attraction of a protein to silica, coating the capillary wall is a solution.²²

Other forms of CE for proteins, such as capillary gel electrophoresis (CGE) follow capillary zone electrophoresis. CGE was first implemented by using the same cross-linked polyacrylamide chemistry of traditional SDS-PAGE, this time in a capillary to separate monomers, dimers, and oligomers of bovine serum albumin.²³ However some early work indicated that using crosslinked gels was not very satisfactory in capillary columns due to inhomogeneity, bubble formation, limited column lifetime and poor reproducibility. When choosing the crosslinked gel filled capillaries for protein

separation, another problem is that polyacrylamide absorbs at low UV wavelengths, making high sensitivity detection difficult.

To solve the problems related to using cross-linked gel in SDS-CGE, replaceable non-crosslinked entangled polymer has been used. It is a solution of hydrophilic polymers dissolved in an appropriate buffer. The first proposed application was for the separation of DNA.²⁴ An attractive characteristic of the gel solution is that it is easy to be rinsed out from the capillary and regenerated using pressure.

It is anticipated that the electroosmotic flow (EOF) still exists in the capillary and would be a problem in the use of the non-crosslinked gel solution. EOF is the result of ionized silanol groups on the inner capillary wall that cause a net positive charge for the buffer. Under an electric field, the bulk solution will be pushed toward cathode.²⁵ Any existing EOF will drive the negatively charged SDS-proteins to the opposite direction of electrophoretic separation. Therefore the surface must be treated to suppress EOF. A permanent modification of the capillary wall using silylation was the most attractive alternative in controlling protein-wall interactions as well as the extent of electroosmotic flow.²⁶ The reactive surface silanol groups provide opportunities for a chemical modification. (3 glycidoxypropyl)-trimethoxysilane was first reported to bond to the capillary wall as early as 1983.²¹ A similar silylation method was also reported by Hjerten for controlling electroosmotic flow in capillary isoelectric focusing.²⁷ However, covalent coatings have a limited lifetime, and reliable coverage of the capillary surface is both difficult and crucial for high efficiency separations.^{28,29}

In theory, the electroosmosis value will approach zero when the viscosity in the double layer close to the tube wall approaches infinity.²⁷ This leads to the dynamic

coating method to suppress EOF and also prevent molecule adsorption. Dynamic coating is usually carried out by small ionic, zwitterionic, or nonionic molecules and especially by low concentrations of certain water-soluble nonionic polymers.²⁸ It is well documented on coating the walls with a polymer such as polyethylene glycol (PEG)³⁰ and methyl-hydroxyethyl cellulose (MHEC)³¹.

To summarize, in a typical CGE experiment for protein separation, the capillary wall is first treated with either permanent coating or dynamic coating to suppress EOF. The capillary is then filled with a replaceable polymer solution as separation media. Next, either untreated proteins (native-CGE) or SDS-treated protein sample (SDS-CGE) is loaded using pressure or electrokinetic force for separation. In SDS-CGE, proteins are separated based on their different molecular weights, just like the SDS-PAGE, while in native-CGE the separation also relates to the mass to charge ratio of the protein.

Compared to SDS-PAGE on a slab gel platform, capillary gel electrophoresis enables sample size reduction, automation, repeatable separations of one sample, and faster analysis time. These features make the technique of potential use for improve the electrophoresis and blotting step of a Western blot.

CGE for Western Blots.

Recently CE has been used for Western blotting. In one approach, Anderson et al. describes combining CE separation and immunoassay on a traditional blot membrane. (Figure 1-4).³²

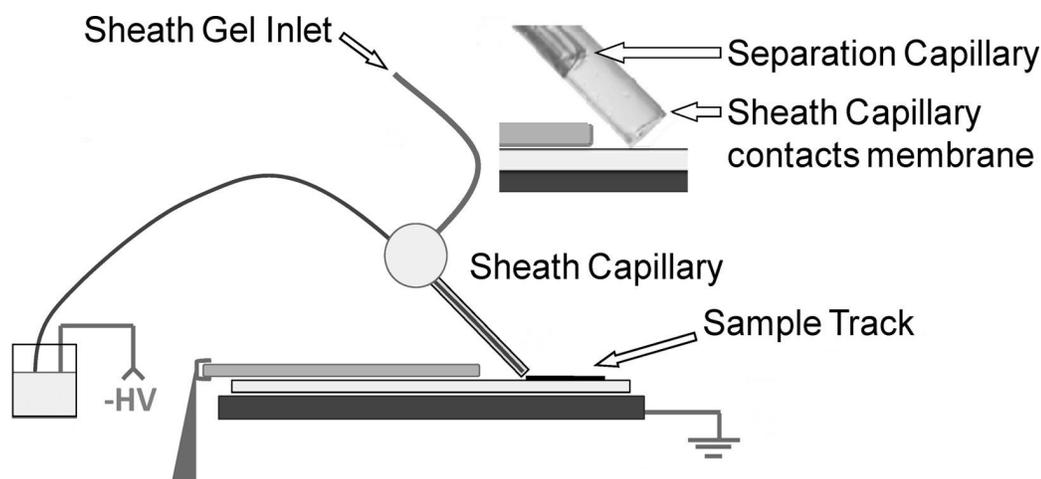


Figure 1-4. Western blotting using capillary electrophoresis instrument overview.³² Sample is injected at the inlet of the separation capillary. The protein mixture migrates the gel-filled capillary under an electric field. Proteins exit the capillary as it drags over the surface, and deposit on the blotting membrane. A translational stage moves the blot past the end of capillary to preserve the protein separation on the membrane. Gel is pumped through a sheath capillary that surrounds the latter portion of the separation capillary and makes direct contact with the blotting membrane.³² Reprinted with permission from Anderson, G. J.; M. Cipolla, C.; Kennedy, R. T. *Anal. Chem.* **2011**, *83* (4), 1350–1355. Copyright (2011) American Chemical Society.

In this approach, the exit of sieving electrophoresis capillary was interfaced to a blotting membrane so that separated proteins were captured on a moving PVDF membrane as they exit the column. The time of analysis is reduced through faster separation (40 min for up to 155 kDa proteins), owing to the higher electric field possible on CGE compared to conventional slab gels, and elimination of the electro-blotting step. Using a fast immunoassay method, a full Western blot for lysozyme was completed in one hour with 50 pg mass limit of detection (LOD). The low mass LOD was due to the low volume injection and confinement of the separated zones to a small spot. Because the proteins are captured on a membrane, they are accessible for other chemical tests besides immunodetection. For example, capillary electrophoresis and matrix-assisted laser desorption/ionization mass spectrometry (MALDI/MS) have been combined to provide separation and mass analysis of peptide and protein mixtures in the attomole range using

similar membrane capture^{33,34}.

Another way that CE has been used for Western blotting is based on work described O'Neill and coworkers.³⁵ Figure 1-5 shows a commercially available instrument developed based on this work that automates Western blotting. CGE was used to separate proteins in a 400 nL capillary. Typically the injection volume is 1/10 of the whole capillary volume. The separated proteins were then immobilized on the pretreated capillary wall surfaces by photoactivated cross-linking. The captured target proteins can be probed by flowing primary antibodies through the capillary. This method combined with rapid photochemical capture method has low sample consumption and the potential for automation. However proteins are only immobilized on the surface of capillary wall. The low protein-capture efficiency, $\sim 0.01\%$ ³⁶ limits the sensitivity of this method.

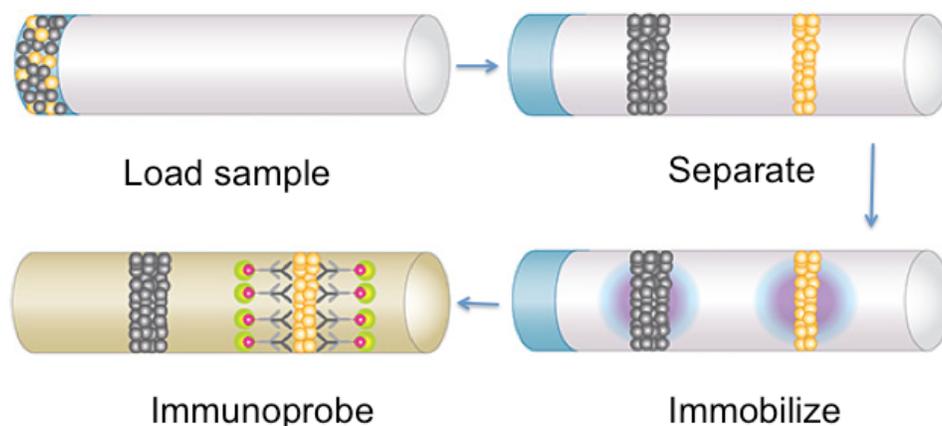


Figure 1-5. Workflow of ProteinSimple®'s Simple Western™ Assay. Capillary is pre-filled with sieving media. As little as 40 nL of sample is loaded into the capillary automatically and separated by size as they migrate through a stacking and separation matrix. The separated proteins are then immobilized to the capillary wall via a proprietary, photoactivated capture chemistry. Target proteins are identified using a primary antibody and immunoprobed using an HRP-conjugated secondary antibody and chemiluminescent substrate.³⁷ Reproduced with permission from publishers.

Microfluidic Devices for Performing Western Blotting

Microfluidics is a platform to manipulate fluids in channels with dimensions of tens of micrometers. Advantages of using microfluidic platforms for analysis include short analysis time, relatively low cost, low sample consumption, ability to integrate complex network, and potential for multiplexing and easy automation. Western blotting involves multiple steps and is of mediocre repeatability possibly due to lack of precise control. One main benefit of performing Western blotting using microfluidic devices is the possibility to integrate multiple steps on a single device so sophisticated procedures can be carried out. For example on a hybrid CE-Western blotting apparatus,²⁷ band broadening occurred when protein transferred onto the membrane. Using microchips, the dexterity of fluidic manipulations would provide more precise control of the peak shape during transfer.

These advantages were also convincingly demonstrated in one of the initial reports on using a microfluidic chip for protein sieving. In this study, high-speed separation (1 min over 1.25 cm long channel) and high separation efficiency (10^7 plates/m or 3750 plates/s) were achieved for a protein size ladder.³⁸ Injection of a well-defined sample plug (about 25 pL) contributed to such excellent separation results. Some other work focused on testing different chip materials, for example poly methyl methacrylate (PMMA), for protein separation.³⁹ Despite these early successes with protein size separations by microfluidics, it was several years for a microfluidic Western was demonstrated.

In this regard, integrated microfluidic devices for Western blotting are developed. These systems enable protein sieving on a chip and then directing towards an antibody

capture region on-chip.⁴⁰⁻⁴² This method is fast but requires covalent antibody immobilization on the chip before separation. Typically only one protein is analyzed per use of the chip. Another approach uses photoactivated cross-linking method to immobilize proteins on gel permanently through covalent bonds after size-based separation.^{36,43} Immunoassay is then performed by flowing primary and secondary antibodies through the separation region, followed by conventional imaging. These systems demonstrate promising analysis speed but rely on single use chips, which may increase cost. In some recent work, free-standing polyacrylamide gel microstructures are developed to support multiple assays. For example, 72 unlabeled native protein samples were separated and stained with SYPRO Ruby in 7.5 h.⁴⁴ In most of the work, resolution is not optimized. For example, only protein peaks with molecular mass differences >19% were resolvable ($R_s = 1$).³⁶

Another demonstration of the potential of using microfluidic devices for multiplexing Western blotting was recently reported.⁴⁵ In this method, a noncontact microarray printer was used to deposit cell lysates into slab gel. After separation of many protein spots, proteins were transferred to a membrane using a traditional semi-dry transfer method. A 96-well gasket was then used as a guide to apply different antibodies on different lanes of separations. High-throughput assessment of protein was achieved but resolution was poor. Variable transfer efficiency issue was also not addressed.

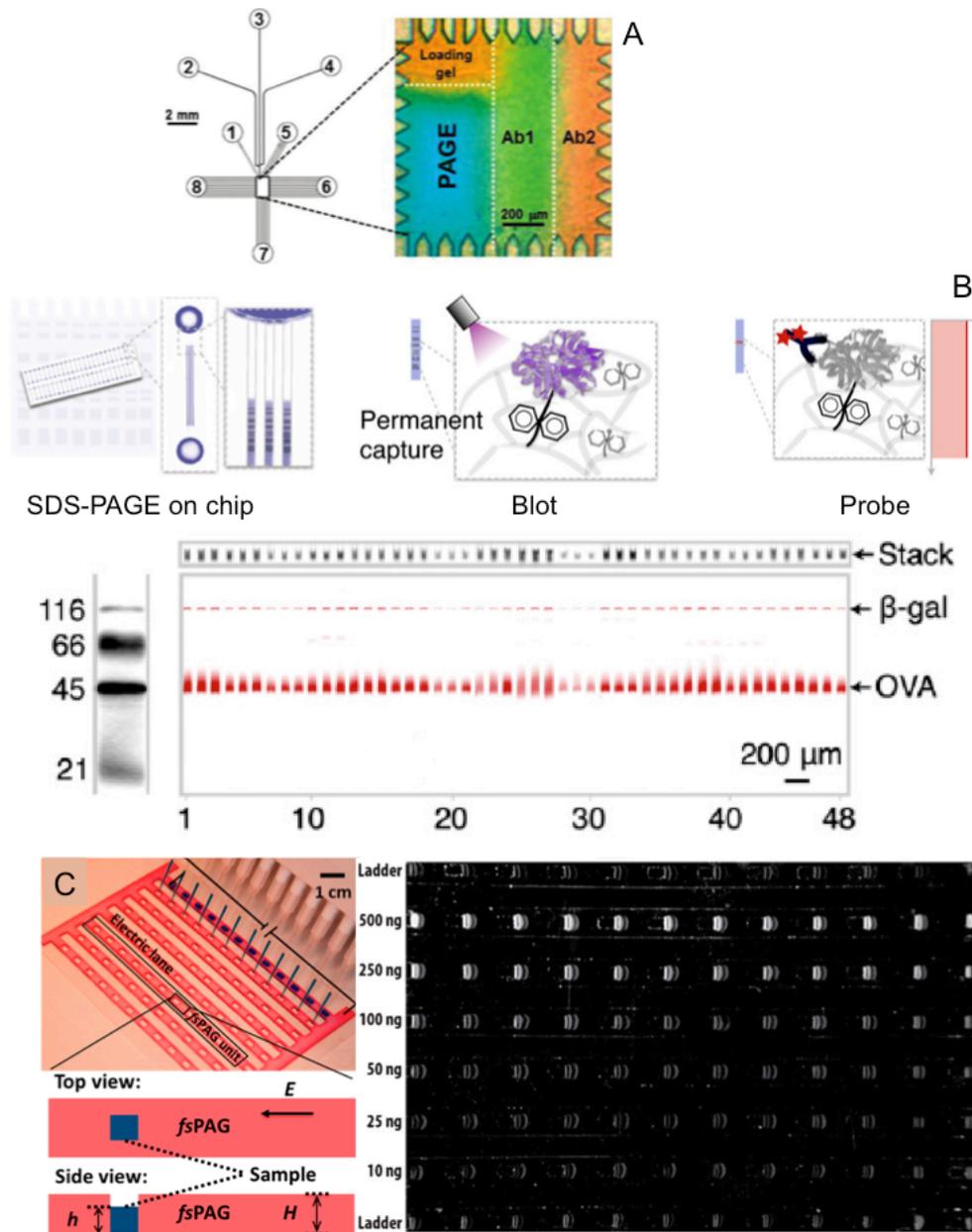


Figure 1-6. Examples of doing Western blotting on chip. (A) Multiplexed native Western blotting integrated in a microfluidic device. Proteins are separated in PAGE separation region before transferred in the antibody capture regions by lateral electrophoresis. Antibodies are pre-integrated in the blotting regions.⁴² (B) Single-microchannel μ Western assay enables 48 Western blots per chip in an hour. The microfluidic workflow is comprised of SDS-PAGE, permanent capture, and electrophoretic introduction of fluorescently labeled primary antibody.³⁶ (C) Image of a 96-plex polyacrylamide gel electrophoresis array. A schematic of a single unit is shown. The sample well is embedded in the gel. Gel structure and sample fluid heights are indicated by H and h . The array separates and detects 72 unlabeled native protein samples (BSA and OVA standards mixture) in a 7.5 h assay using SYPRO Ruby staining solution. Figures are reproduced with permission from publishers.

Online Sample Preconcentration

Although microfluidic devices allow fast analysis and offer potential for seamless integration of functions at the chip scale, from a detection perspective the inherently small dimensions of microchannels generate difficulties with conventional detection techniques, especially for trace or low-abundant species.

To solve the problem, high sensitivity detectors such as laser-induced fluorescence (LIF), chemiluminescence or electrochemical detectors could be used. It is also common to load more sample into the system. However without compressing the large sample bands, separation resolution and efficiency will be affected. Therefore on-line preconcentration or sample stacking methods are usually necessary for CE Western blotting or microchip Western blotting applications, especially when detecting low-abundant proteins.

There are two domains for preconcentrating samples in CE or MCE, electrokinetic preconcentrating and physical preconcentrating. Methods like fraction collection, solid phase extraction and preconcolumn sample enrichment fall within physical preconcentration category.⁴⁶ Electrokinetic preconcentrating includes methods such as capillary isoelectricfocusing (CIEF), isotachopheresis (ITP), and field amplification sample stacking (FASS). In this section, we focus on coupling electrokinetic preconcentration methods to Western blotting.

ITP involves the movement of charged molecules in an electric field at same speed. It is a separation technique but also recognized as one of the most effective on-line preconcentration techniques in CE and MCE, especially for ionic analytes. In ITP, sample is placed between two buffer systems. The front buffer is called a leading

electrolyte (LE) and the rear buffer is called the terminating/tailing electrolyte (TE). The LE has a faster mobility at a certain pH environment than the sample, while the TE has a slower mobility than the sample. Once the voltage is applied across these three subjects, the LE, sample, and TE ions will search for their mobility position. After the rearrangement, they will move through the capillary or microchannel at a fixed velocity, which is determined by LE. In other words, sample ions will be sandwiched between LE and TE (Figure 1-7). From the Kohlrausch regulating function,^{47,48} the concentration of species i in the stacked zone, C_i can be approximately related to C_L , the concentration of the leading electrolyte,

$$C_i = \frac{C_L \mu_i (\mu_L + \mu_{LC})}{\mu_L (\mu_i + \mu_{LC})}$$

where C_i is the concentration of the leading electrolyte and μ_i , μ_L , μ_{LC} are the mobility of sample, leading and counter ions, respectively. Based on this equation, it can be concluded that the final concentration of the analyte is largely proportional to the molarity of the leading ions.

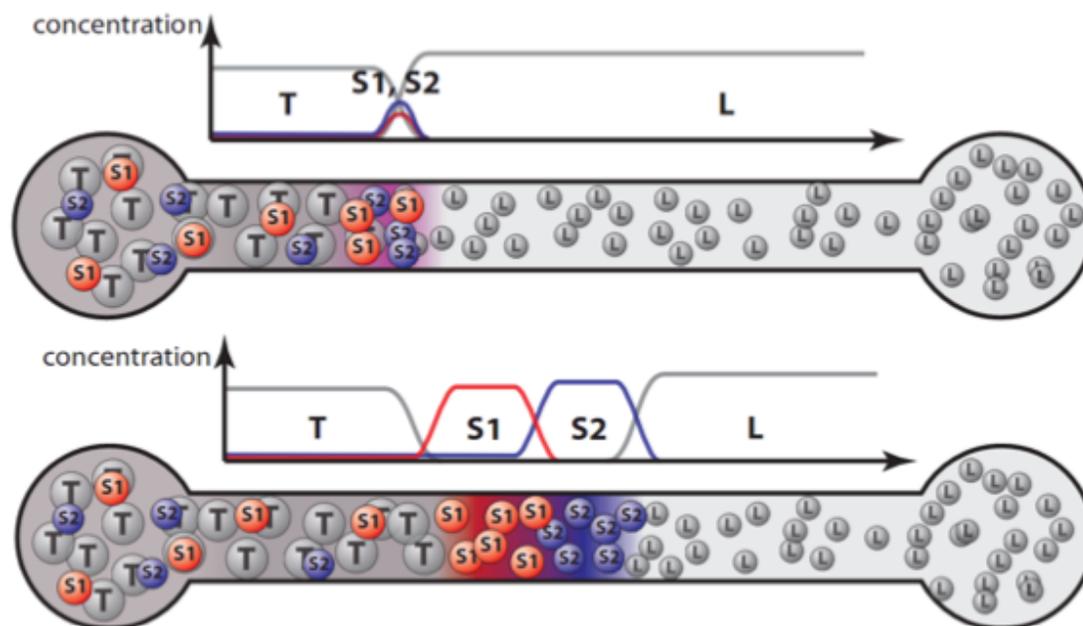


Figure 1-7. Mechanism of ITP in capillaries. Leading electrolyte (L) has the highest mobility, terminating electrolyte (T) has the lowest, and sample has mobility in between. After applying electric field, S1 and S2 will preconcentrate in different zones with distinctive electric field and then move through the capillary at same velocity until leading electrolyte dissipates.

To obtain both effective preconcentration and separation, ITP is often coupled with another separation mode, e.g., CZE or CGE. Such combination is called transient ITP (tITP). tITP is very popular since it is not necessary to have a complicated channel network and voltage setup to build three successive zones. Some experiments have achieved a concentration increase of 10^5 to 10^6 fold relative to the initial sample concentration, using Alexa Fluor 488 as the test compound.⁴⁹ Others also reported 800-fold signal enhancement of human serum albumin following the preconcentration on standard cross-channel microchips made of PMMA using tITP. In this case, all the channels were filled with a desired concentration of methylcellulose in the LE, and sample was also prepared in LE. After injection, a plug of TE was introduced from the back of sample plug, initiating tITP process.⁵⁰

In ITP, the choice of buffer system and the pH are extremely critical in order to get several orders of magnitude improvement in detection sensitivity. It is also important to be aware that the length of the injection is not taken into account of the effective separation length. In other words, the amount of time spent for separation (CZE or CGE) is decreased. The migration time will vary from injection to injection, and needs to be calibrated using some internal standards.

Another simple and powerful tool to preconcentrate sample is field amplification sample stacking (FASS).⁵¹ In the simplest form of FASS, a long sample plug prepared in a lower ionic strength environment is injected into a capillary or microchannel equilibrated with a higher ionic strength buffer system. When an electric field is applied across the capillary, sample ions experience a higher electric field strength and move faster until they hit the boundary of different ionic strength buffers. Once the ions pass that boundary, they will experience a lower field therefore velocity decreases. Sample from low concentration buffer environment will accumulate at the boundary and accomplish preconcentration.

Lower conductivity in sample buffer often results in a higher FASS preconcentrating factor. To obtain higher preconcentration efficiency in FASS, one needs a large difference in the conductivity between the two solutions and a large volume injection of the sample. Usually 10 times lower concentration in sample buffer than in separation buffer is used for FASS. Due to the focusing process in FASS, this helps injecting close to 10 times more sample without deteriorating separation resolution and efficiency.^{52,53}

Dissertation Overview

The main focus of this thesis is to describe a novel microfluidic based Western blotting technique. The goals are achieving fast protein sizing, automated protein transfer with minimum efficiency loss, comparable resolution to traditional methods, and multi-analyte detection.

Chapter 2 reports a fast Western blotting approach by integrating microchip gel electrophoresis, direct protein blotting, and fast immunoassay together. In this work, proteins are separated on a microchip based on sizes and transferred directly onto a moving membrane as they exit the chip. Protein ladder ranging from 11 kDa – 155 kDa are baseline separated in 2 min. The method has good sensitivity and is compatible with analysis of proteins in complex samples. The use of conventional Western blot membranes and immunoassay allow rapid and versatile assay development illustrated by the 4 different assays used in the report.

In Chapter 3, we investigate improving separation resolution as well as multiplexing by producing multiple blots from a single sample. Resolution is improved by using longer electrophoresis channels. ERK1 (44 kDa) and ERK2 (42 kDa) proteins are baseline resolved. This allows multiple cross-reactive proteins to be detected in one blot. To prove the capability of multiplexing, multiple injections are made from a single sample. The blots are then immunoassayed together. We demonstrate detection of 11 proteins from 400 ng total protein sample using this approach.

Chapter 4 presents work on improving throughput by running multiple protein separations on a parallel channel network interfaced to a moving membrane. 21 western blot assays are finished in 30 min using the microchip-SNAP i.d. apparatus. Different

sieving polymer solutions are also investigated to further increase the separation speed and achieve better throughput. 20% dextran, 10% glycerol, with 1% SDS gel buffer can operate at a high electric field up to 1200 V/cm and allows for separation of 11 kDa - 155 kDa protein ladder in 1 min.

Chapter 5 discusses future improvements to microfluidic Western blotting system. In this thesis most of the work has been focused on improving separation performance and transfer efficiency. Conventional immunodetection methods still apply. A faster immunoassay approach with low reagent consumption is desired. We present some preliminary work that by miniaturizing the size of incubation well, one blot could be analyzed with only 0.2 mL reagents. Another fast immunoassay method is also under development. Reagents are sequentially deposited onto the separation track. Analysis time and reagents cost are both significantly reduced.

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Chapter 2 Western blotting using microchips

Introduction

Western blotting combines electrophoretic sieving with immunoassay to provide semi-quantitative assay of proteins.^{1,2} The method has become a workhorse in biochemical research and clinical laboratories because it provides high selectivity of both separation and immunoassay. Fractionation prior to immunoassay overcomes interference by non-specific binding of antibodies, even in complex mixtures, and allows probing of multiple isoforms of a protein with a single antibody. Western blotting is also reliable, robust, and easily optimized. The primary weaknesses of the method are that it is slow (often taking 8-20 h), requires significant manual intervention, and has no established microscale counterpart preventing application when sample is limited.

The past few years have seen significant success in improving different aspects of Western blotting³⁻¹² Methods to improve the application of blocking agents and antibody have decreased time required for the immunoassay step.³ Integrated microfluidic systems in which proteins are sieved on a chip and then directed towards an antibody capture region on-chip have demonstrated Western blot-like results in a rapid, miniaturized format.^{4,6} While fast and sensitive, these systems require covalent antibody immobilization on the chip and/or use of pre-labeled proteins which may be limiting in some settings and result in single-use chips. Another approach has been to separate

proteins by capillary isoelectric focusing (IEF) or chip sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then immobilize them by photo cross-linking to the capillary wall or gel.^{7,9} Immunoassay is performed by passing primary and secondary antibodies through the separation channel followed by imaging detection. These methods show good performance and replicate the selectivity of Western blot. Further, they allow parallelization (e.g., 48 channels for the sieving example) for good throughput.⁸ These systems are highly promising, but rely on single use chips or capillaries which may increase cost.

We have previously described a hybrid approach in which sieving capillary electrophoresis is interfaced to a blotting membrane so that separated proteins are captured as they exit the column.¹⁰ The resulting immobilized proteins are then detected using a rapid immunoassay. The method uses relatively fast CE separation (40 min) and blotting during separation to improve the speed of the overall assay.

In this work, we extend this approach by using a microfabricated chip for sieving separation. The use of a microfluidic system greatly improves the speed of separation, automates injection, and improves reliability and performance of interface to membrane. Multiple injections and separations using the same channel and capture membrane are demonstrated showing potential of improved throughput and economical use of chips by reuse.

Experimental Section

Materials and Reagents. Fluorescein isothiocyanate (FITC), FITC protein ladder, and Alexa Fluor® 532 goat anti-rabbit secondary antibody were purchased from Invitrogen (Carlsbad, CA). Polyvinylidene fluoride (PVDF) membranes were purchased

from Millipore (Billerica, MA). Rabbit anti-lysozyme and anti-actin were purchased from Sigma (St. Louis, MO). Rabbit anti-AMPK was purchased from Cell Signaling (Danvers, MA). Rabbit anti-carbonic anhydrase was from Genway Biotech (San Diego, CA). All solutions were made using Milli-Q (Millipore) 18 M Ω deionized water. Borofloat glass slides were purchased from Telic Company (Valencia, CA).

Sample Preparation. FITC-labeled bovine serum albumin (BSA) was prepared by mixing 10 mL of 24 mg/mL FITC in dimethyl sulfoxide with 300 mL of 4 mg/mL of BSA in 100 mM Na₂HPO₄ adjusted to pH 7.5 with 100 mM NaH₂PO₄ (PBS) and incubating for 1 h at room temperature. Proteins to be analyzed were denatured by heating at 75° C for 5 min in denaturation buffer consisting of PBS, 3% SDS and 5% β -mercaptoethanol (BME). Denatured protein samples were diluted from stocks with sample buffer (50 mM PBS, 200 mM GABA, and 0.01% Tween 20). FITC-BSA was diluted to 0.5 mg/mL protein for assay. Protein ladder was diluted 1:10 from supplier for assay.

INS-1 cells were grown on cell culture plates in Roswell Park Memorial Institute (RPMI) 1640 culture medium. RPMI was poured off and radio-immunoprecipitation assay buffer (RIPA) from Thermo Fisher Scientific, Inc. (Rockford, IL) was added to lyse cells. Cell lysate was incubated on ice for 30 min before transfer to a tube and centrifuging at 1,000 \times g for 5 min. Supernatant was desalted using Micro Bio-Spin 6 chromatography columns from Bio-Rad (Hercules, CA) and proteins denatured as described above. Chicken eggs were purchased from a local grocery store. Egg whites were stirred using magnetic stirrers until clear (~40 min). Clarified egg white was diluted in 20 mM Tris buffer and then prepared as described for cell lysate.

Chip Fabrication. Chips were fabricated from borofloat glass slides using photolithography and wet etching methods.¹³⁻¹⁵ Briefly, slides coated with a layer of chrome and photoresist were exposed to UV light (Optical Associates, Inc., Milpitas, CA) for 5 s through a photomask (Fineline-imaging, Colorado Springs, CO) that had the desired channel network patterned in it (see Figure 2-1). The exposed glass was etched in 17:24:79 (v/v/v) HNO₃/HF/H₂O for 25 min resulting in channels that were 15 μm deep and 50 μm wide except the post-column channel which was 90 μm wide. Access holes (0.4 or 1 mm) were made in the etched glass using drill bits from Kyocera (Costa Mesa, CA). The resulting slides were soaked in piranha solution (H₂SO₄ / 30% H₂O₂ 3:1 v:v) for 20 min and then RCA solution (NH₄OH / 30% H₂O₂ / H₂O 1:1:5 v:v:v) for at least 40 min. A borofloat glass slide was thermally bonded, as a cover, to the etched glass slide using a programmable furnace.^{13,15} The chip was cut using a dicing saw (Diamond Touch Technology, Inc. Golden, CO) to create a point at the post-separation channel as shown in Figure 2-1. Polyimide-coated fused silica capillaries were connected to the sheath channels using PEEK Nanoports (IDEX Health & Science, Oak Harbor, WA).

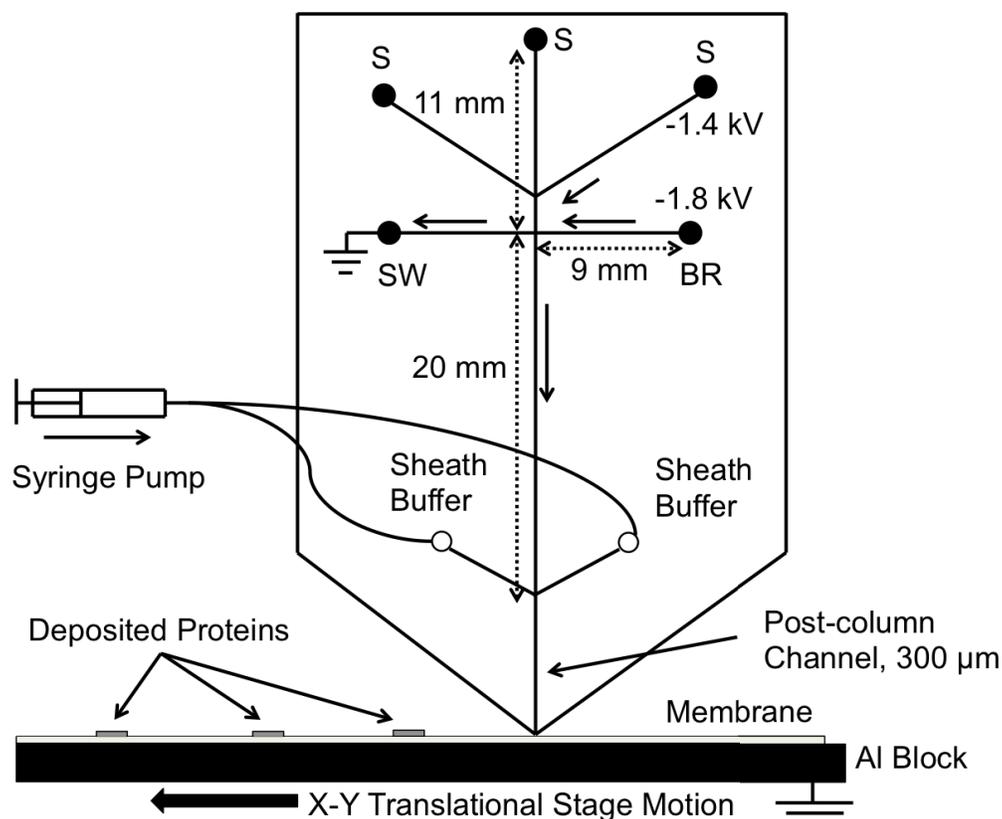


Figure 2-1. Microchip overview. Samples are loaded in different sample reservoirs (S). Samples are injected by floating the buffer reservoir (BR) and sample waste (SW) with voltage applied between the desired sample reservoir and the Al block at the exit. During separation, flow from the sample reservoir is gated to the sample waste reservoir (SW) using the voltages as shown. During these operations other sample reservoirs are floating. Size based separated SDS-protein complexes are captured in discrete zones on the PVDF membrane moving beneath the chip outlet to preserve separation information. Sieving media is pumped through the sheath channels to give stable current. Channel lengths are indicated by double arrow lines and direction of flow during separation is indicated by solid, single arrows.

Microchip Gel Electrophoresis Preparation and Performance. Prior to use channels were conditioned by sequential rinsing with 0.1 M NaOH, water, 0.1 M HCl, and water for 10, 2, 5, and 2 min respectively, followed by pumping sieving media (an entangled polymer solution of proprietary composition, AB Sciex, previously Beckman-Coulter part 390953) through channels for 10 min. Reservoirs, except sample reservoir, were filled with the same sieving media. The chip was mounted on a lab jack and

positioned over a PVDF membrane that was wetted with a 50:50 v:v mixture of methanol and 100 mM Tris buffer at pH 8.8. The membrane was supported by a grounded Al block mounted on an automated X-Y translational stage (Thomson-Linear, Radford, VA) as shown in Figure 2-1. The chip, with voltage applied as in Figure 2-1, was lowered towards the membrane while monitoring current. When current was detected, indicating contact, lowering was stopped and the chip held in position for separation(s). Gated injection with -1400 V applied for 3 s was used to inject samples. During separation, sieving media was pumped through the two sheath channels at 30 nL/min using a syringe pump (Chemyx Inc., Stafford, TX) and the stage was moved at 6 mm/min, unless otherwise stated, so that separated protein bands migrated out of the chip and were captured on membrane. Fluorescent proteins were detected on membrane by direct imaging using a Typhoon 9410 variable mode imager (GE Healthcare). In some cases, separations were detected directly on the chip using an inverted fluorescence microscope (Olympus, Melville, NY) equipped with a Xenon arc lamp (Sutter instrument company, Novato, CA) and an electron multiplier CCD camera (Hamamatsu, Bridgewater, NJ).

Immunoassay. Immunoassay of captured protein was performed using a SNAP i.d. (Millipore) according to manufacturer instructions.³ Membranes with separated proteins were wetted with methanol and placed on a blot holder. Vacuum was used to pull blocking buffer (0.3% dry milk with 0.1% Tween 20), antibody solutions, and wash buffer (TBS buffer with 0.1% Tween 20) sequentially through the membrane. Primary antibodies were diluted to 1:1000, 1:1666, 1:30, and 1:256 for AMPK, lysozyme, carbonic anhydrase II, and actin, respectively. Secondary antibody, conjugated with

Alexa Fluor® 532, was diluted to 1:2000. All antibody solutions were diluted using blocking buffer.

Results and Discussion

Chip Design and Experimental Overview. The microfluidic device used for separation and blotting is illustrated in Figure 2-1. Protein-SDS complexes are injected and separated by sieving electrophoresis using a tee arrangement.^{16,17} As proteins migrate from the separation channel they are transported via a sheath flow to a membrane that is slid past the exit of the chip to capture separated zones. Blotting occurs as compounds exit, thus saving time relative to a traditional Western blot which has a separate blotting step. Sheath flow of sieving media was found to be necessary to achieve stable current as previously reported for CE Western blot.¹⁰ (The unstable currents without sheath flow may result from migration of methanol from the blotting membrane or hydroxide ion electrochemically formed at the exit into separation channel¹⁸. With a sheath gel, these potentially interferences are prevented from entering the separation channel and instead flow to the membrane.) A significant advantage of microfabrication is that sheath channels are precisely formed and integrated with the device with minimal extra column volume whereas a capillary-based system required manually aligned coaxial capillaries to have a sheath flow. As a result, microfabrication increased robustness and reproducibility of operation.

Separation. We first examined separations of fluorescent protein-SDS complexes on the gel-filled chip. Size-based separations of proteins from 11 kDa to 155 kDa were obtained in 5 min at 240 V/cm over a 2 cm length (Figure 2-2).

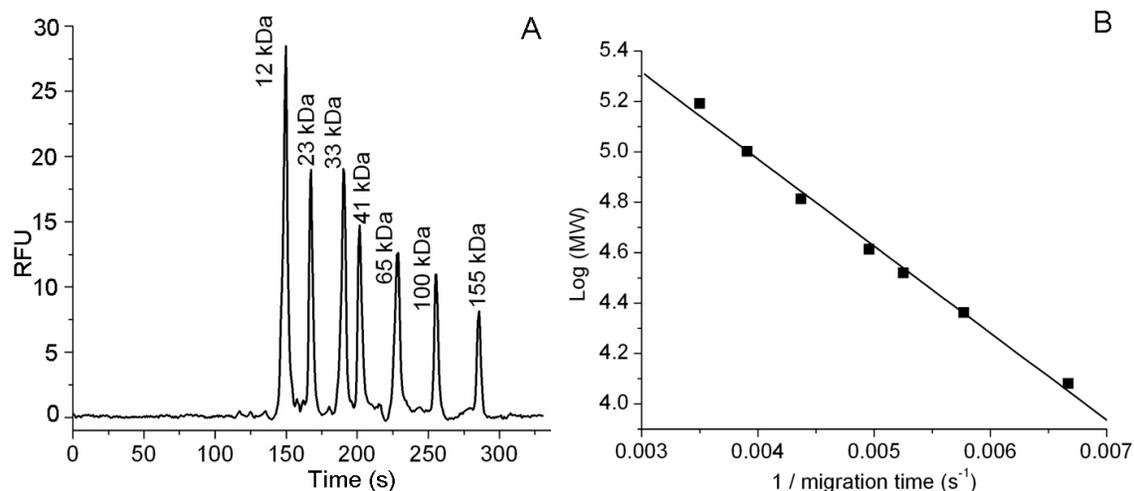


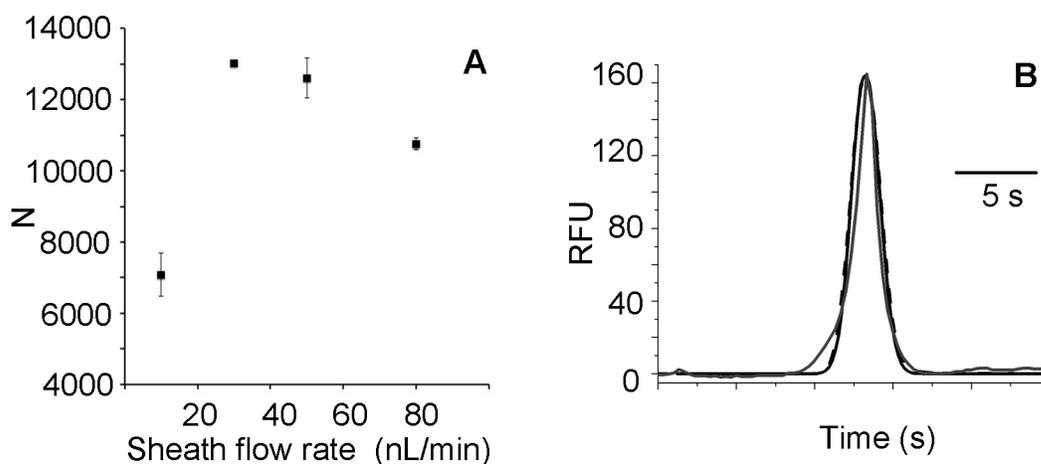
Figure 2-2. Size dependent separation of FITC-labeled protein ladder using microchips. (A) Size dependent separation of FITC-labeled protein ladder in microchips. Detection window was set at the end of separation channel, 300 μm away from the chip outlet. Electric field during separation was 240 V/cm. (B) Relationship of MW to migration time.

Separation efficiency ranged from 5×10^4 to 1.8×10^5 theoretical plates and peak capacity was 45 (using the average zone width and resolution = 1 for calculation) when detecting at the exit of the separation channel. Separation times could be reduced to 2 min with 4×10^4 plates (average for ladder) at 460 V/cm, but above this field bubble formation was common. The separations are substantially faster than what has been achieved with capillaries where typical separation times are 20 min.¹⁹ The shorter time is due to miniaturization of the separation channel and illustrates another advantage of microfabrication for the Western blot.

It may be possible to further improve the separation time. Previous chip-based work using a lower injection volume, pinched injection, narrower channels, and less viscous sieving media achieved 7×10^4 to 1.2×10^5 plates and peak capacity of 55 in 1 min separation on a 1.25 cm long channel.²⁰ The larger channels and injection volumes used here were to accommodate a less sensitive detector (Xe-arc lamp source rather than

laser) while the sieving media was used because it is commercially available. Thus, use of other media and narrower channels are likely to yield faster separations.

Transfer to Membrane. After migrating from the separation channel, band broadening may occur both in the sheath flow channel and during deposition on the membrane. The post-column length was just 300 μm to minimize broadening during transfer. Sheath flow rate had to be above 20 nL/min to avoid degrading separation efficiency (Figure 2-3A). Higher flow rates both decreased plates, likely to due flow-induced zone broadening, and diluted zones during transfer. 30 nL/min was chosen as the best value. At this flow rate, little difference could be seen in peaks detected at the end of the capillary compared to end of the sheath flow (Figure 2-3B).



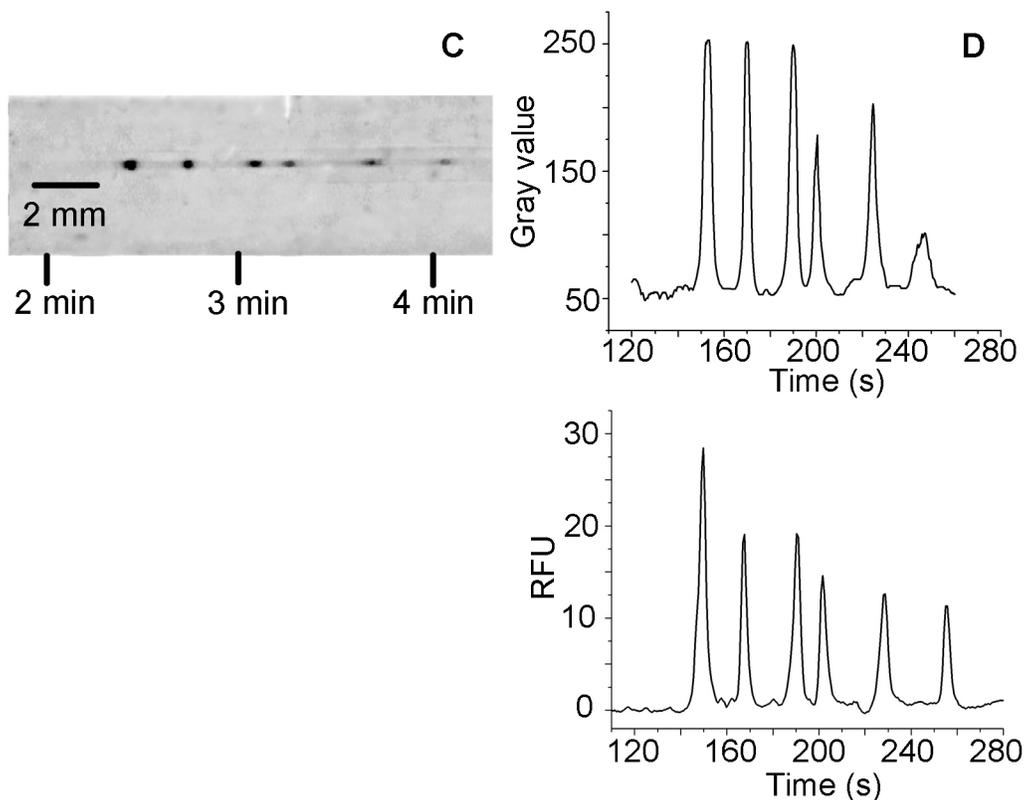


Figure 2-3. Transfer of separated proteins to blotting membrane. Electric field was 240 V/cm, separation channel length was 2 cm, sheath flow rate was 30 nL/min, and membrane was moved at 6 mm/min. (A) Effect of sheath flow rate on theoretical plates (N) for SDS-FITC-BSA. Error bar is ± 1 standard deviation ($n = 4$). (B) Comparison of peak width for on-chip detection (solid line), in post-channel 300 μm beyond the end of separation (dashed line), and on membrane (gray line) for electrophoresis of 0.5 mg/mL SDS-FITC-BSA. (C) Gray scale fluorescent image of membrane that captured size-dependent separation of FITC-labeled protein ladder. (D) Comparison of protein ladder separation on membrane (upper) to detection in channel (lower). Membrane trace is a line scan through membrane with x-axis converted to separation time.

During protein deposition the membrane velocity also had an effect on separation. It is necessary to move the membrane fast enough to capture resolved proteins in different spots (i.e., a velocity that is too low would allow separated zones to stack together on the membrane); however, increasing the speed also spreads the zone out on membrane and dilutes signal (see Figure 2-4). For 240 V/cm, we typically used 6 mm/min, which decreased efficiency by 28% on-membrane relative to on-channel

(Figure 2-3B). As shown in Figure 2-3C, using these conditions, separated proteins were captured in discrete zones on the membrane. A line scan of the membrane allows comparison to separation detected on column (Figure 2-3D). The electropherogram retains an average of 5×10^4 theoretical plates; but, the overall separation shows lower resolution than detected on chip, e.g. resolution between peaks at 2.5 and 2.8 min decreased from 1.81 to 1.67 and between 3.2 and 3.4 min from 1.18 to 1.04.

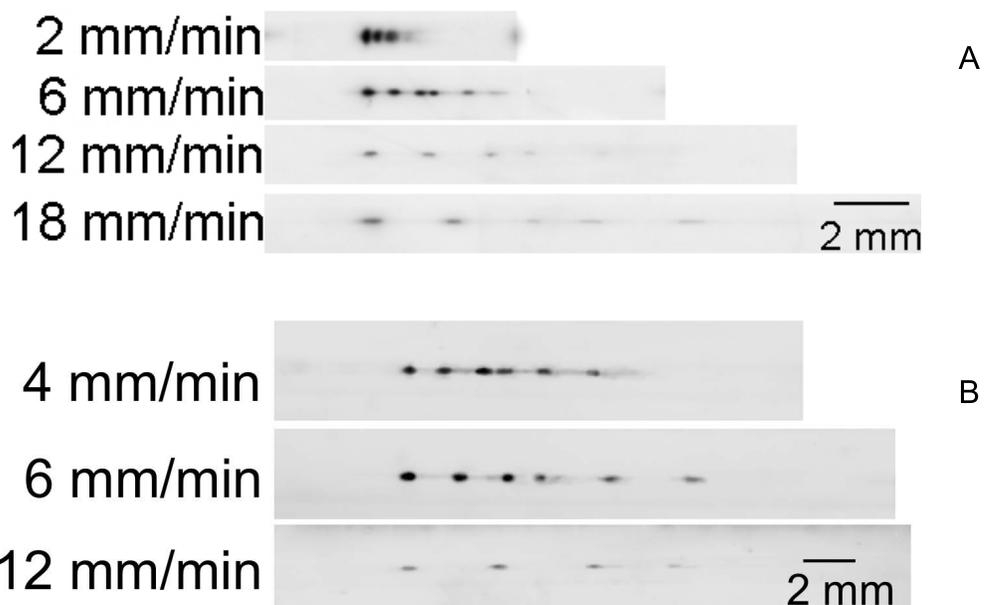


Figure 2-4. Effect of stage moving rate on separation resolution. Protein ladder was captured on membrane moving at rates indicated. (A) Separation field was 460 V/cm. Under this condition, peaks overlapped if the membrane was moving at a rate lower than 3 mm/min. First two peaks resolution increased from 1.1 to 1.7 when stage moving rate doubled (from 6 mm/min to 12 mm/min). Further increasing the stage moving rate did not improve resolution and but resulted in broadening and dilution of zones. (B) Separation field was 240 V/cm.

Some variability in spacing and intensity was observed on the membrane relative to the on-column detection, especially for the largest (slowest) protein. These effects were not investigated thoroughly. The intensity effect could relate to quenching on the membrane or transfer and capture efficiency. The spacing effect may be due to an effect

of sheath flow speeding up the slowest migrating protein; however, this requires a formal study to reach a firm conclusion. Conditions can be varied to aid sensitivity, separation time or resolution depending on requirements of the assay. For most assays described below we used 460 V/cm and 6 mm/min membrane rate yielding 2 min separation times for the complete protein ladder with 3×10^4 plates on membrane (see Figure 2-5).

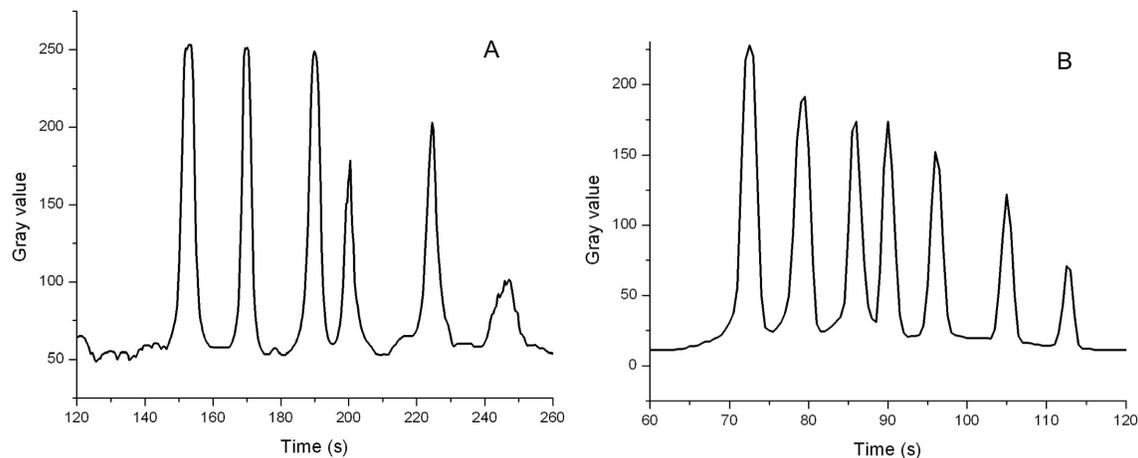


Figure 2-5. Effect of electric field on separation captured on membrane. Illustration of separation captured on membrane using line traces. Traces are line scans through captured zones with separation at 240 V/cm (A) and 460 V/cm (B).

Figures of Merit. Sensitivity and limits of detection (LOD) were determined for several proteins by Western blot analysis. For these experiments, chips with three sample reservoirs were used and samples at different concentrations injected sequentially onto the separation channel so that separation of samples was recorded in different lanes on a single membrane. The entire membrane was processed for immunoassay at once. Blots for calibration are shown in Figure 2-6. Using actin as a test protein, we found a linear relationship ($R^2 = 0.98$; see Figure 2-7) between concentration (from 2.4 nM to 240 nM) and fluorescent signal (measured as peak area for a line scan through the protein spot). LOD was 0.03 $\mu\text{g/mL}$ (0.7 nM) using a S/N of 3 as the lowest detectable signal (see Figure 2-6 and line scan membrane for 2.4 nM actin in Figure 2-7). We estimate 200 pL

injected suggesting a 25 fg mass LOD. Using a similar approach, the LOD for carbonic anhydrase II was 0.05 $\mu\text{g/mL}$ (2 nM) and lysozyme was 0.06 $\mu\text{g/mL}$ (4 nM). These detection limits compare well to other microfluidic Western blots, e.g. 2.5 nM for protein G.⁶

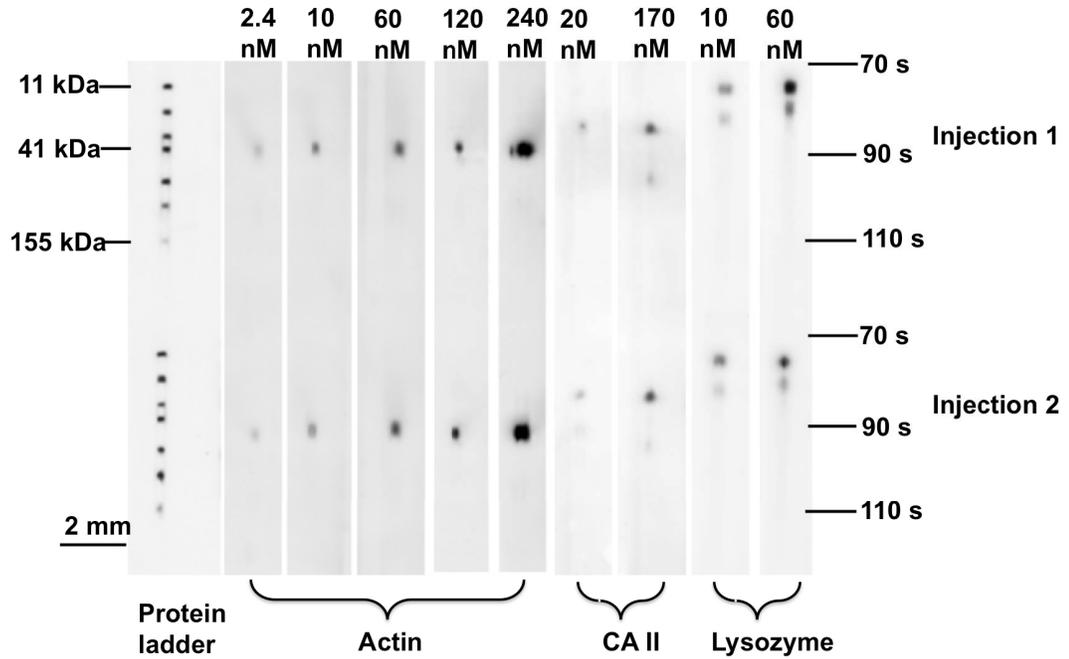


Figure 2-6. Microfluidic Western blot of unlabeled actin, carbonic anhydrase II (CA II), and lysozyme at different concentrations. Each trace shows repeated injection of a sample captured on the same membrane. Separations were performed using 460 V/cm electric field. Samples were injected every 90 s. Time axis on right indicates time since injection. Protein ladder was injected separately to provide size calibration. (Line scans of actin injections shown in Figure 2-7).

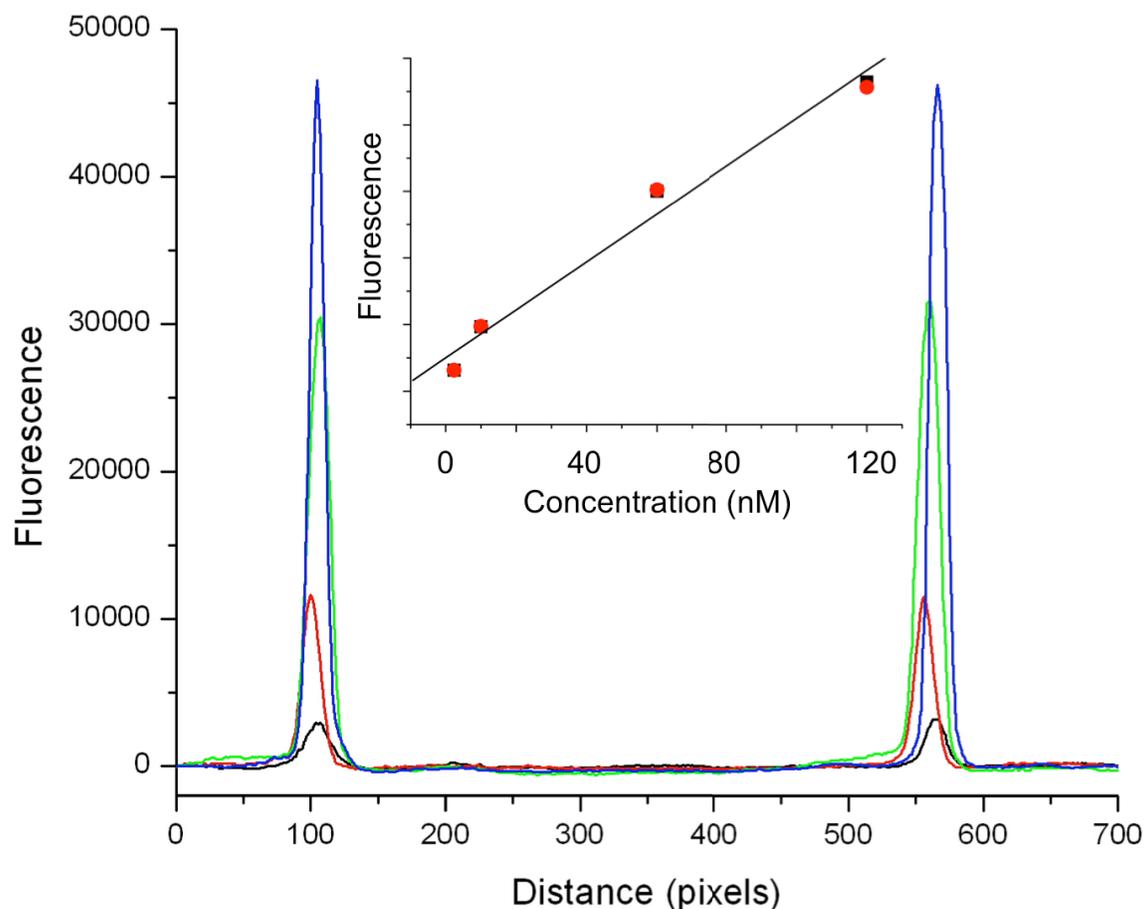


Figure 2-7. Determine detection limit of actin. Line scans for 2.4 nM (black), 10 nM (red), 60 nM (green), and 120 nM (blue) actin injections from image in Figure 2-6. The inset figure shows a linear relationship between actin concentration and fluorescent intensity with R^2 equals to 0.989. Black squares indicate the peaks at 110 pixels position. Red circles indicate the peaks at 565 pixels position. Signal to noise ratio from 2.4 nM actin trace (black) was used to calculate LOD of 0.7 nM.

An advantage of the method described here is that any secondary antibody and detection method could be used since the separation and blotting step are decoupled. For these experiments, we used Alexa Fluor® 532 labeled secondary antibodies and fluorescence detection; however, the same approach could utilize other detection methods such as chemifluorescence. Fluorescent antibodies allow the same detector to be used to detect the calibration proteins as illustrated in Figure 2-6.

For most Western blots, it is desirable to use a standard ladder for size calibration. In this system the ladder could be mixed with the sample or injected and analyzed on a separate lane. Less interference occurred by injecting in a separate lane so this approach was generally used. Reproducibility of migration time is important for sequential injections. For multiple injections the RSD of migration time was 3 to 6% for actin (89 ± 3 , $n = 10$), carbonic anhydrase II (82 ± 2 s, $n = 4$), and lysozyme (75 ± 5 s, $n = 4$). Possible sources of variability are inconsistent moving rate of the stage holding the membrane (it was operated at lower end of its range and resolution), and lack of thermal control.

Analysis of Complex Samples. To assess the utility of this approach for Western blotting in biological samples we measured actin and AMP-activated protein kinase (AMPK) in INS-1 cell lysate and lysozyme in egg white as demonstrations. Actin was readily detected at its expected molecular weight (42 kDa) in cell lysate samples that contained 600 $\mu\text{g/mL}$ protein. Peak area RSD was 0.2% for multiple injections. Using a calibration curve made from actin standards, the actin concentration in INS-1 cell lysate was estimated to be 10 $\mu\text{g/mL}$ or 0.7% of total protein. This value is lower than previously reported estimates of 2-3% of total protein content reported for hamster ovary cells.²¹ The difference may relate to differences in cells or differences in the response in binding of antibody to bovine (used for standard) and murine (INS-1 cells) actin.

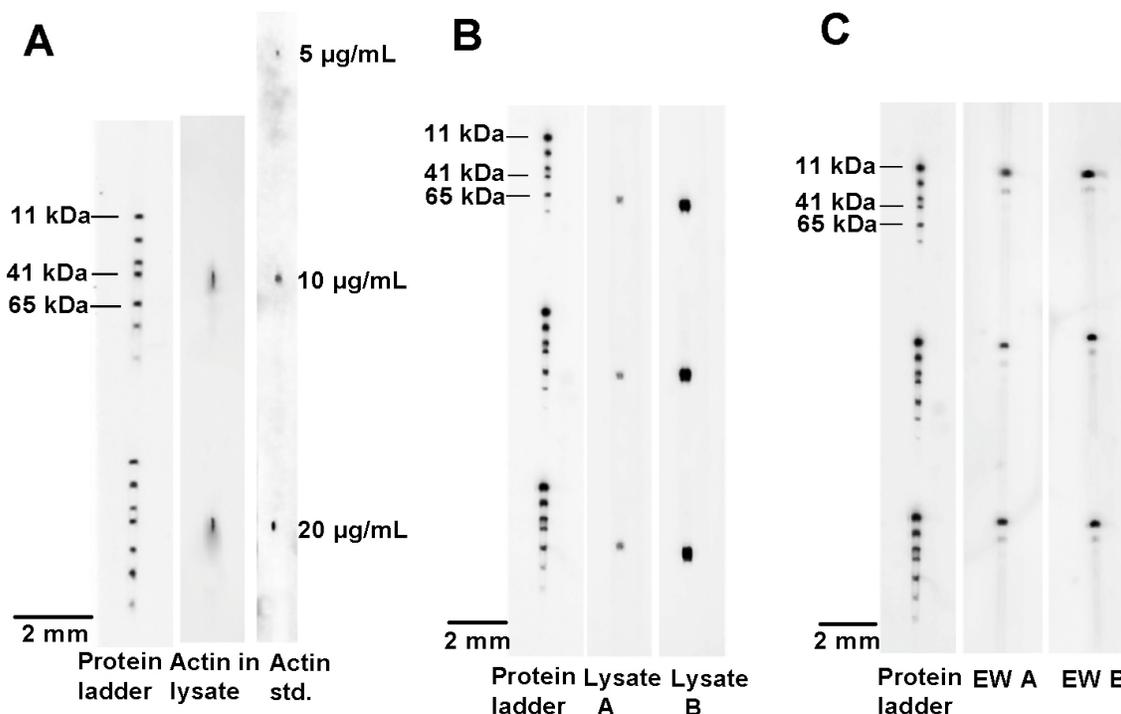


Figure 2-8. Western blot assay of biological samples. (A) Assay of actin in 1.5 mg/mL INS-1 cell lysate (duplicates are shown). Standard actin assays used for calibration are shown to the right. Separation field was 460 V/cm. (B) Western blot assay of AMPK from INS 1 cell lysates. Lysate was diluted to 1 mg/mL (Lysate A) and 2 mg/mL (Lysate B) before analysis. (C) Immunoassay of unlabeled lysozyme from egg whites (EW) with initial fluorescent scanning of labeled size standards. Egg white sample A (EW A) had a concentration of 1/100 of original homogenate and sample B (EW B) had a concentration of 1/40.

While actin is a commonly used standard protein, we also sought to demonstrate detection of a more typical target, such as a signaling enzyme. AMPK is a “master” regulator of metabolism and of strong interest for its control of b-cell growth, metabolism, and insulin secretion.²² AMPK was readily detected as a 66 kDa protein in INS 1 cell lysate (1 and 2 mg/mL total protein) as shown in Figure 2-8 and Figure 2-9. Literatures demonstrate AMPK as a 63-64 kDa protein.²² Multiple injections from the same reservoir had peak area RSD of 0.4%. Although standards were not readily available for quantification, the good reproducibility and results from assay of different dilutions of cell lysate show the potential for relative quantification.

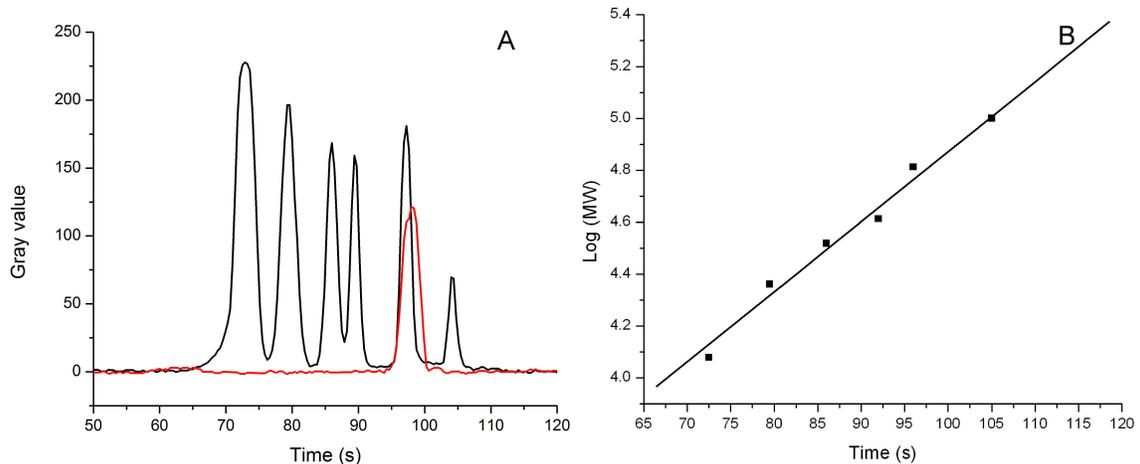


Figure 2-9. Estimate AMPK molecular weight. (A) Size standards (black) are overlaid with AMPK (red). Line scans are from Figure 2-8. (B) Relationship of migration times to log (molecular weights). AMPK is detected at 98 s, and the molecular weight is calculated to be 66 kDa.

As another demonstration of the assay, lysozyme was detected as a 14 kDa protein in egg white samples (Figure 2-8C). Lysozyme is a major protein component of egg white (3.4%). As with the AMPK assay, repetitive samples were reproducible (RSDs of area were 5% and 2% for 1/40 and 1/100 diluted samples). Using comparison to lysozyme standards, the estimated concentration in egg white was 4 mg/mL, which falls in the expected range of 2.2 to 4.5 mg/mL.²³

With multiple sample reservoirs and use of a common secondary antibody, it is possible to determine different proteins from different samples on the same membrane. This is illustrated in Figure 2-10 where AMPK in cell lysate and lysozyme in egg white were both determined by sequentially separating them with capture on different lanes and then processing the membrane for immunoassay. To avoid carry-over between injections, sample was driven towards the injection cross for 30 s to rinse the cross region when switching sample reservoirs.

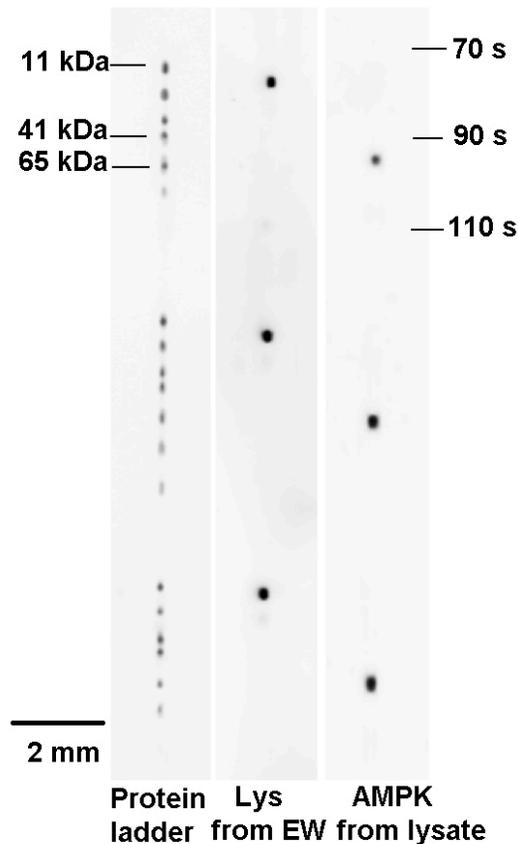


Figure 2-10. Analysis of different samples on one membrane. AMPK from 1.5 mg/mL INS 1 cell lysate and lysozyme (Lys) from egg white (EW) homogenate were immunoassayed at the same time after separations from different sample reservoirs.

Throughput and Analysis Time. As shown by the above demonstrations, the microfluidic Western blot described here has good sensitivity and reproducibility. The assay also retains the information content, versatility (different proteins in different sample types) and flexibility of a conventional Western blot (i.e., it uses the same detection methods and antibody treatment steps). Perhaps the most significant advantage is the potential for good throughput. Using this approach a single Western blot requires 22-32 min (2 min separation and another 20-30 min for immunoassay). This time is much faster than conventional gel Westerns; but slower than a fully integrated chip which has 5 min total analysis time⁶. The rapid assays on an integrated chip however used antibodies covalently attached to the chip or pre-labeled proteins whereas the system reported here

used unlabeled sample proteins and conventional membranes. (In discussing these times, we are ignoring set-up and sample preparation that is required for all approaches).

The current approach has the potential for considerably higher throughput, when multiple samples are analyzed. The rate limiting step of the method described here is the immunoassay; however, multiple separations can be captured on a single membrane and immunoassay performed on the entire membrane allowing for improved throughput. Several approaches for laying multiple separation tracks on a single membrane can be envisioned, some of which were illustrated here. As shown in Figures 2-8 and 2-10, using multiple sample reservoirs, different samples can be separated sequentially through one channel and collected in different tracks on one membrane for subsequent analysis. In Figures 2-8B, 2-8C, and 2-10, nine separations (3 separate samples, including ladder, in triplicate) were collected on a single membrane which was processed in 25 min for a total analysis time of 43 min or ~5 min/sample. In principle it is feasible to scale up the number of sample reservoirs coupled to a single separation channel to further increase the throughput. As shown by the examples here, the samples may be different and may have the same or different protein targets. A related approach would be to use a single sample reservoir that could be rapidly reloaded for sequential separations. The limiting factor on scale up of samples would be the number of separations that can be acquired from a single separation channel. In a pilot study, we found that 60 injections could be achieved through a single channel before the separation times began to drift (Figure 2-11). This sequence required 100 min suggesting a potential throughput of 60 samples/130 min or slightly over 2 min/sample. After 60 injections migration time increased 10%. Replacing the gel completely regenerated the separation performance. These results illustrate

another important feature which is reusability of the chip. The ability to pump the entangled polymer solution in and out of the chip as needed would also allow extended operation time for many samples. Other methods to improve throughput, such as separation from channels operated in parallel, are also in readily within reach.

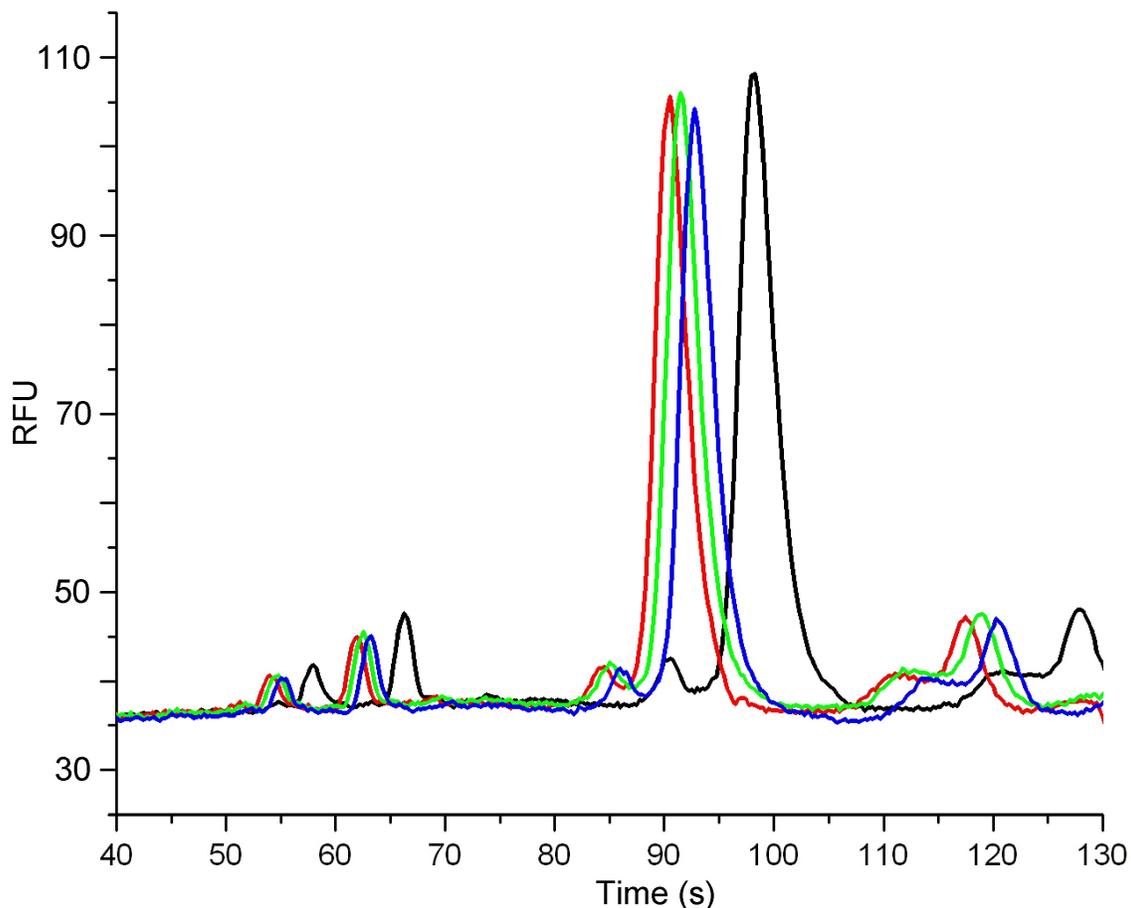


Figure 2-11. Peak migration time shifts over a series of injections. 60 repetitive injections of FITC-BSA were made every 100 s with detection on chip by fluorescence microscopy. Electropherograms of 1st injection (red), 20th injection (green), 40th injection (blue), and 60th injection (black) are overlaid. BSA peak migration time after 60 injections and separations (a total time of ~100 min) shifted for 10% compared with the first electropherogram.

Needed Improvements. This work is a significant step forward from the original hybrid method CE interfaced to membrane because it increased the speed of separation 8-fold, demonstrates potential for rapid serial injections through the same sample, increases

robustness and reduced band broadening by using a microfabricated sheath-flow, and demonstrates utility for biological sample analysis. Despite these advances, further improvement is essential to make this a practical tool. To achieve the throughput advances described above, it is necessary to integrate more sample reservoirs and parallel channels. Improved separation conditions will allow faster separations (e.g., different gels).²⁴ Detection limits may be improved by better preconcentration. A modest isotachophoretic effect was achieved by adding leading and terminating electrolytes to the sample; but more effective approaches have been described.^{25,26}

Comparison to Other Approaches. Previous work has recently demonstrated excellent throughput (e.g., 48 samples in 60 min) for a microfluidic Western blot.⁹ Besides demonstrated throughput, the prior work also offers advantages of minimal moving parts and integration within the chip. The system described here, seems to have the potential to achieve comparable levels of throughput. The method here may offer other advantages such as repeated injections from a single chip, simple regeneration of chip (rinse of gel rather than overnight incubation of aggressive solvents), potential for long term operation, capturing protein external to the chip for other processing (e.g., mass spectrometry analysis or stripping and re-blotting of different proteins), and compatibility with existing blotting and immunoassay protocols. These differences suggest that both approaches extend the capability of Western blots and offer new opportunities for protein analysis.

Conclusion

These results demonstrate a new approach to microfluidic Western blot. The microfluidic chip used allows fast size-based separation and direct delivery of proteins

onto blotting membranes. Microfabrication also allows well-controlled sheath flow for the blotting step ensuring low loss of separation efficiency and robust performance. The method has good sensitivity and is compatible with analysis of proteins in complex samples. The use of conventional Western blot membranes and immunoassay allow rapid and versatile assay development illustrated by the 4 different assays used in this report. Preservation of protein on separate membrane also offers the potential of other physical and chemical tests on the sample. Because a single membrane can capture multiple rapid separations, potential for good throughput exists.

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Chapter 3. Multiplexed Western Blotting Using Microchips

Introduction

Western blotting¹ is a semi-quantitative protein assay that combines sieving electrophoresis and immunoassay. Western blotting has become a routine tool for biochemistry research because it is robust, easily optimized, inexpensive, and provides high specificity. Even though Western blotting is a workhorse method, it has a number of limitations such as long analysis time (~20 h), requirement of several manual steps, and sample consumption of 10 μg total protein so that it is not compatible with small samples. Microfluidic-based Western blot systems have been developed to improve the speed, automation, and mass sensitivity of Western blotting. In some cases, sieved proteins are captured by photo-induced cross-linking to the sieving gel or capillary walls allowing for subsequent in situ immunoprobing.⁸⁻¹² Our lab has developed a method where proteins separated by microchip electrophoresis (MCE) are blotted onto a membrane as they exit the separation path.^{13,14} In this work we show that MCE interfaced to membrane can also be used to improve the information content of Western blotting by enabling multiplexed assays.

Traditional Western blotting is usually confined to measuring one protein per assay. To overcome this limitation, reblotting techniques have been developed that allow more than one protein to be detected on a blot.² In such procedures, after a membrane is probed for the first protein, the blot is “stripped” to remove the antibody, often with

surfactant solutions at extreme pH. The membrane is then re-probed with a second antibody. Only a few proteins can be measured this way because the stripping steps remove not only antibody; but, also a significant amount of target protein from the membrane surface.³ Re-probing also increases the total assay time because the slow immunoassay steps are performed in series.^{4,5} An emerging alternative for multiplexed analysis is to use multiple antibodies, sometimes with different color emission, on one blot^{6,7}; however, this technique also has limited potential to scale up beyond a few proteins and it requires high resolution electrophoresis and high quality antibodies with no cross-reactivity.

Little of the microfluidic work has emphasized improving content. One approach was to incorporate 2 antibody capture regions on a chip and drive dye-labeled proteins through these regions by electric field.¹² In another example, unlabeled ovalbumin (45 kDa) and beta-galactosidase (116 kDa) were resolved by MCE and then probed with different primary but the same secondary antibody to provide assay of 2 proteins per blot.¹¹

In this work, we investigate improving content by both increasing resolution, to allow cross-reactive proteins to be detected, and by producing multiple blots from a single sample for multiplexed assays. Resolution is improved by using longer electrophoresis channels. The added separation resolution is useful not only for multi-protein assays but also when cross-reactivity occurs or antibody selectivity is not known. We also demonstrate that multiple injections can be made from a single sample and deposited in separate tracks that can be probed with different antibodies in parallel. Because MCE uses such small amounts per injection, it is possible to make several

injections from a single sample to increase information content. We demonstrate detection of 11 proteins from 400 ng total protein sample using this approach; although in principle even more proteins could be measured.

Experimental Section

Materials and reagents. FITC-protein ladder containing 7 proteins from 11 kDa-155 kDa was from Invitrogen (Part number LC5928, Grand Island, NY). Polyvinylidene fluoride (PVDF) membranes were from EMD Millipore (Billerica, MA). Rabbit anti-extracellular-signal-regulated kinases (ERK) 1/2, rabbit anti-phospho-ERK1/2, rabbit anti-phospho-mitogen activated protein kinase kinase (MEK) 1/2, rabbit anti-phospho signal transducer and activator of transcription 3 (STAT3), and rabbit anti-phospho-protein kinase B (AKT) were from Cell Signaling (Danvers, MA). Mouse anti-MEK1/2, mouse anti-AKT, and mouse anti-STAT3 were from Santa Cruz Biotechnology (Dallas, TX). Mouse anti- β tubulin was from Sigma Aldrich (St. Louis, MO). Jurkat cell lysates, A431 cell lysates, goat anti-rabbit secondary antibody and goat anti-mouse secondary antibody were from LI-COR Bioscience (Lincoln, NE). Cell lysate was prepared in lysis buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 25 mM NaCl, 10 mM NaF, 2 mM Na_3VO_4 , 1% Triton-x 100 and 10% glycerol. Jurkat cells were stimulated by 0.2 ng/mL phorbol 12-myristate 13-acetate (PMA) for 15 min before making the lysates. Unless stated otherwise, all solutions were made using Milli-Q (Millipore) 18 M Ω deionized water.

Sample Preparation. Cell lysates were denatured by heating at 90 °C for 5 min in denaturation buffer consisting of 3% sodium dodecyl sulfate (SDS) and 5% β mercaptoethanol (BME). For most assays, 1 μL of the denatured lysate was mixed with 1

μL FITC-protein ladder, 2 μL of 100 mM Na_2HPO_4 buffer pH 7.5, and 6 μL water. Unless stated otherwise, the final total protein concentration was 0.24 mg/mL.

Chip Fabrication. Chips were fabricated from borofloat glasses by using standard wet etching methods.¹⁵⁻¹⁷ Briefly, slides coated with a layer of chrome and photoresist were exposed to UV light (Optical Associates, Inc., Milpitas, CA) for 5 s through a photomask (Fineline-imaging, Colorado Springs, CO) that had the desired channel network patterned in it (see Figure 3-1). The exposed glass was etched in 17:48:55 (v/v/v) $\text{HNO}_3/\text{HF}/\text{H}_2\text{O}$ for 12.5 min resulting in channels that were 15 μm deep and 50 μm wide except the post-column channel which was 90 μm wide. Turns were added to the design in order to fit an 8.6 cm separation channel on a 5 cm long glass slide. The measured width of the channel through the arc on photomask was 10 μm . After HF wet etching, the dimensions were 40 μm wide x 15 μm deep at the arc. Access holes (0.4 or 1 mm) were made in the etched glass using drill bits from Kyocera (Costa Mesa, CA). The resulting slides were soaked in piranha solution (H_2SO_4 / 30% H_2O_2 3:1 v:v) for 20 min and then RCA solution (NH_4OH / 30% H_2O_2 / H_2O 1:1:5 v:v:v) for at least 40 min. A borofloat glass slide was thermally bonded, as a cover, to the etched glass slide using a programmable furnace. The chip was cut using an ADT 7100 series dicing saw (Horsham, PA) to create a point at the post-separation channel as shown in Figure 1. Channel lengths are indicated on Figure 1. Polyimide-coated fused silica capillaries were connected to the sheath channels using PEEK Nanoports (IDEX Health & Science, Oak Harbor, WA). Glass tubes of 0.05" i.d. were glued on the surface and served as sample reservoirs.

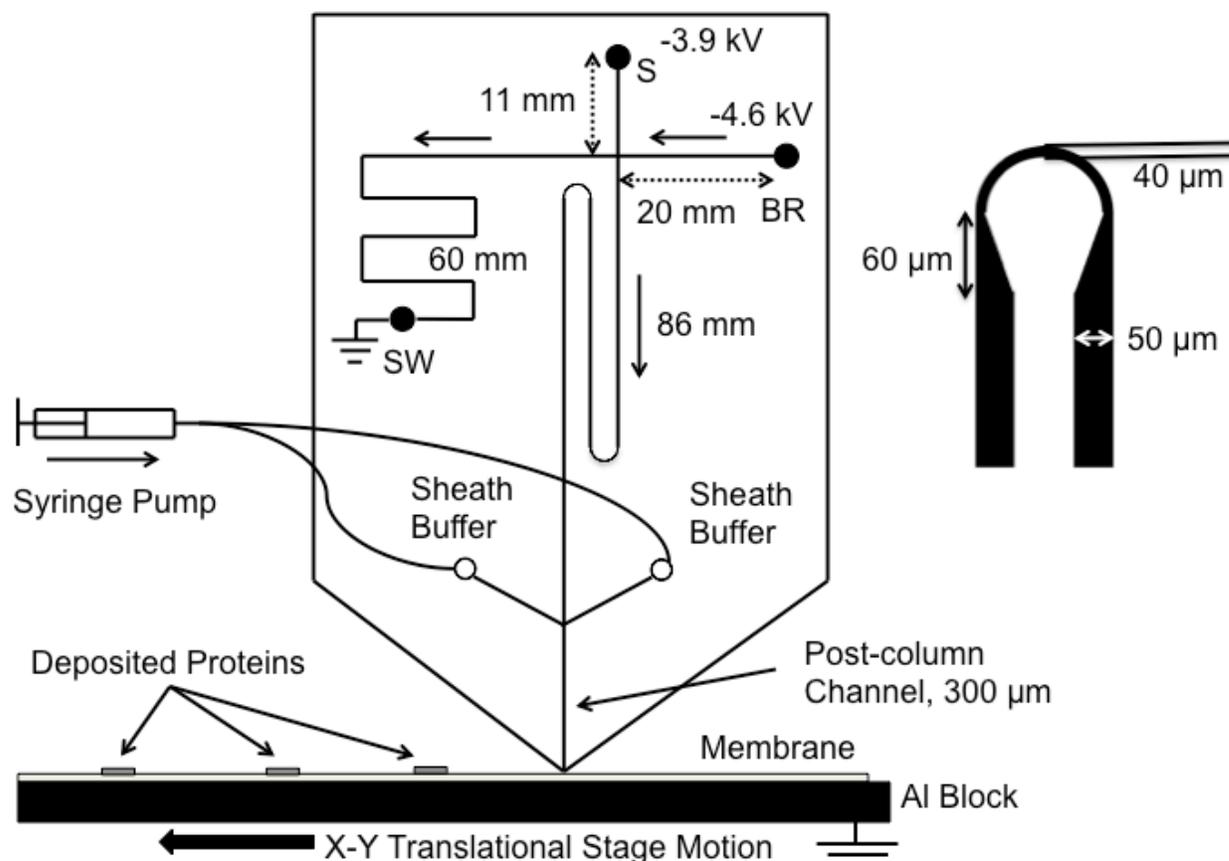


Figure 3-1. Microchip overview. Sample was injected using gated injection method. SDS-protein complexes separated by size were captured in discrete zones on the PVDF membrane moving beneath the chip outlet to preserve separation information. Sieving media was pumped through the sheath channels to give stable current. Channel lengths are indicated by double arrow lines and direction of flow during separation is indicated by solid, single arrows. The inset figure shows the asymmetric turn used to reduce geometric dispersion.

Microchip gel electrophoresis preparation and operation. Channels were conditioned by sequential rinsing with 0.1 M NaOH, 0.1 M HCl, and water for 20, 15, and 10 min respectively, followed by pumping sieving media (AB Sciex, part number 390953, Framingham, MA) through the channels for 25 min. The chip was mounted on a custom-built x-y-z positioner using a 3D printed holder made of acrylonitrile butadiene styrene (ABS) (Figure 3-2). The chip was positioned over a PVDF membrane that was wetted with a 50:50 v:v mixture of methanol and 100 mM Tris buffer at pH 8.8.

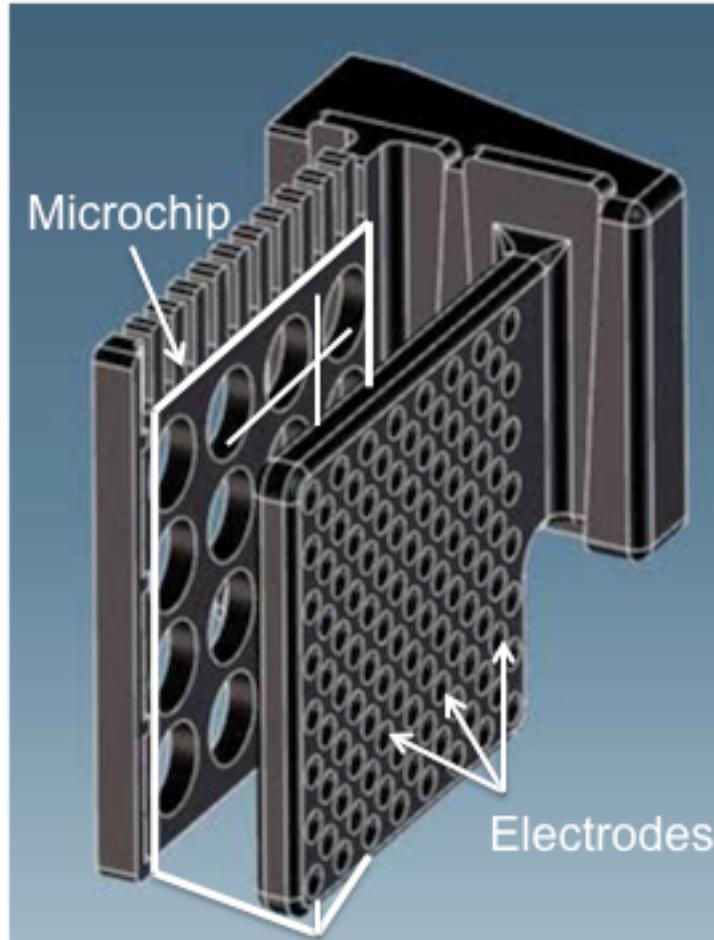


Figure 3-2. Overview of a chip holder. Chip was held on the left and electrodes were held through the holes on the right piece. There were additional holes on the left piece just to save materials during 3D printing.

To perform a separation, the chip with voltage applied as in Figure 3-1 was lowered toward the membrane while monitoring the current. Once contact was made, the chip was lowered another 20-40 μm . If the chip was not lowered, the electrical connection was unstable resulting in current fluctuations. Pushing the chip 20-40 μm lower from initial contact ensured stable connections and well-formed peaks on the membrane (Figure 3-3). If the chip was lowered 80-100 μm further into membrane, the current remained stable but the protein peaks captured on membrane were split (Figure 3-

3) and resolution was lower. These effects were due to the glass tip interfering with protein deposition.

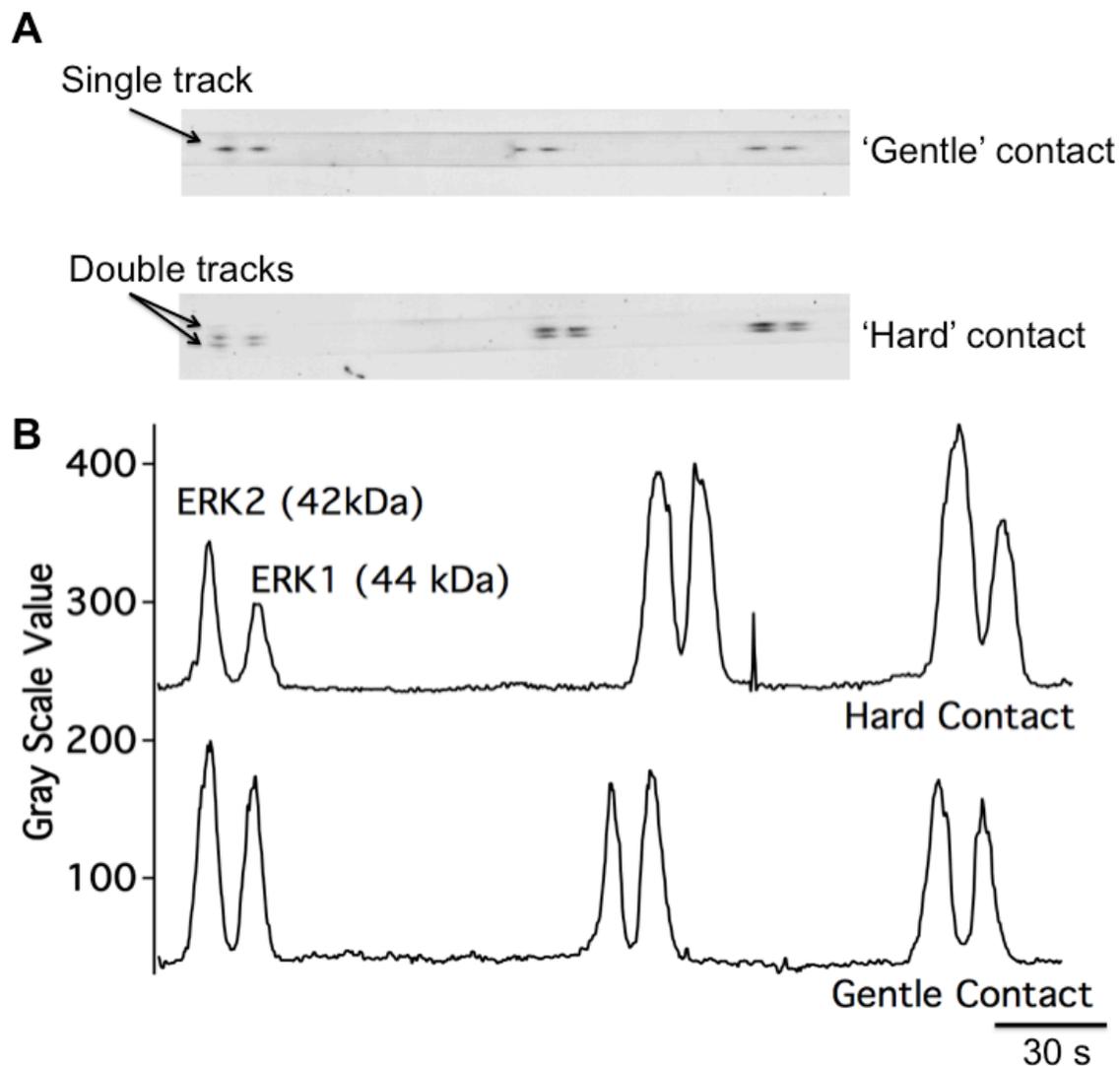


Figure 3-3. Comparison of ERK1/2 separation on membrane when the chip was pushed 80-100 μm into the membrane (considered a hard contact) to pushed 20-40 μm into membrane (considered gentle contact). (A) Direct comparison of two scanned membranes. Proteins were split into a double traces under hard contact. (B) Line scans of ERK1 and ERK2 separation on membrane.

A gated injection method was used to introduce sample into separation channel.¹⁸

Sample was loaded in the sample reservoir (S). Sample was injected by floating the buffer reservoir (BR) and sample waste (SW) with voltage applied between the desired

sample reservoir and the Al block at the exit. During separation, flow from the sample reservoir was gated to the sample waste reservoir (SW) using the voltages as shown. Unless stated otherwise, on a chip with 8.6 cm long separation channel, gating voltage was -4.6 kV. -3.9 kV was applied in the sample reservoir for 10 s while floating the two side channels to inject the sample. During separations the sieving polymer solution was pumped through sheath flow channels at 30 nL/min. The membrane was moved at 4 mm/min during the separation.

Conventional Western blots. For some experiments, proteins in cell lysates were separated using Ready Gel® Precast Gels (Biorad, Hercules, CA). Separation voltage was 100 V and during transfer voltage was 50 V.

Immunoassay. Immunoassay of the captured proteins was performed following protocols for conventional Western blot assays. Briefly, membranes were incubated in blocking buffer (LI-COR Bioscience, part number 927-40000, Lincoln, NE) for one hour to prevent non-specific binding. The membrane was then incubated in the primary antibody solution overnight in a 4 °C fridge. After washing away free antibodies on the membrane surface, it was treated with secondary antibody for one hour before scanning. Unless stated otherwise, primary antibodies were diluted to 1:1000 and secondary antibodies conjugated with 700 nm dye were diluted to 1:5000. All antibody solutions were diluted using blocking buffer.

Detection on Membrane. Fluorescent ladder proteins were detected on the membrane by direct imaging using a Typhoon 9410 variable mode imager (GE Healthcare) in fluorescence mode. For immunoassay, the membrane was dried at room

temperature and then imaged using Odyssey near IR imager (LI-COR Bioscience, Lincoln, NE).

Multiplexing. When analyzing multiple proteins from one cell lysate sample, sequential injections were made from a single sample. Each separation was deposited on a different moving membrane or they were captured on a single membrane that was then cut so that each piece could be immune-probed separately. Each membrane strip that preserved a full separation was scanned under a fluorescent imager before immunoassay for later size calibration. All membranes were then blocked and treated with different antibodies for the target proteins.

Results and discussion

Chip Design. Figure 3-1 illustrates the microchip used in this work. SDS-protein complexes were loaded into the sample reservoir and injected onto the 8.6 cm long electrophoresis channel for separation. The chip outlet was dragged along the surface of the membrane so that as proteins migrated from the separation channel they were deposited directly onto the membrane. In this way, blotting occurred automatically as the compounds migrated, which saved time compared to a traditional Western blot.

The chip was designed with asymmetric side channels (one was 20 mm and the other 60 mm) connecting the sample waste and buffer reservoir to the injection cross. These different lengths prevented over-heating of the chip when applying high voltage for the separations. For example, when the separation electric field was 400 V/cm, the electric fields in the two side channels were calculated to be 573 V/cm and 579 V/cm (Figure 1). On a chip with symmetric side channels of 20 mm long, to maintain the same separation electric field, the electric field in side channels would be higher than 1000

V/cm. Under such conditions air bubbles were formed in the channels preventing stable operation.

Improving separation resolution. Prior work with this general approach emphasized achieving rapid separations on a microchip with short separation length. We previously showed for example that a FITC-protein ladder containing 7 proteins from 11 kDa to 155 kDa could be separated with baseline resolution within 2 min on chips with 15 μm deep by 50 μm wide by 2 cm long channels with 460 V/cm applied.¹³ In this work we examined increasing resolution by optimizing the channel dimensions and injection conditions. Resolution of ERK1 and ERK2 (42/44 kDa) was used a critical pair for testing resolution.

We first investigated the effects of channel depth and width on separation performance. Channels that were 9 x 28 μm , 15 x 50 μm , 19 x 58 μm , and 32 x 84 μm , all 4 cm long, were evaluated by separating a protein ladder containing 7 proteins from 11 kDa to 155 kDa using the same injection and separation voltage. Inspection of the resulting electropherograms (Figure 3-4) revealed several trends. Increasing channel size yielded more signal for early eluting peaks but worse separation resolution and loss of signal for larger proteins. For example, resolution between 32 kDa and 40 kDa decreased from 1.6 ± 0.1 ($n = 3$) to 0.6 ± 0.1 ($n = 3$) when channel dimensions increased from 9 x 28 μm to 32 x 84 μm . More injection bias is also observed when using deeper and wider channels. For example, the 6th (98 kDa) and 7th (155 kDa) peak, which were detected in 9 x 28 μm and 15 x 50 μm channels, were not observed in 19 x 58 μm and 32 x 84 μm channels. These results were attributed to electric field effects within the larger injection crosses.¹⁹ We chose chips with 15 x 50 μm channels for further optimization. Although

the smaller 9 x 28 μm channels yielded better performance, their high flow resistance made filling the channels inconvenient, e.g. conditioning time was at least 50 min compared to 10 min for the 15 x 50 mm chips. In principle, less viscous sieving media would allow smaller channels to be used.

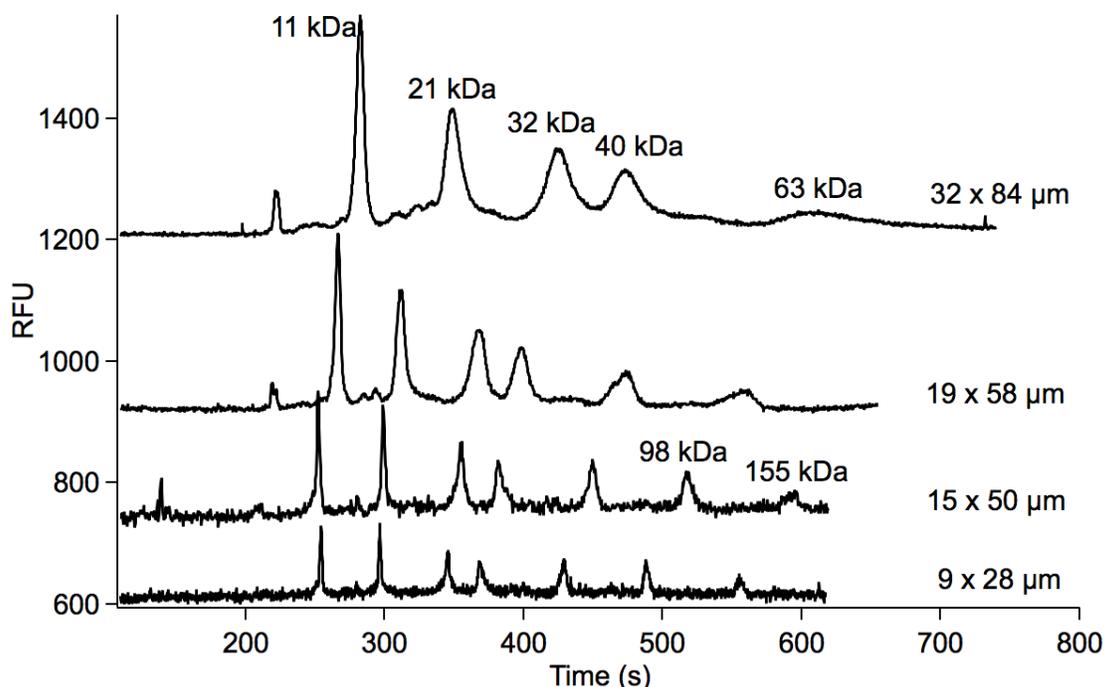


Figure 3-4. Comparison of separation performance on chips with different dimensions. RFU on the Y-axis stands for relative fluorescent unit. Overlaid electropherograms are offset in Y-axis for clarity. All channels were 4 cm long and width and length indicated by the trace. FITC-labeled protein ladder at 1 mg/mL was used for the sample. The ladder had 7 proteins, but on chips with 19 x 58 μm or 32 x 84 μm channels, only 5 protein peaks were detected. Peak capacities were 40 ± 2 ($n = 3$), 34 ± 2 ($n = 3$), 17 ± 1 ($n = 3$), and 12 ± 2 ($n = 3$) in 9 x 28 μm , 15 x 50 μm , 19 x 58 μm , and 32 x 84 μm channels, respectively. Resolution between 32 kDa and 40 kDa peaks were 1.8 ± 0.1 ($n = 3$), 1.5 ± 0.2 ($n = 3$), 1.0 ± 0.1 ($n = 3$), and 0.8 ± 0.3 ($n = 3$) in 9 x 28 μm , 15 x 50 μm , 19 x 58 μm , and 32 x 84 μm channels.

We next compared resolution of a protein ladder for separation lengths from 1 to 4 cm (Figure 3-5A). Based on the trends (which showed the expected resolution increase with square root of length²⁰, see Figure 3-5B), we estimated that the length needed to resolve proteins with a 5% difference in molecular weight (e.g. ERK1/2) was 8.6 cm. To

fit an 8.6 cm long separation channel on a 5 cm long chip requires adding turns to the channel; however, turns cause geometric dispersion by the “race-track” effect²²⁻²⁴ where molecules move faster along the inner wall than the outer side of the channel when making the turn. In this work, an asymmetrically tapered turn was applied to minimize this source of dispersion.²⁴

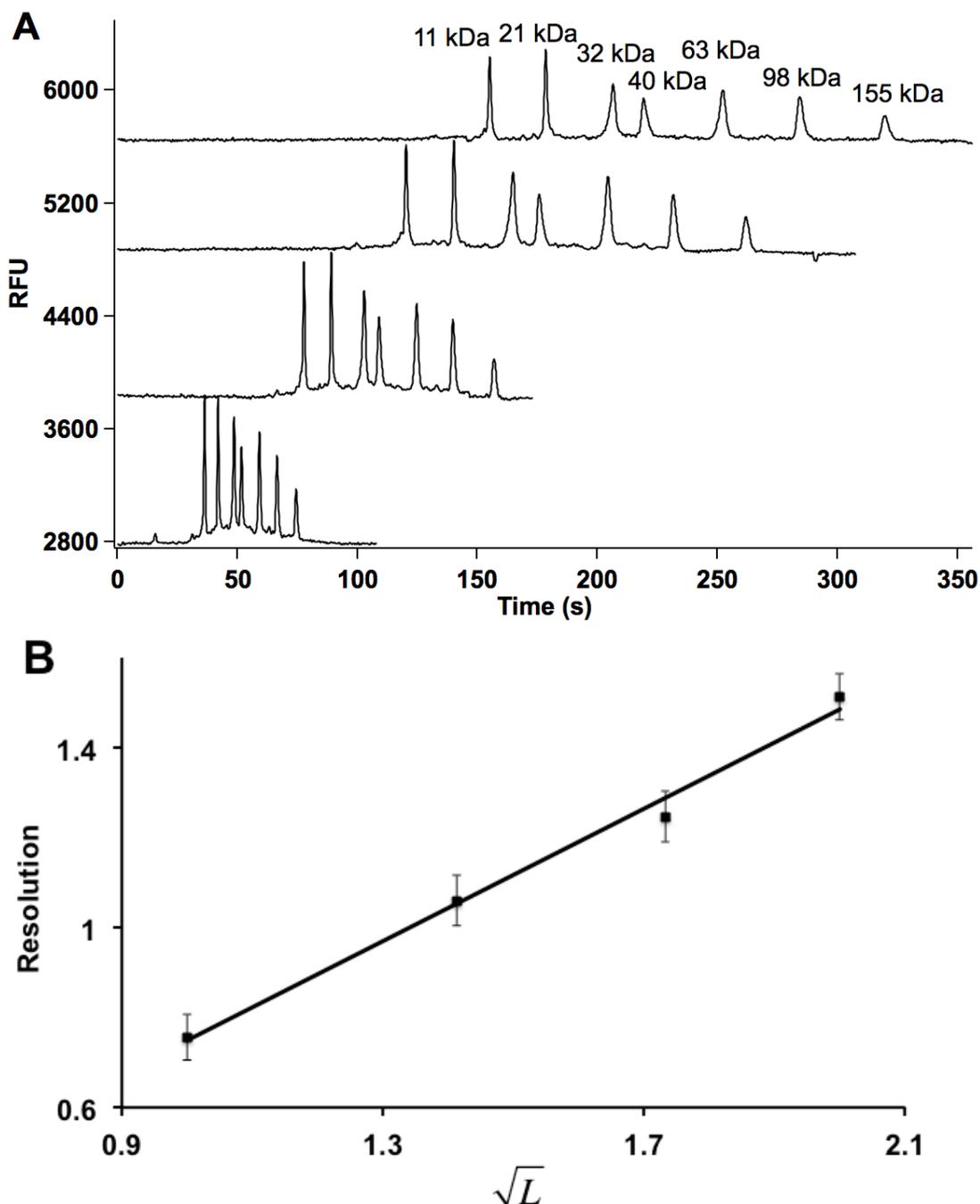


Figure 3-5. Compare separation resolution between two proteins from the protein ladder at different channel lengths. RFU on the Y-axis stands for relative fluorescent unit. Overlaid electropherograms were offset in Y-axis. (A) Electropherograms were recorded 1 cm, 2 cm, 3 cm, and 4 cm from injection cross, respectively. (B) A linear relationship between the square root of separation length and resolution between 32 kDa and 40 kDa proteins, $R^2=0.99$. Separation field was 400 V/cm and sample was injected using gated injection method. 3 replicates at each separation length were recorded.

To verify the effectiveness of this approach to reduce band broadening during sieving electrophoresis, FITC-lysozyme was injected and detected at various points along the separation channel. Figure 3-6 shows that the number of theoretical plates as a function of length using a serpentine channel chip with and without tapered turns. As anticipated the slope of the line decreased with each turn when using constant channel width, indicating added zone broadening after the turns; but, when adding asymmetric turns, the slope was constant over the entire length of separation channel, indicating little contribution to band broadening due to the turns. Based on these experiments, a chip with 15 μm deep x 50 μm wide x 8.6 cm long separation channel containing 15 μm deep x 40 μm wide arcs was considered the best dimension for achieving the desired resolution.

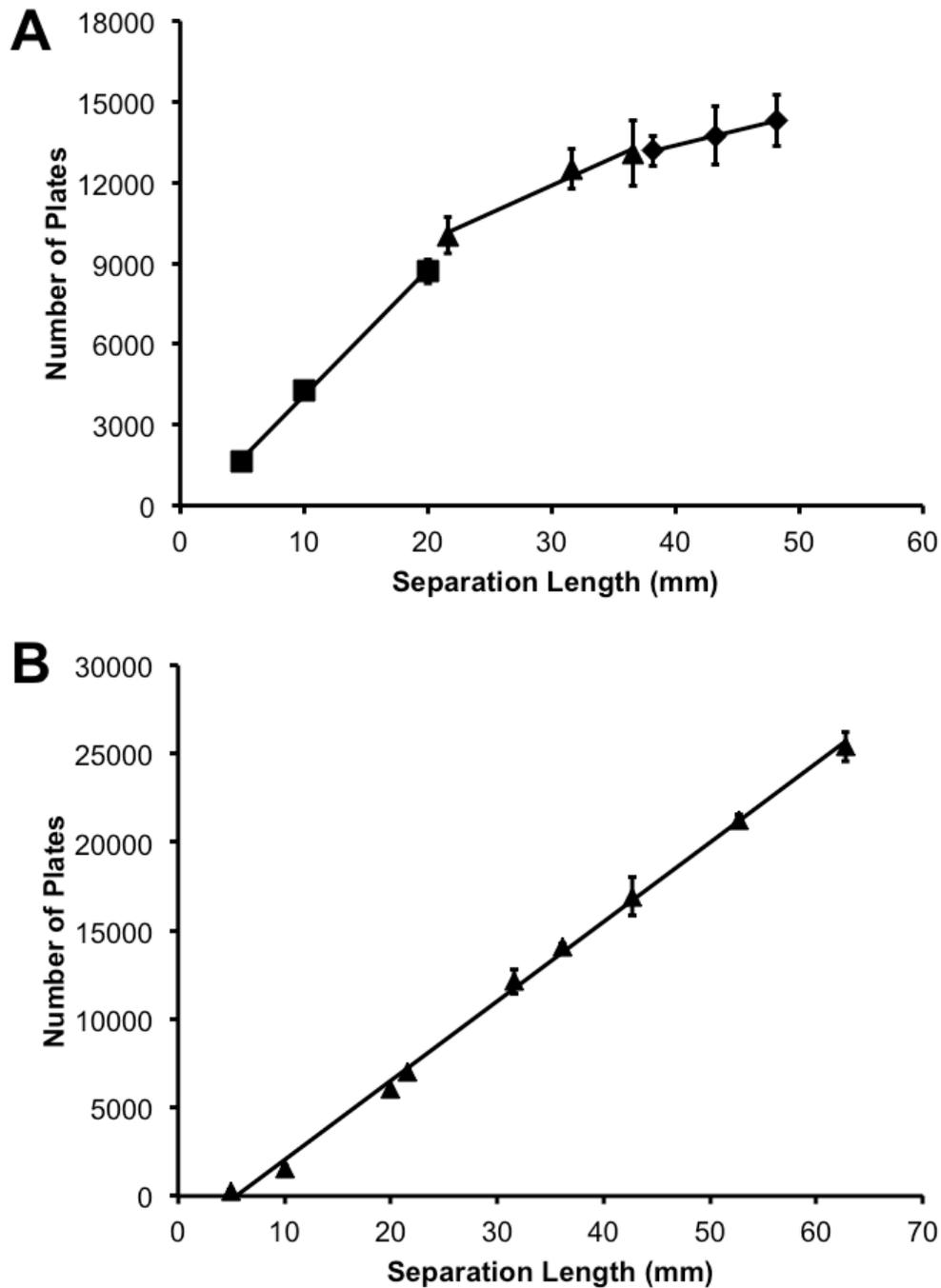


Figure 3-6. Variation of theoretical plates of FITC-lysozyme with separation distance. In A, a chip with turns of same width as separation channel were used. In B, a chip with asymmetric tapered turns indicated in Figure 3-1 were used. The symbols with different shapes signified different regions of the channel that corresponded to the asymmetric turns. Squares indicate the channel region before the first 180 degree turns. Triangles show the region between first and second turn. Diamond symbols indicate regions after the second turn. Three injections were averaged to produce each data point. Error bar is ± 1 standard deviation ($n = 3$)

Injection for Real Samples. After determining the channel dimensions that gave the desired resolution, the system was tested for detection of ERK1/2 in cell lysate. Initial results with a 5 s gated injection, which had been used for development, yielded faint or inconsistent signals on the membrane from Jurkat cell lysate at 2.4 mg protein/mL. Using 10 s injection times allowed ERK1/2 to be detected; but resolution was less than expected ($R_s = 0.6 \pm 0.2$, $n = 3$), likely due to over injection (Figure 3-7A).

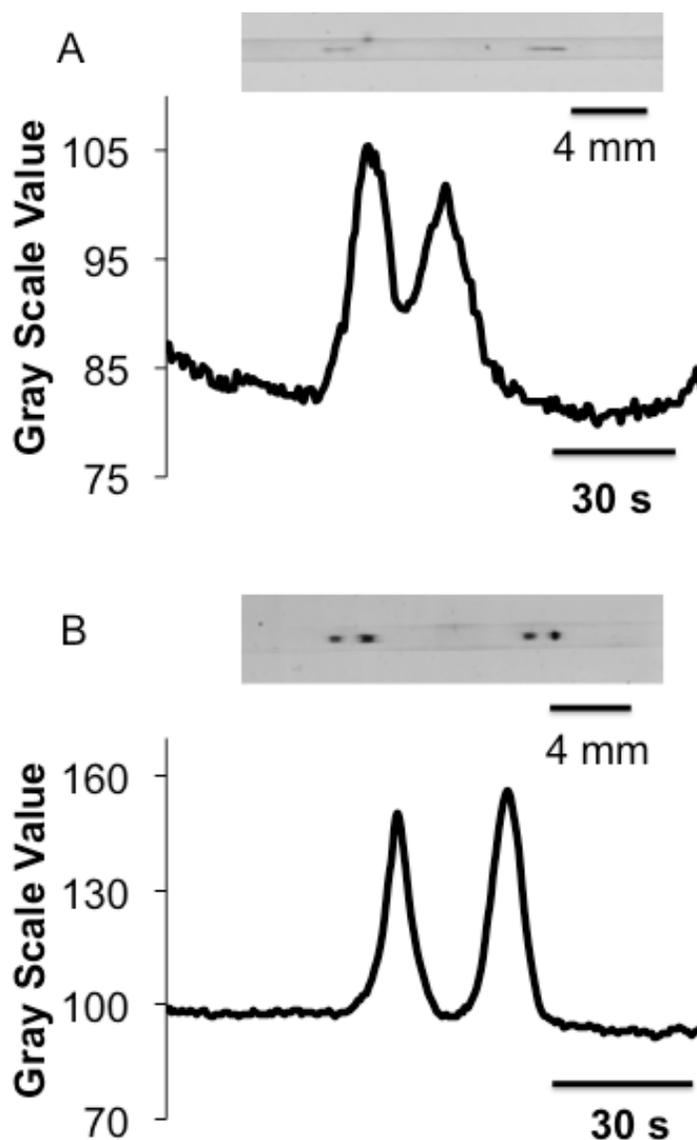


Figure 3-7. Western blot assay of ERK1/2 from Jurkat cell lysate with different injection conditions. (A) 2.6 mg/mL cell lysate (duplicates are shown in blot image) injected for 10 s at 399 V/cm using gated injection. Separation field was 400 V/cm. (B) Assay of

ERK1/2 from Same sample as (A) but with with original lysate diluted 1:10 (v:v) with 20 mM phosphate buffer pH 7.5. Traces below each membrane were line scans through membrane with x-axis converted to separation time.

To address these issues, we diluted samples 1:10 in 20 mM Na₂HPO₄ buffer which allowed both field amplification sample stacking (FASS) and isotachophoretic (ITP)²⁵ focusing. The injection conditions were optimized using FITC-BSA as the analyte which allowed visualization of the injection by fluorescence microscopy. Figure 3-8 shows that when FITC-BSA was dissolved in low conductivity solvent (water), signal increased an average of 15 ± 2 fold ($n = 8$) compared to diluting sample in gel. This effect can be attributed to FASS as sample migrates from the sample reservoir to the injection cross. Adding 20 mM Na₂HPO₄ to the diluent, to act as leading electrolyte for on-line ITP preconcentration, resulted in compression of the zone within 500 to 600 μ m of the injection cross (Figure 3-8). As a result, peak width decreased from 18.4 ± 1.2 s ($n = 8$) to 9.6 ± 0.3 s ($n = 8$) when diluted in phosphate. The combined effect allowed a 38 ± 1 fold ($n = 8$) peak height increase when FITC-BSA was diluted in phosphate rather than in gel buffer.

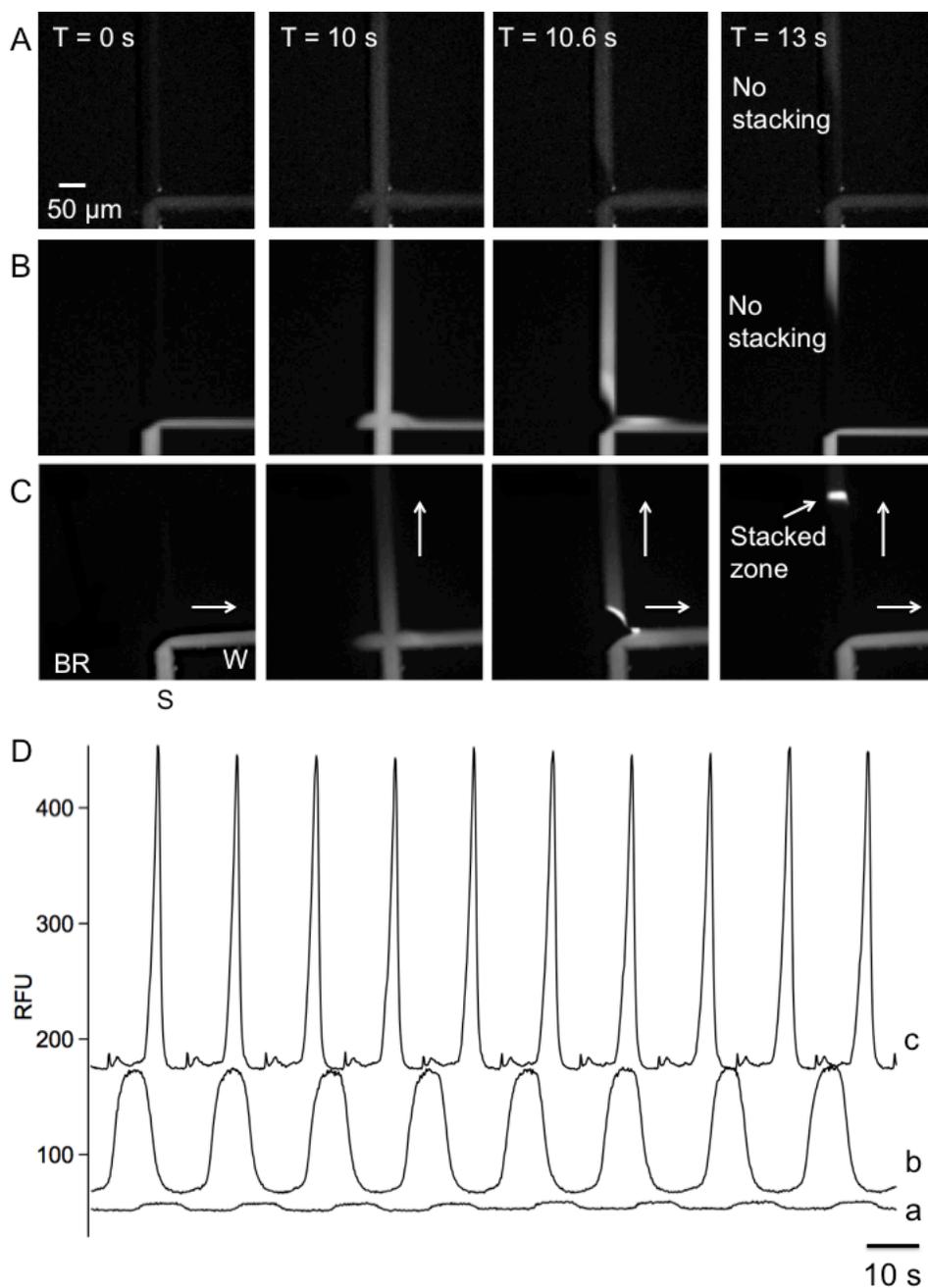


Figure 3-8. Sample stacking effect on injection and separation. 0.01 mg/mL FITC-BSA was used and all injections were 10 s long at 400 V/cm. Overlaid electropherograms were offset in Y-axis for clarity. White arrows showed the sample flow direction. At $T = 0$, voltage was applied in buffer reservoir (BR) and sample reservoir (S). Waste reservoir (W) was grounded. During the 10 s injection, both BR and W were floated. After injection, voltage was applied on BR again to gate sample to the waste. A) FITC-BSA was diluted 1:10 in the same gel buffer used for separation B) FITC-BSA was diluted in water 1:10. C) FITC-BSA was diluted in 20 mM Na_2HPO_4 buffer 1:10. 600 μm long sample plug was focused into a 20 μm wide band within 3 s. D) Compare electropherograms when sample was diluted in (a) gel, (b) water, (c) 20 mM Na_2HPO_4 .

As shown in Figure 3-7B, when cell lysate samples were diluted 1:10 in 20 mM Na_2HPO_4 buffer, peaks had higher signal and were better resolved than injections made without dilution. For example, ERK2 (42 kDa) peak area was 3.5 ± 0.5 ($n = 3$) fold higher than using original lysate. Resolution of ERK1/2 was improved to 1.4 ± 0.1 ($n = 3$) compared to 0.6 ± 0.2 ($n = 3$) when analyzing original lysate.

The resulting separations can be compared to traditional Western blotting (Figure 3-9). For these experiments, 10 μg total protein was loaded onto a 10 cm long gel and separation was carried out at with 100 V for 1 h with an additional hour required for protein transfer. Under these conditions, ERK1 and ERK2 were separated with a resolution of 0.9. Thus, the microchip approach yielded better resolution in less time (8 min for separation and transfer), and used much less sample (400 ng loaded onto the chip).

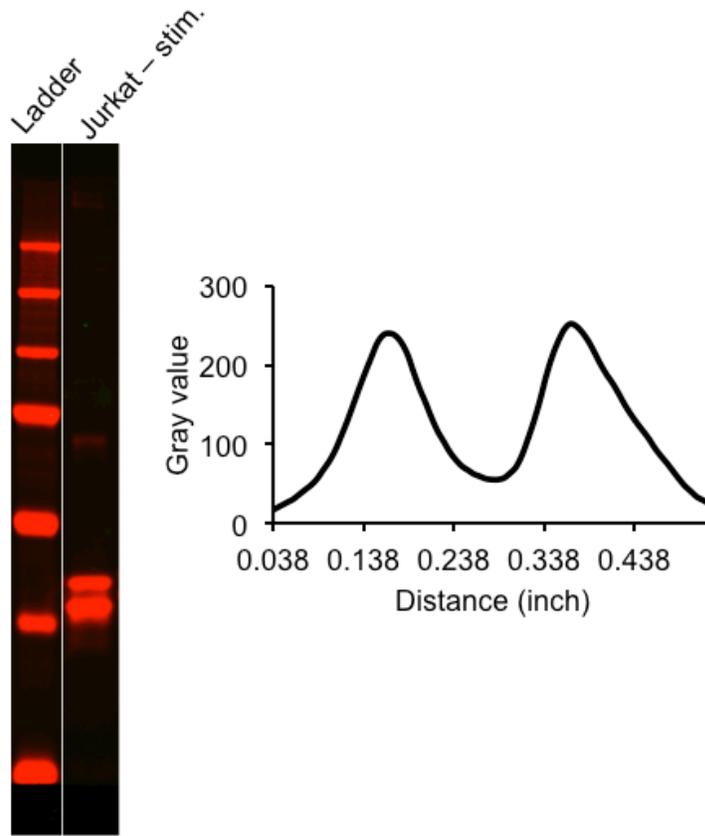


Figure 3-9. Western blot of ERK1 and ERK2 using a traditional protocol. Jurkat cell lysate was stimulated by 0.2 ng/mL PMA for 15 min. 10 μ g total protein was used for the assay. Separation was carried out on a slab gel at constant 100 V. Resolution between ERK1 and ERK2 was 0.9.

Multiplexing Using Microchips. To assess the utility of the microfluidic, direct-blotting technique for detecting multiple target proteins from single cell lysate sample, we performed assays for ERK1/2, MEK1/2, AKT, STAT3, their phosphorylated forms, and beta-tubulin in Jurkat cell lysate samples (Figure 3-10). For these experiments, 2 μ L of sample, corresponding to 400 ng of total protein, was loaded onto the chip. The sample was injected 9 times with each separation being deposited on a different portion of the membrane. The membrane tracks were then treated separately for each protein. ERK1 and ERK2, phospho-ERK1 and phospho-ERK2 were resolved so that a total of 11 proteins were detected in the assay. For comparison, a conventional Western blot would

have ~90 μg total protein or repetitive stripping and reblotting process, to achieve a comparable number of proteins detected.

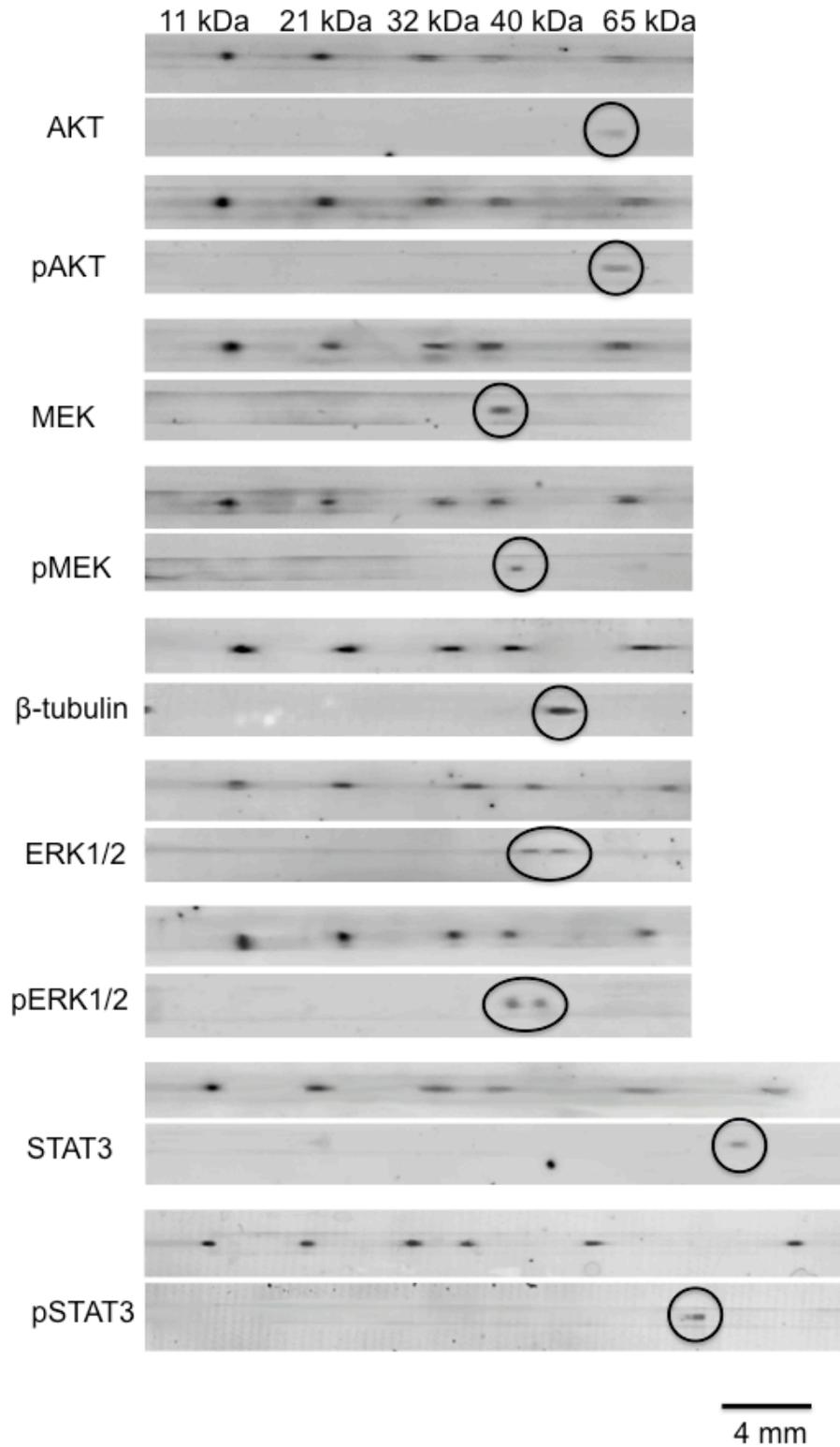


Figure 3-10. Multiplexed Western blot assays of Jurkat cell lysate by MCE. Details on sample preparation were mentioned in the experimental section. 2 μ L of 0.2 mg total protein/mL cell lysate was loaded onto the chip. The same sample was injected 9 times with each trace laid on a different membrane. . Each membrane was probed with a

different antibody as indicated in the figure. For each assay, the membrane was scanned using a fluorescent scanner first at 488 nm excitation wavelength to record protein ladder separation. This information would be used to calibrate the size of unlabeled protein after immunoassay. After incubating with different antibodies, membranes were scanned at 700 nm excitation wavelength for the target protein.

These results show how a combination of added resolution and multiple injection allow multiplexed assay from a single sample. Although 11 proteins were detected in these experiments, further enhancement of the multiplexing is possible. The only limitation so far is that we need to regenerate the chip after 2.5-3 hours operation, after which the gel usually becomes very viscous and dries out. The current also becomes unstable after long time operation and air bubbles are easily formed, which could be attributed to Joule heating and could be further improved by adding external cooling assistant, like heat sinks and air-cooling.

Reproducibility was evaluated by measuring STAT3 signal from 4 different types of cell lysates in triplicate within a day. Figure 3-11A shows that for multiple injections, the RSD of STAT3 peak area was 2.6%, 8%, 7%, and 3.8% for EGF-stimulated A431, resting A431, PMA-stimulated Jurkat, and resting Jurkat lysates, respectively (n = 3). The peak area ratios are comparable to those obtained for a conventional Western blot (3-11B). Figure 3-11C shows the peak area standard deviation for STAT3 in each sample type using microchip based Western blotting system. This result shows that similar antibodies and protocols for the immunoassay step can be used with both the MCE and conventional gel-based Western. This experiment also illustrates the efficiency of sample usage. To achieve the triplicate injections would have required 30 mg of total protein compared to 400 ng loaded for these samples.

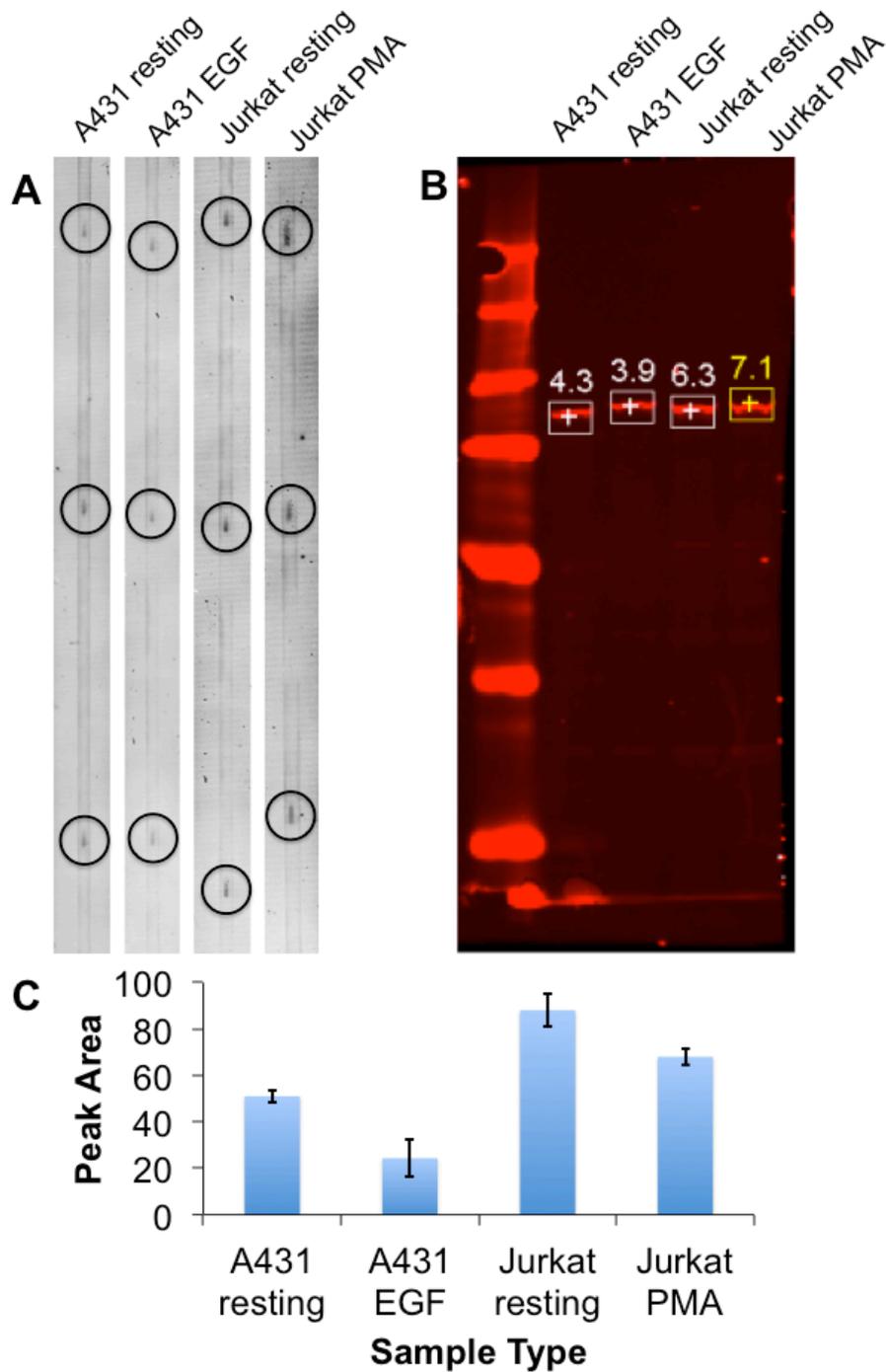


Figure 3-11. Microchip based Western blotting system repeatability study. Repeated injections for determination of STAT3 from EGF -stimulated A431, resting-A431, PMA-stimulated Jurkat, and resting Jurkat cell lysate using A) microchip based Western blotting system and B) conventional Western blotting methods. Error bar is ± 1 standard deviation (n = 3)

Day-to-day repeatability was also reasonable. For example, resolution between ERK1 and ERK2 was 1.3 ± 0.1 , and ERK 2 (42 kDa) peak area was 105 ± 22 gray scale value for repeated assays on three different days (Figure 3-12). The high peak area standard deviation may be due to the difference in antibody incubation time on different days.

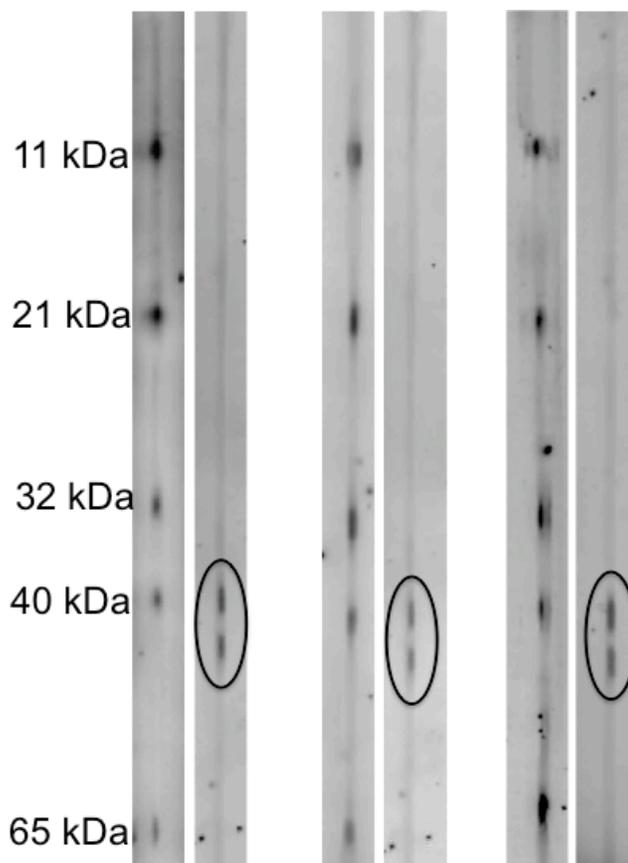


Figure 3-12. Western blot assays of PMA stimulated Jurkat cell lysates conducted on different days. ERK1 and ERK2 were used to demonstrate the repeatability.

Conclusion

We demonstrated the use of a microfluidic Western blot system to achieve multiplexing and better separation resolution at shorter times and with less sample consumption than conventional methods. 2 kDa different (4.5%) proteins were baseline

separated within 8 min. The sample consumption is also significantly reduced due to the microscale channel size. By preserving proteins on separate membranes, multiple analytes from a single lysate sample were tested. 9 Western blots with a total of 11 proteins were accomplished using 0.4 ug of total proteins.

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Chapter 4. Fast and High-throughput Protein Analysis Using Parallel-Channel Microchip Gel Electrophoresis

Introduction

Western blotting technique¹ is benefiting a lot of clinical diagnostics and fundamental biological applications. However some crucial drawbacks, such as long analysis time, low data throughput, and mediocre repeatability, hinder the progress in more life science applications.

Since the first introduction of microfluidics for chemical analysis,² lab-on-a-chip devices have been greatly developed in the area of chemical separations. One important attractiveness of using microfluidics-based separation is its ability to demonstrate rapid and high-throughput analysis while keeping the cost of fabrication essentially the same. Accommodating parallel channel manifolds for separations will help to achieve this goal.

A wide variety of microchips, with most applications involving parallel separations of bio-molecules, affinity assays, and DNA analysis have been reported.³ For example, chips with 36 electrophoresis units were developed for rapid G protein GTPase activity enzyme assays. 36 electrophoretic assays were performed in 30 s suggesting the potential throughput up to 4320 assays per hour.⁴ There were also techniques developed to separate proteins on a microchip with 50 parallel channels by isoelectric focusing.⁵ Another 768-lane DNA sequencing system based on microfluidic plates has been

designed. The system throughput exceeded 4.0 Mbases of raw sequence per day and can be compared to 0.5–1.5 Mbase per day for commercial 96 lane capillary array machines.⁶

There are also commercial instruments developed to achieve fast and high-throughput protein sizing with good repeatability using microfluidic technology. Agilent 2100 Bioanalyzer is suitable for analyzing up to 10 protein samples on a single device within approximately 30 min.⁷ The product is developed based on an early work about protein sizing on microchips.⁸ Another type of device designed for higher-throughput analysis is called LapChip from PerkinElmer. Protein sample is sipped from a standard well plate by vacuum and separated based on size in a microchip. With sample acquisition time under 40 seconds the instrument can analyze 96 protein samples in less than an hour.

Limited work has been done to couple high-throughput features to Western blotting analysis. One recent work introduced a parallel microchannel network to specifically recognize the immobilized proteins on a membrane after SDS-PAGE. The microchannels were placed orthogonally to the protein bands and 7 proteins could be detected.^{9,10} Another microfluidic system that enabled unlabeled ovalbumin (45 kDa) and beta-galactosidase (116 kDa) protein separation, immunoblotting, and detection on a single device was also available. This system was compatible with 48-sample per hour throughput within a standard 1-inch by 3-inch microscope slide footprint.¹¹ However, the resolution was not optimized to separate closely related proteins. Multiplexing was not reported possibly due to mediocre resolution and antibody cross-reactivity.

Our group has developed Western blotting using a microfluidic chip for fast sieving separation while blotting the proteins on a moving membrane.¹² In this report, we

present work on improving throughput by running multiple separations on a parallel channel network interfaced to a membrane. 21 western blot assays are finished in 30 min using the microchip-SNAP i.d. apparatus. Different sieving polymer solutions are also investigated to further increase the separation speed and achieve better throughput. 20% dextran, 10% glycerol, and 1% SDS in 100 mM Tris-borate buffer is chosen as the separation media. Protein ladder ranging from 11 kDa to 155 kDa is separated within 1 min when applying a 1200 V/cm electric field

Experimental Section

Material and Reagents. Fluorescein isothiocyanate (FITC) and FITC protein ladder containing proteins ranging from 11 kDa to 155 kDa were from Invitrogen (Grand Island, NY). Polyvinylidene fluoride (PVDF) membranes were from Millipore (Billerica, MA). Rabbit anti-lysozyme and anti-actin were from Sigma (St. Louis, MO). Goat anti-rabbit antibody labeled with 700 nm near IR dye and goat anti-mouse antibody labeled with 700 nm dye were from LI-COR Biosciences (Lincoln, NE). All solutions were made using Milli-Q (Millipore) 18 M Ω deionized water. Borofloat glass slides were from Telic Company (Valencia, CA). Dextran was from Sigma (St. Louis, MO). Electroosmotic flow (EOF) suppressor, UltraTrol pre-coat LN was from TargetDiscovery (Palo Alto, CA). Entangled polymer solution was from AB Sciex (Framingham, MA).

Sample Preparation. FITC-labeled bovine serum albumin (BSA) and lysozyme was prepared by mixing 10 μ L of 24 mg/mL FITC in dimethyl sulfoxide with 300 μ L of 4 mg/mL of protein in 100 mM Na₂HPO₄ adjusted to pH 7.5 with 100 mM NaH₂PO₄ and incubating for 1 h at room temperature. The FITC-labeled proteins were purified using PD-10 desalting columns from GE Healthcare (Piscataway, NJ). Proteins to be analyzed

were denatured by heating at 95° C for 5 min in presence of 3% SDS and 5% β -mercaptoethanol (BME). Denatured protein samples were diluted from stocks with sample buffer (20 mM phosphate). Protein ladder was diluted 1:10 from supplier for assay.

Chip Fabrication. Detailed fabrication process was described before.¹³⁻¹⁵ Briefly, slides coated with a layer of chrome and photoresist were exposed to UV light through a photomask that had the desired channel network patterned in it (see Figure 4-1). HF wet etching chemistry was used and channels were 15 μ m deep and 50 μ m wide. After drilling the access holes on the etched side of the glass slides, they were washed by piranha solution and RCA solution before thermally bonded to blank borofloat glass slides. The chip was cut using a dicing saw to create a point at the post-separation channel as shown in Figure 4-1. Glass tubes with 3/16" i.d. and 5/16" o.d. were used to serve the purpose of buffer reservoirs. Distance between sample reservoir and the injection cross was 18 mm. Separation channels were 20 mm long. The channel connecting injection cross and buffer reservoir or waste reservoir was 10 mm long. The chip was cut using a dicing saw (ADT 7100, Advanced Dicing Technology Inc. Horsham, PA) to create points at the post-separation channels as shown in Figure 4-1. Chip tips were ground using a micropipette beveler (Sutter Instrument Company, Novato, CA). The chip was placed at 45° to the grinding paper surface. Typically after 10 s grinding, the chip thickness was checked under a microscope to make sure the channel outlet was not ground. Final tip thickness was 0.8 mm. Microchips that were used for testing the dextran gel performance had different dimensions. Those chips had single separation channel also 20 mm long. The two side channels connecting injecting cross

were 9 mm long and the channel length between sample reservoir and injection cross was 11 mm.

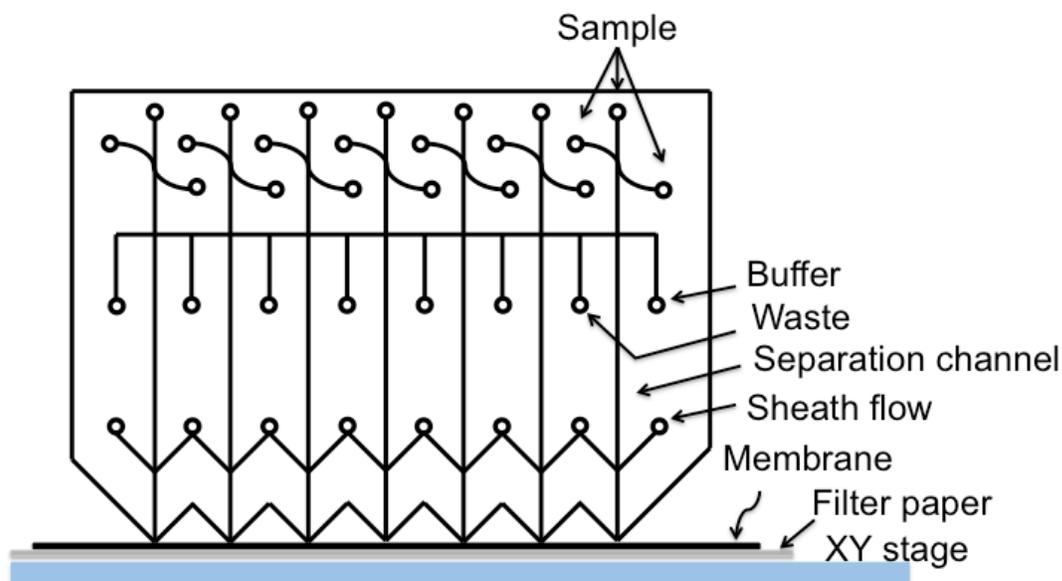


Figure 4-1. Microchip overview. The drawing is not to scale. Separation channel is 2 cm long. Post separation channel is 300 μm long. The channel connecting injection cross and buffer reservoir or waste reservoir is 1 cm long. Sieving media is pumped through the sheath channels to stabilize current. Separation field was 230 V/cm.

Operating chips with parallel channels. Prior to use channels were conditioned by sequential rinsing with 0.1 M NaOH, 0.1 M HCl, and water for 20, 10, and 5 min at -0.6 bar using a vacuum pump, followed by filling channels with sieving media (an entangled polymer solution of proprietary composition from AB Sciex) for 20 min at -0.6 bar. The condition time was longer than on a single channel chip because the complicated channel network added to the backpressure. Reservoirs, except sample reservoir, were filled with the same sieving media.

After chip conditioning, voltage was applied following gated injection schemes. During injection, all channels connected to sample waste reservoir and buffer reservoirs were floated and -800 V was applied in all the sample reservoirs. During separation, the voltage in sample reservoirs was kept the same, and -1000 V was applied in all buffer

reservoirs while all the waste reservoirs were ground. For two adjacent separation channels, they share a buffer reservoir or a sample waste reservoir. Separation field was calculated to be 230 V/cm based on the voltage applied in each reservoir and the length of channels.

For on-chip detection, FITC-lysozyme and FITC-BSA separations were recorded using an inverted fluorescence microscope (Olympus, Melville, NY) equipped with a Xenon arc lamp (Sutter instrument company, Novato, CA) and an electron multiplier CCD camera (Hamamatsu, Bridgewater, NJ). Unless stated otherwise, sample was injected for 5 s at 200 V/cm, and separations were recorded at 2 cm detection point.

When capturing proteins on membranes, same sieving media used for separation was pumped through 8 sheath channels at 30 nL/min to stabilize current. The chip was mounted on an acrylonitrile butadiene styrene (ABS) 3D printed chip holder. PVDF membrane was wetted with a 50:50 v:v mixture of methanol and 100 mM Tris buffer at pH 8.8. The membrane was supported on a filter paper placed on the XY translational stage as shown in the Figure 4-1. The stage moved at 6 mm/min so that the protein separation was captured on the membrane. When running experiments on a chip with 7 parallel channels, in order to prevent overlapping of the 7 tracks, it was placed at a 25° angle (Figure 4-2). The chip was then lowered towards the membrane while monitoring the current. When current was detected, chip was lowered for another 20-40 μm to ensure stable contact between the tip and the membrane during experiments.

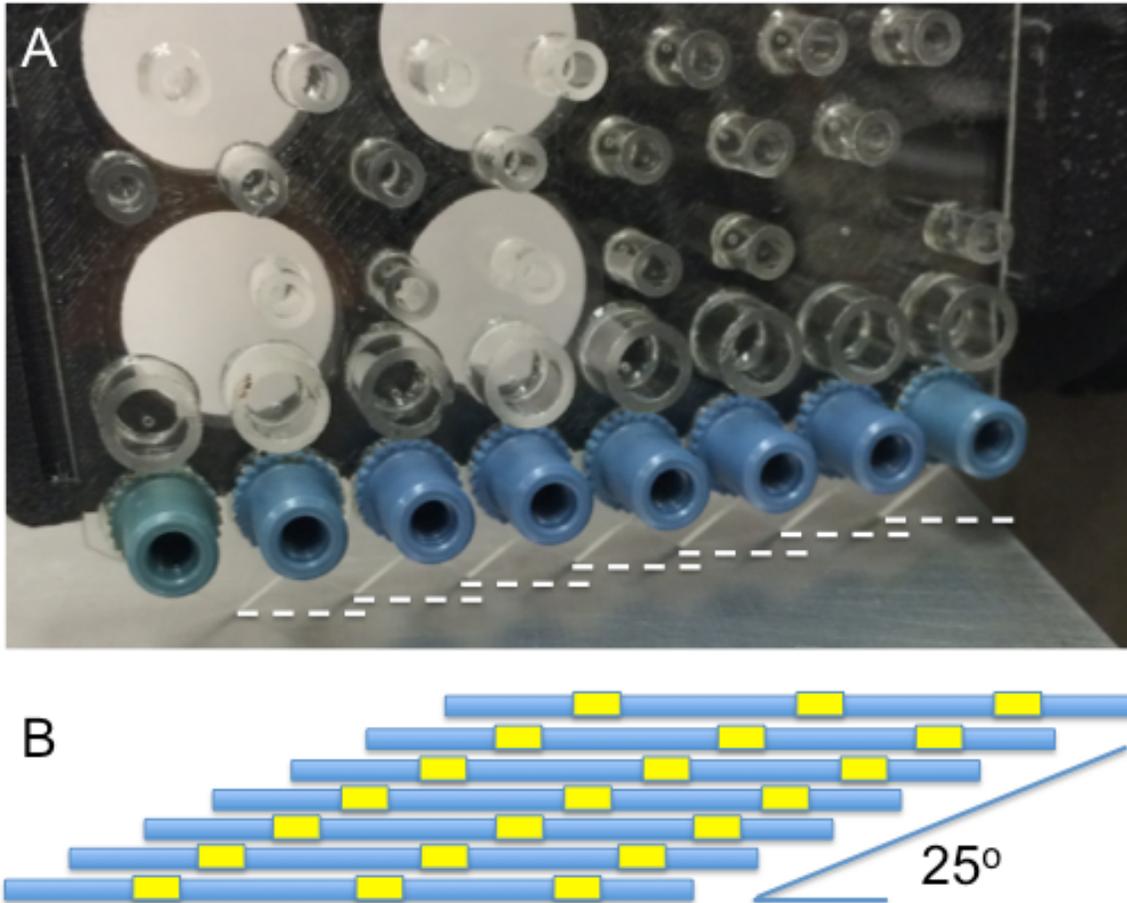


Figure 4-2. Chip orientation when operating parallel separations. A) Chip was placed in a 25° angle to X-axis. White dashed line indicated the separation tracks that were not overlapped. B) A schematic drawing of 7 separation tracks on membrane. 7 lanes were not overlapped. Blue rectangles showed the tracks from each channel and yellow bars indicated protein peaks from each separation.

Immunoassay. After protein sizing on a chip with 7 parallel channels, the membrane was immunoblotted using a SNAP i.d. (Millipore, Billerica, MA) system following manufacturer's instructions.¹⁶ Lysozyme antibody was diluted to 1: 1666. Goat anti-rabbit secondary antibody, conjugated with Alexa Fluor® 532, was diluted to 1:2000. All antibody solutions were diluted using blocking buffer.

Protein sizing using dextran gel. 100 mM Tris-borate buffer containing 20% dextran, 10% glycerol and 1% SDS (named dextran gel) was prepared and ultra-sonicated for 30 min to ensure complete dissolving. Dextran gel had a viscosity of 70 ± 2 cP (n = 3)

at 40 °C while commercial gel viscosity was 78 ± 4 cP ($n = 3$). Channels were conditioned with 0.1 M NaOH, 0.1 M HCl, H₂O, and EOF suppressor UltraTrol sequentially for 10 min, 5 min, 2 min, and 10 min respectively. The channels were then filled with dextran gel. When comparing performance using commercial gel from AB Sciex with home-made dextran gel, -2.5 kV and -2 kV were applied in the gating and sample reservoir. The separation electric field was 460 V/cm. When performing faster separation at a higher electric field using dextran gel e.g. 1200 V/cm, -5 kV and -4.3 kV were applied in the gating buffer reservoir and sample reservoir. When using pinched injection method, membrane was ground, and -5 kV, -1.5 kV, and -1.5 kV were applied in the buffer reservoir, sample reservoir, and waste reservoir. Separation was recorded on-chip using a fluorescent microscope or captured on a moving membrane before scanned using a Typhoon 9410 variable mode imager.

Results and Discussion

On-chip multi-analyte analyses. Typical SDS-PAGE allows one separation in each gel lane. To analyze another sample, it needs to be loaded to another lane or casting new gels. The preparation time is relatively long and thus reduces the analysis throughput. Performing multiple separations in series from a single channel gains a throughput advantage. To analyze different samples, 3 sample reservoirs are coupled to the same separation channel. When analyzing different samples, carryover could happen when switching injection voltage from the previous sample reservoir to a new one. To test carryover, three injections were made from 260 µg/mL, 800 µg/mL, and 260 µg/mL FITC-lysozyme and FITC-BSA mixture. Figure 4-3 shows electropherograms collected

at the end of separation channel. We compared the peak areas of FITC-lysozyme and FITC-BSA and calculated the carryover using the following equation:

$$carryover = \frac{A_{Sig} - A_{Expect}}{A_{Sig'} - A_{Expect}}$$

A_{Expect} was average peak area from the first 260 $\mu\text{g/mL}$ sample injection. A_{Sig} was the average peak area from second 260 $\mu\text{g/mL}$ injection. $A_{Sig'}$ was average peak area from 800 $\mu\text{g/mL}$. After the first injection (260 $\mu\text{g/mL}$) was made, voltage was switched to 800 $\mu\text{g/mL}$ sample. The new sample rinsed the 260 $\mu\text{g/mL}$ sample to the waste and stabilized within 110 s before the next injection was made. FITC-lysozyme carryover when switching from 800 $\mu\text{g/mL}$ to 260 $\mu\text{g/mL}$ was $4.5 \pm 0.4 \%$ ($n = 5$) and FITC-BSA carryover was $4.1 \pm 0.3 \%$ ($n = 5$). When switching between 260 $\mu\text{g/mL}$ and 80 $\mu\text{g/mL}$ sample, FITC-lysozyme carryover was $1.8 \pm 0.1 \%$ ($n = 5$) and FITC-BSA carryover was $0.6 \pm 0.03 \%$ ($n = 5$). The results showed small carryover when switching injection voltage among different samples, which allowed multiple samples to be analyzed using the same separation channel.

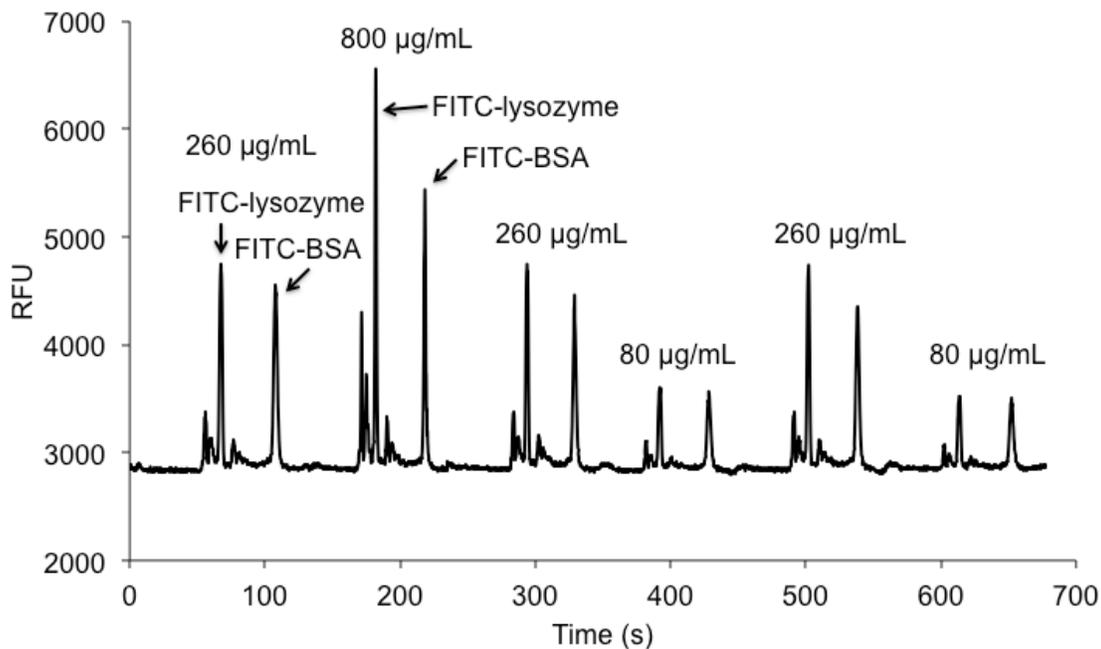


Figure 4-3. Separation of FITC-BSA and FITC-lysozyme mixture on a 3-sample reservoir chip. 260 µg/mL, 800 µg/mL, 260 µg/mL, 80 µg/mL, 260 µg/mL, and 80 µg/mL FITC protein mixtures were injected sequentially. RFU is relative fluorescent unit.

To further improve throughput, 7 parallel separation channels were accommodated on a 3" by 3" chip (Figure 4-4). On-chip separation performance was tested under a fluorescent microscope using 7 FITC-lysozyme standards with different concentrations (concentrations are labeled in Figure 4-5).

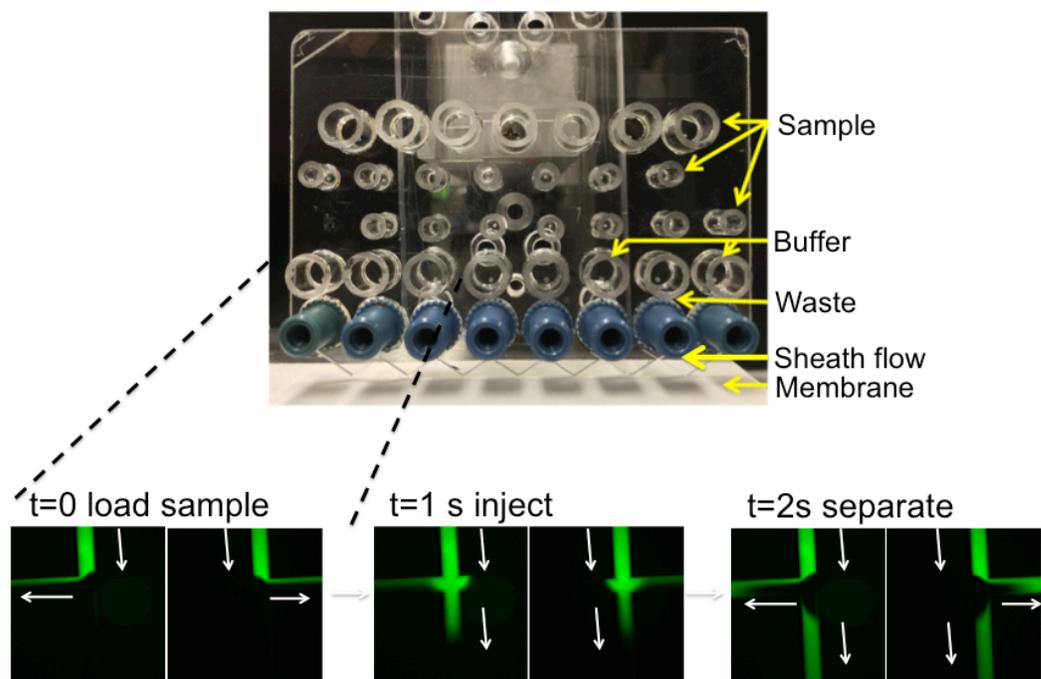


Figure 4-4. An example of simultaneous injection from two adjacent channels. Two channels share a sample waste reservoir or a buffer reservoir. During sample loading and separation, -800 V was applied in seven sample reservoirs, -1000 V was applied in all buffer reservoirs, and all waste reservoirs were grounded. During injection, buffer and waste reservoirs were floated, and samples were injected electrokinetically at 210 V/cm for 5 s . The white arrows show the direction of the flow during different operations.

Figure 4-4 shows sample injection at two adjacent channels. When floating side channels, samples were injected into separation channels simultaneously. When gating voltage was turned on after 2 s injection time, samples from both channels were pushed to the waste. Separations of repetitive injections from 7 samples were recorded (Figure 4-5). Each trace with different colors indicated three repetitive injections from different channels. The system showed good repeatability among injections within one channel, for example peak area RSD for 400 nM FITC-lysozyme (black trace in Figure 4-5) was 2.5% ($n = 3$). A calibration curve showed a linear relationship between average peak area and sample concentration across different channels (Figure 4-6).

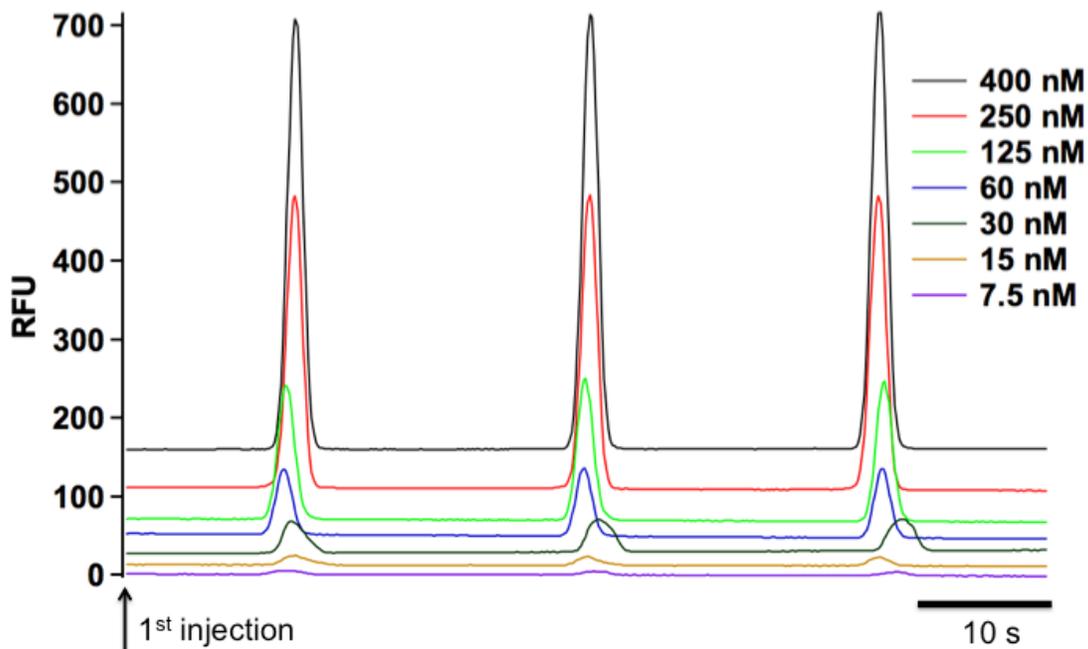


Figure 4-5. Parallel separation on microchips. The figure shows electropherograms for 7.5 nM, 15 nM, 30 nM, 60 nM, 125 nM, 250 nM, and 400 nM FITC-lysozyme separations. Separation electric field was 230 V/cm. Samples were injected at 210 V/cm for 5 s. Overlapped injections were made every 20 s. Electropherograms were recorded at end of separation channels using a CCD camera. Calibration curve is shown in Figure 4-6. Electropherograms were offset in Y-axis for clarity.

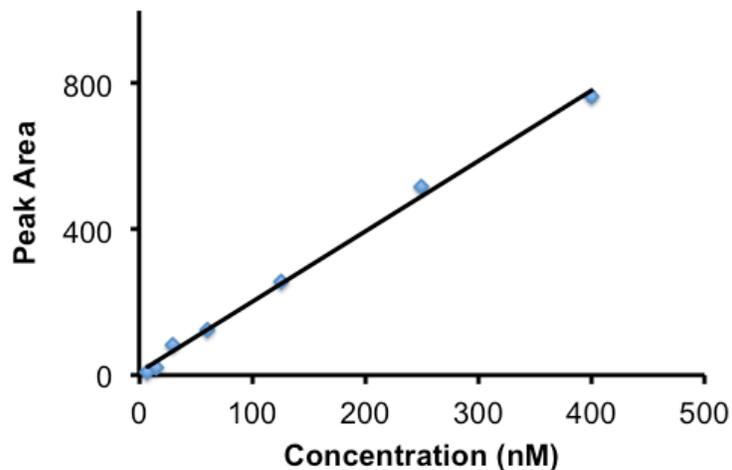


Figure 4-6. A linear relationship between FITC-lysozyme concentration and average peak area of the three peaks from each concentration. Error bars, which are smaller than the points, are ± 1 standard deviation ($n = 3$). $R^2 = 0.99$. Average peak area RSD within each channel is 2.5 % ($n = 3$).

Comparison to other approaches. Previous work has recently demonstrated excellent throughput (e.g., 48 samples in 60 min) for a microfluidic Western blot.¹¹ The system reported here has the potential to achieve comparable levels of throughput and also provides advantages such as repetitive injections, easy regeneration of the chip, and capability of multiplexing. Another published work^{19,20} reported 96 separations in 10 min run time using 8 parallel channels. Due to the short separation length (9 mm) of each unit, there was little room for improving resolution. Downstream immunoassay analysis was also not reported possibly due to the difficulty of driving and rinsing antibodies through the target proteins. The parallel channel system reported here captured eluted proteins on membrane for further processing. In conclusion, this system offers opportunities for high throughput Western blots. Better channel network organization and larger chip dimensions would help to accommodate more parallel channels and improve throughput more.

Capture parallel separations on membrane. To prevent 7 separation tracks from overlapping together, the chip is placed in 25 degree to the direction of membrane movement. However this chip orientation may affect peak width upon deposition. Figure 4-7 shows different chip orientations and the protein ladder separations. Comparing individual peaks from the ladder, peak intensity decreased by an average of $40 \pm 4\%$ ($n = 7$), when the chip moved in Y direction compared to X direction. Peak widths were also broadened by $35 \pm 7.7\%$ ($n = 7$). Resolution between 32 kDa and 40 kDa peaks decreased from 0.9 ± 0.1 ($n = 3$) to 0.5 ± 0.1 ($n = 3$) when moving in X direction compared to Y direction. Our hypothesis is that when the chip is moving in Y direction, any unbound or

weakly bound proteins get dragged along the edge of the chip on the membrane resulting in extra peak spreading which affects resolution on membrane.

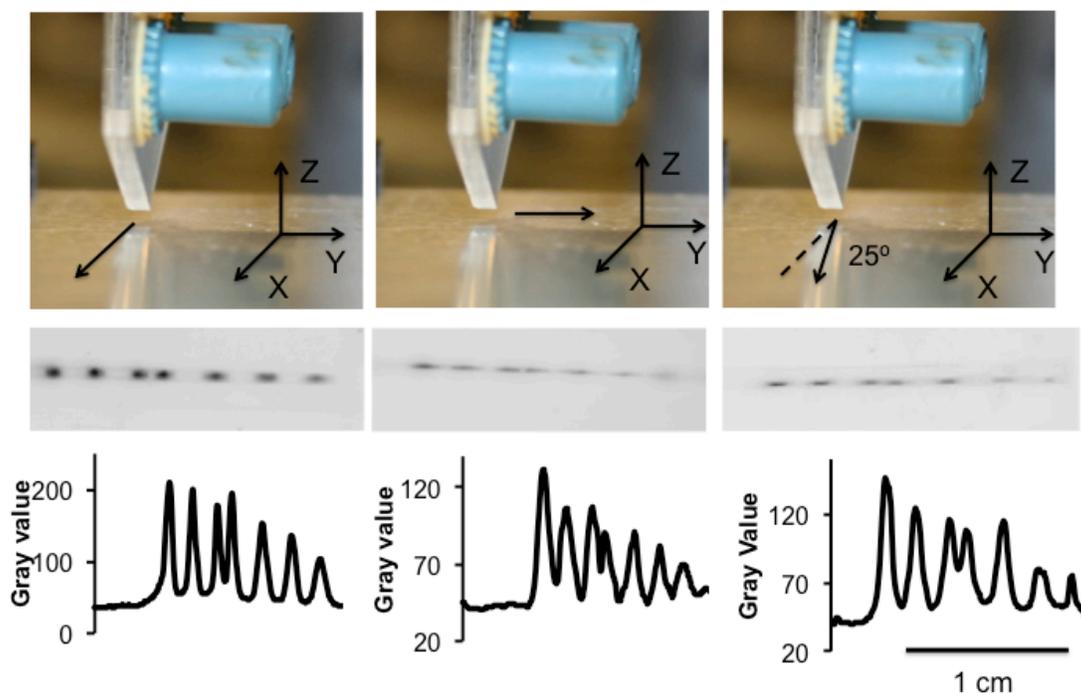


Figure 4-7. Effect of stage moving direction on peak shape. Channel opening at the end of chip is $15\ \mu\text{m}$ in depth (Y direction) and $80\ \mu\text{m}$ in width (X direction). The chip is 1.4 mm thick. Arrows show the direction chip moves relative to the stage. Ladder separations were captured on membrane. Line scans show comparison of peak shapes among different movements.

When moving the chip in a small angle, i.e. 25° relative to the X direction (Figure 4-7), it causes less band broadening comparing to moving in Y direction. Average peak width only increased $20 \pm 3\%$ ($n = 7$) compared to moving in X direction. Separation resolution between 32 kDa and 40 kDa was 0.9 ± 0.1 ($n = 3$) when moving in X direction, and it decreased to 0.6 ± 0.1 ($n = 3$) when moving in 25-degree direction.

Another way to reduce band broadening is to reduce the thickness of the chip. Using a 1.4 mm thick chip, peak widths were broadened by $35 \pm 7.7\%$ ($n = 7$) when moving in Y direction than moving in X direction. When the thickness of the outlet was reduced to 0.8 mm using a pipette beveler, peak width was only broadened by $11 \pm 1\%$

(n = 3) when moving in Y direction comparing to moving in X direction. To conclude, we moved the chips with ground outlets at a 25-degree angle for running parallel separations.

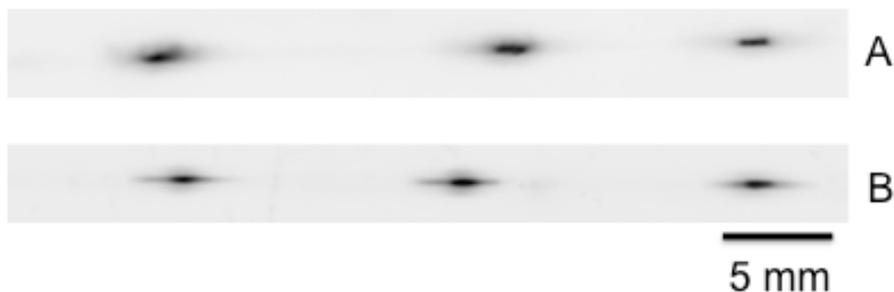


Figure 4-8. Blotting actin standards on membrane using ground chips. The tip is ground using a micro pipette beveler and the total thickness is 0.8 mm. Chip is moving in A) X direction and B) Y direction. Average peak width when moving in X direction is 3.3 ± 0.06 mm (n = 3). Average peak width when moving in Y direction is 3.7 ± 0.2 mm (n = 3).

Figure 4-9 shows an example of using parallel separation for high-throughput Western blotting. 21 unlabeled lysozyme standards were pipetted into different sample reservoirs and 7 standards were injected and separated at same time. 3 injections from different reservoirs containing the same concentration lysozyme were made in each lane. Separations of 7 samples were finished in 2 min. Followed by 25 min immunoassay, a total of 21 lysozyme Western blot assays were finished in 35 min. A linear relationship was observed between concentration and peak area (each with 3 replicates, except the 0.5 mg/mL trace) in Figure 4-10 with $R^2 = 0.98$. Migration time RSD of the first injections from all seven lanes was 2% (n = 7).



Figure 4-9. Microfluidic Western blot of seven different concentration unlabeled lysozyme. Concentrations are labeled next to the lane. Each sample was injected three times. Separation was performed using 230 V/cm electric field. Samples were injected every 2 min. Calibration curve is shown in Figure 4-10. Lysozyme isoforms were detected, especially from high concentration samples.

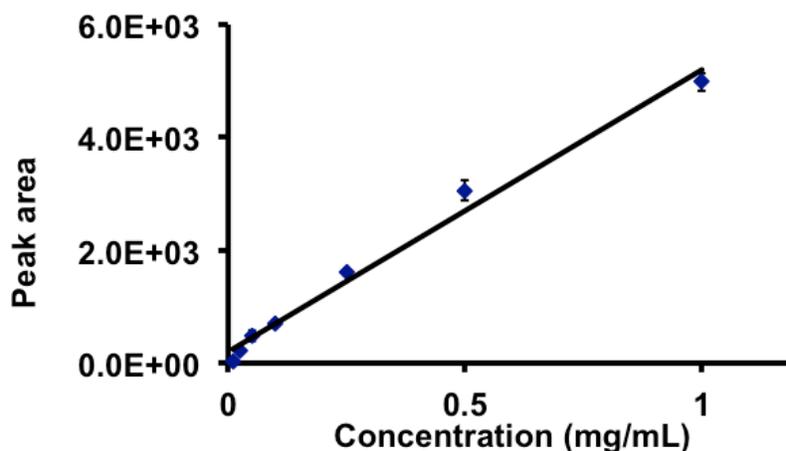


Figure 4-10 A linear relationship between lysozyme concentration and average fluorescent intensity of the three peaks from each concentration. Error bar is ± 1 standard deviation. $R^2=0.98$. Line scans through the traces in Figure 4-9 were used to calculate peak areas.

Unstable connections between outlets and membrane could be the reason for some smearing on membrane, for example the very left peak from 0.5 mg/mL lysozyme in Figure 4-9. Chip outlets that push membrane too much could leave a deep imprint, which causes some non-specific binding and a high signal. Therefore a flat and leveled surface is critical especially in parallel separations.

Using different gel formulations for faster separation. Using the current parallel chip design and applying 230 V/cm during separation, lysozyme was captured on

membrane in 2 min. For higher speed separations, relative to commercially available sieving media, we studied an alternative solution. Non-crosslinked water-soluble polymers such as linear polyacrylamide, polydimethylacrylamide, and polysaccharide derivatives have gained popularity due to the ease of refreshing compared to crosslinked gel. Dextran for separations would offer a uniform pore distribution for reproducible separation, no adsorption to analyte, low EOF, low viscosity and low cost.^{21,22} Glycerol was added into the buffer to stabilize the mesh structure and increase separation resolution.²³ SDS was added to the media to help maintain the denatured protein analyte chains. Previous work has demonstrated that 10% dextran with 5% glycerol 0.1% SDS in 100 mM Tris borate worked for protein sizing on capillaries.^{23,24,25}

When using the same gel formulation on microfluidic chips for protein sizing, separation performance was poor. As expected, gel buffers of increasing dextran percentage exhibited better separation resolution.²³ Figure 4-11 shows electropherograms from separation of protein ladder containing 7 proteins from 11 kDa to 155 kDa using gel with different dextran concentrations. Higher than 25% dextran was not pursued due to the high viscosity and long chip prime time. Using 20% dextran in the gel buffer, peaks were fully resolved. Resolution between 32 kDa and 40 kDa peaks was 1.1 ± 0.05 (n = 5).

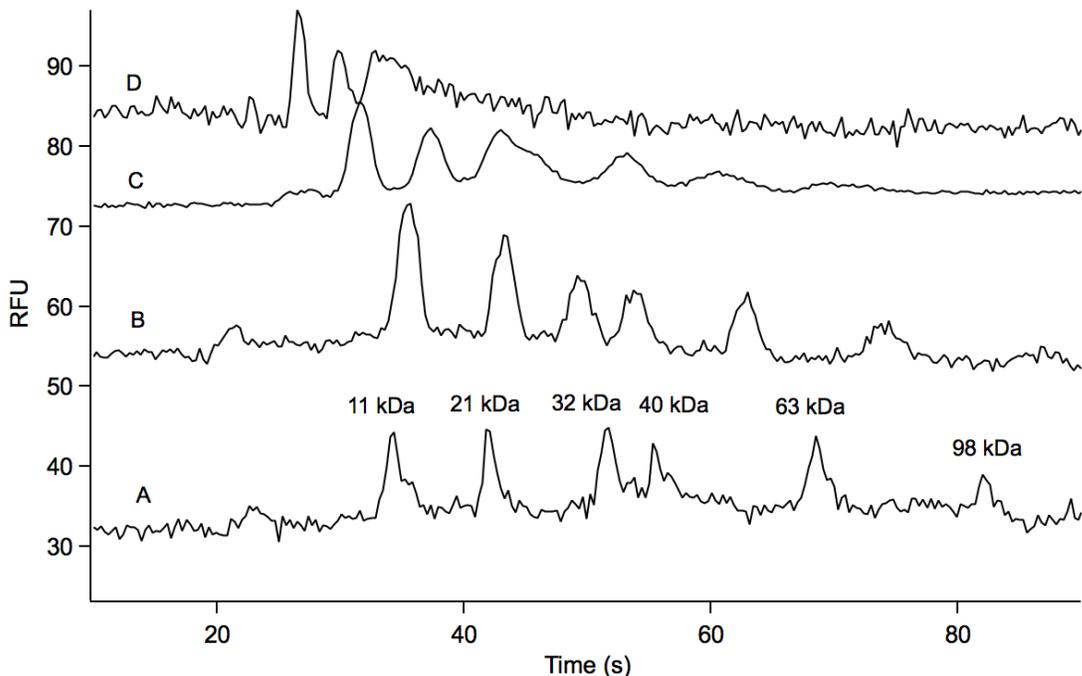


Figure 4-11. Effect of dextran levels on protein sizing in microfluidic chips. Chips have 15 μm deep 50 μm wide channels. Dextran percentage varies in 100 mM Tris-borate, 10% glycerol, 0.1% SDS sieving media. The traces show ladder separation using A) 25%, B) 20%, C) 15%, and D) 10% w/w dextran. Traces are offset in Y-axis for clarity.

Another optimized factor is SDS concentration in gel. Figure 4-12 shows that 1% SDS is sufficient to get good resolution, beyond which there is no additional benefits. Using low SDS concentration (0.1%) in gel buffer may affect the denatured protein structure. For example, migration time for 11 kDa peak was $7 \pm 1.6\%$ ($n = 5$) longer than using 1% SDS in gel. When using 2% SDS in gel buffer, electrophoresis current increased $10 \pm 1\%$ ($n = 5$), which was not ideal when applying high separation fields. In conclusion, 20% dextran with 10% glycerol and 1% SDS in 100 mM Tris-borate buffer was adopted as separation matrix.

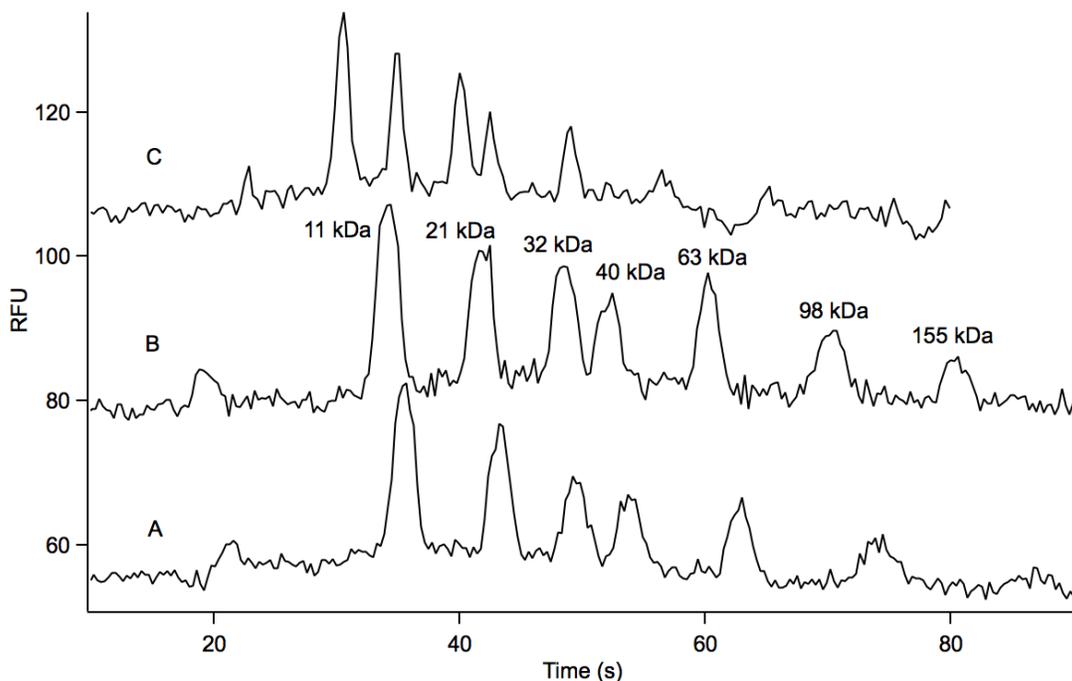


Figure 4-12. Effect of percentage of SDS in gel buffer on ladder separation. Varying SDS in 20% dextran, 10% glycerol in 100 mM Tris-borate sieving media. Representative traces of ladder separations for A) 0.1%, B) 1%, and C) 2% SDS. Traces are offset in Y-axis for clarity.

Separation performance using dextran gel and commercial gel was compared. Figure 4-13 shows ladder separation using both media at a low electric field 460 V/cm. Average migration time decreased $16 \pm 1.2\%$ ($n = 3$) for 11 kDa protein when using dextran gel compared to using commercial gel, possibly due to low viscosity. Peak capacity using commercial gel was 22 ± 2 ($n = 3$), while in dextran gel it was 17 ± 2 ($n = 3$). In conclusion, the two gels had similar performance when applying low electric fields but the home-made dextran gel was much cheaper.

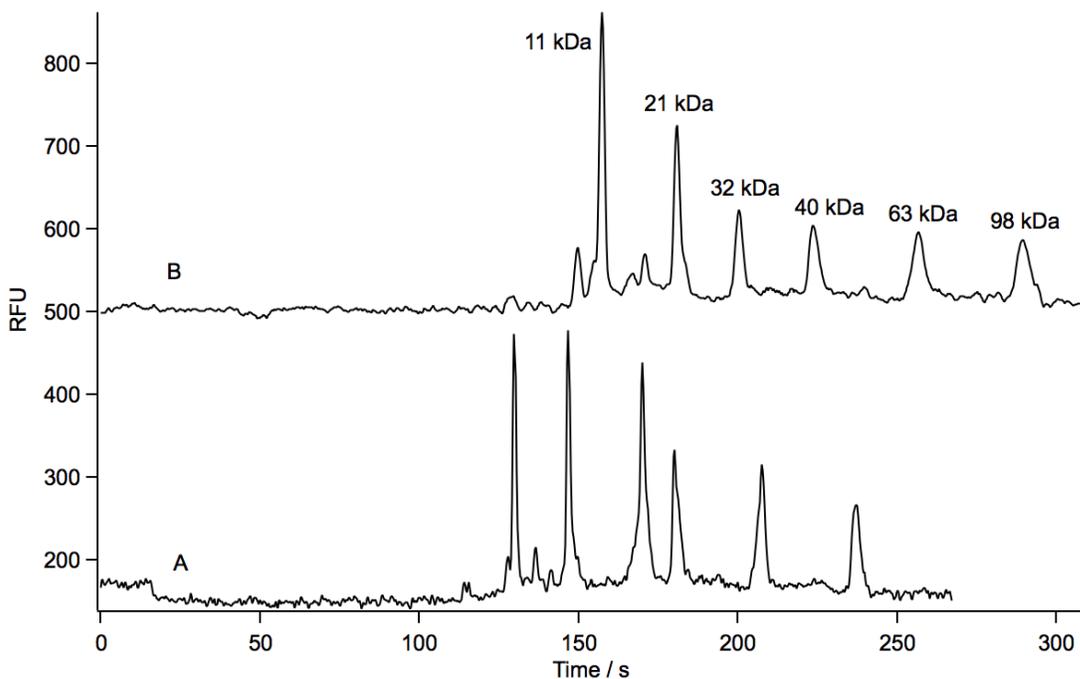


Figure 4-13. Compare separation performance using dextran gel (A) to commercial gel (B). Separation was recorded over 4 cm and separation electric field was 460 V/cm.

To achieve faster separation, it is common to apply a higher electric field. However higher field often causes more Joule heating. Ohm's plot shows the relationship between electrophoresis current and voltage applied across the capillary or microchannels. A linear relationship indicates that no significant amount of Joule heating has been generated. Figure 4-14 shows a linear relationship between current and applied field up to 1200 V/cm on the developed dextran gel, while on commercial gel buffer the linearity only extends to 600 V/cm.

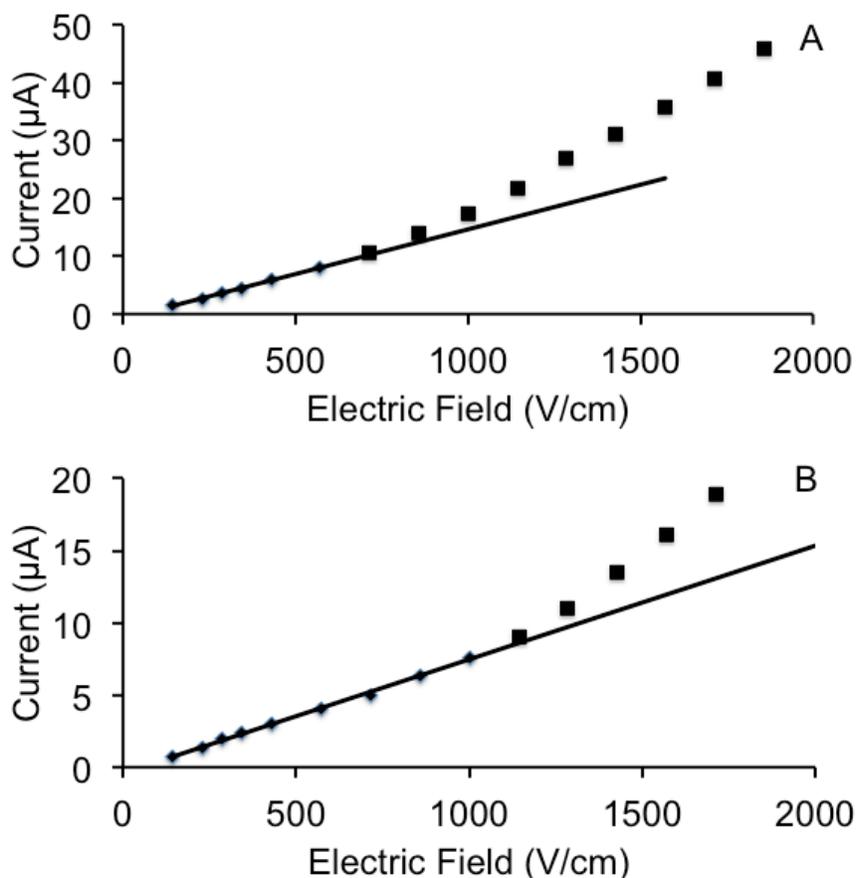


Figure 4-14. Ohm plots for A) commercial gel and B) 20% dextran gel as measured on a glass microchip. The channels on the chip were 15 μm deep and 50 μm wide. Ohm's plot using commercial gel was linear through 600 V/cm, while the plot of 20% dextran, 10% glycerol in 100 mM Tris borate was linear through 1200 V/cm.

We then applied 1200 V/cm across separation channel for protein ladder sizing using the developed dextran gel. Figure 4-15 shows 1 min ladder separation on-chip over a 2 cm long separation channel. Separation efficiency ranged from 5×10^3 to 1.5×10^4 plates when using gated injection method. All peaks were baseline separated, except 32 kDa and 40 kDa proteins with a resolution of 0.8 ± 0.1 ($n = 3$) possibly due to sample overloading. When using pinched injection method, efficiency ranged from 2.7×10^4 to 1.0×10^5 theoretical plates, and peak capacity was 30 ± 1 ($n = 3$), which was slightly lower than a highly cited work using a different type of gel.⁸ The improvement in efficiency was possibly due to the well-defined sample plug injected.

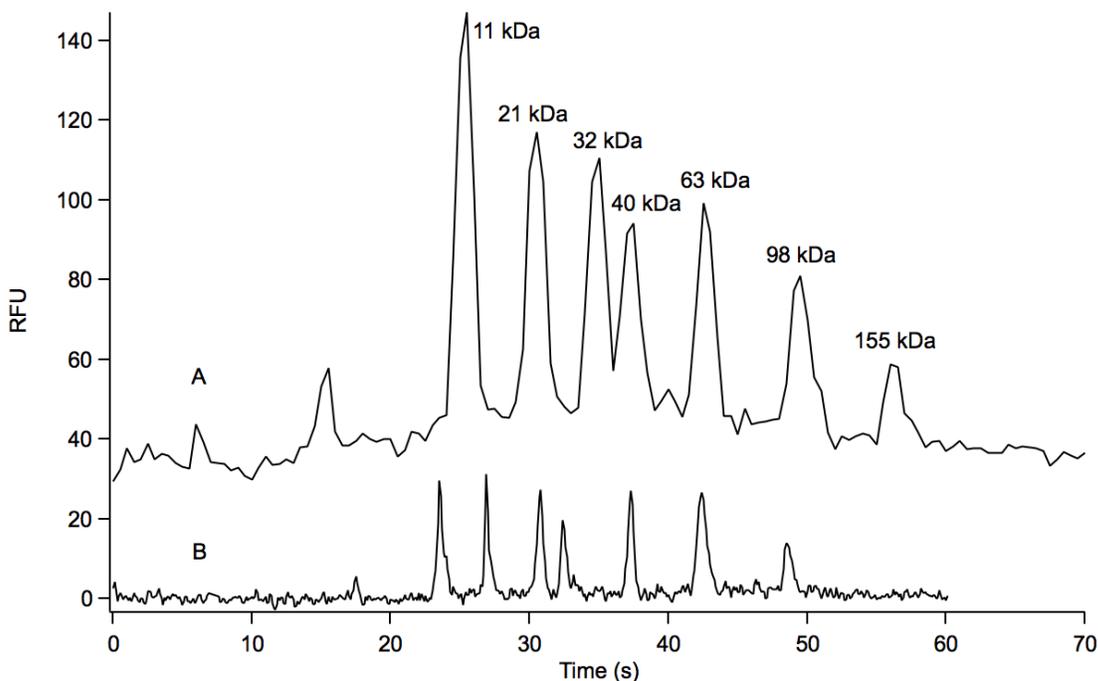


Figure 4-15. Fast size based separation on microchips using 20% dextran, 10% glycerol and 1% SDS in 100 mM Tris-borate buffer. Separation field was 1200 V/cm over 2 cm. Protein ladder with 7 proteins from 11 kDa to 155 kDa were separated within 1 min. A) gated injection method was used. B) pinched injection method was used.

Ladder separation using dextran gel at 1200 V/cm electric field was also captured on membrane (Figure 4-16). Electropherograms showed the separations retained 3×10^3 to 1.1×10^4 theoretical plates using gated injection method. It has been proved that pinched injection method yields a higher separation efficiency. However, current setup for doing pinched injection is not fully automated and does not yield reproducible separation results on membrane. It is probably because we use basic double pole single throw (DPST) switches to change the voltages applied in different reservoirs for pinched injection. We manually throw two switches at the same time when making injections, which could bring variability and affect the separation reproducibility. Future work would focus on using an automated voltage control system to operate pinched injection

scheme. Repetitive pinched injection method is also desired to improve the sample analysis throughput.²⁶

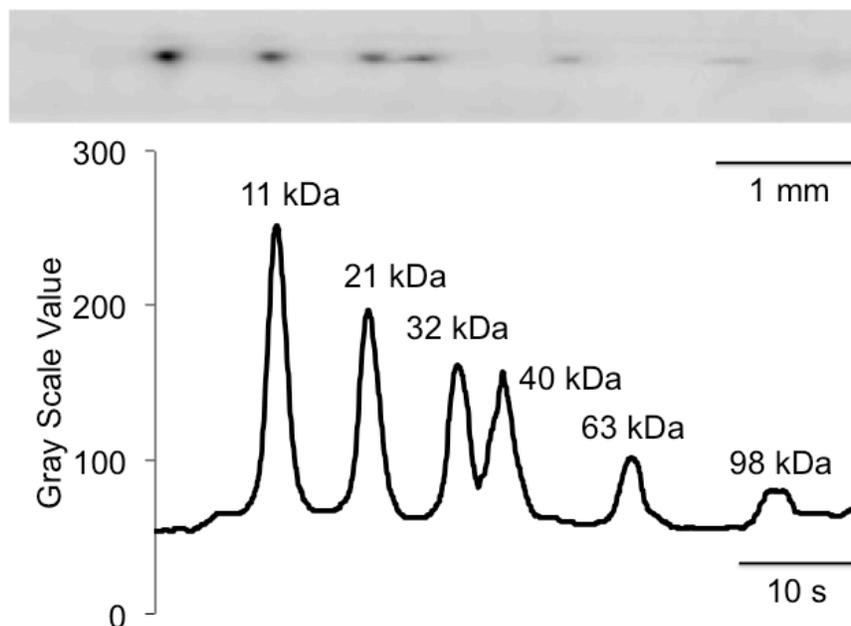


Figure 4-16. Transfer of separated proteins to blotting membrane. Membrane was moving at 6 mm/min. Separation field was 1200 V/cm. Sample was introduced using gated injection method. Separation polymer was 20% dextran, 10% glycerol and 1% SDS in 100 mM Tris-borate buffer. Membrane trace was a line scan through membrane with x-axis converted to separation time.

Perhaps the biggest advantage of using dextran gel is the capability of applying a higher electric field during separation compared to commercial gel. Figure 4-17 shows a direct comparison of protein ladder separation using dextran gel at 1200 V/cm and using commercial gel at 460 V/cm. Resolution between 32 kDa and 40 kDa peaks was 1.05 ± 0.1 ($n = 3$) using commercial gel, and it was 1.20 ± 0.1 ($n = 3$) using dextran gel. We envision better separation resolution and efficiency using dextran gel when extending separation length but in the same analysis time with commercial gel. Currently using dextran gel as separation media and applying high voltage on parallel-channel chips to achieve high-throughput Western blotting is under development.

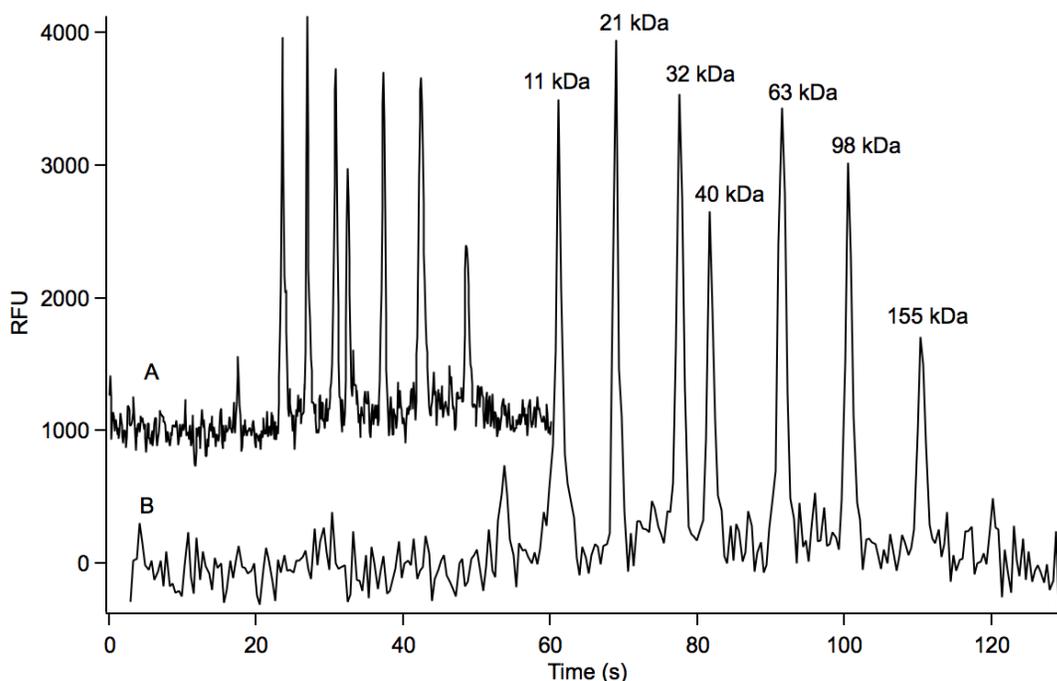


Figure 4-17. Compare protein ladder separation performance using dextran gel and commercial gel. A) applying 1200 V/cm on dextran gel. B) applying 460 V/cm on commercial gel. Both electropherograms were recorded at 2 cm channel.

Conclusion

We reported using parallel separation channels to improve throughput. Currently on a chip with seven parallel channels and 21 sample reservoirs, 21 Western blots could be completed within 30 min. Membranes could be cut into different pieces and treated with different immunoassays so multiplexing is available. 20% dextran with 10% glycerol and 1% SDS in 100 mM Tris-borate was used as a gel alternative to the previously used commercial gel. Electrophoresis current was significantly lower using dextran gel and therefore a high electric field up to 1200 V/cm could be applied for separation. 1 min ladder separation was achieved with 2.7×10^4 to 1.0×10^5 theoretical plates. Using this gel for parallel Western blot analysis at a high electric field is also within reach.

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Chapter 5. Conclusion and Future Direction

Conclusion

A microfluidic based Western blot technology is developed. Fast protein sizing is achieved on chip and directly captured on a moving membrane. The novel microfluidic design also allows a precise control of sheath flow to ensure low protein loss during the blotting step. Although fast analysis is achieved with good sensitivity, resolution between proteins with close molecular weights is often required. By extending separation length and using sample stacking methods, baseline resolution between 2 kDa different (4.5%) proteins is accomplished. Capturing multiple separations on conventional Western blot membranes allows versatile assay development. 11 analytes are detected using 220-fold less sample compared to traditional Western blotting. Analysis throughput is further improved via faster separation at a high electric field and parallelization.

Future Directions

In this dissertation, the implementation of fast protein separation and blotting by microchip CE has been explored. While the feasibility of this method has been validated with a variety of protein assays using different cell lysates, conventional

immunodetection methods still apply. A faster, higher throughput, with low reagent consumption immunoassay approach is desired.

Multiplexing SNAP i.d. analyses. Conventional immunoassay step in Western blotting usually requires a relatively long time possibly due to the slow diffusion of antibodies into the membrane pore for antibody-antigen binding.^{1,2} For example, the antigen and primary antibody incubation usually takes 4-10 hours.³ Millipore developed a vacuum-driven technology and a built-in flow distributor called SNAP i.d. to drive reagents through the membrane, achieving fast immunoassay.⁴ As demonstrated in Chapter 2 and Chapter 4, a complete immunoassay could be finished in 30 min compared to more than 12 hours in a conventional way. The decrease in immunodetection time from hours to minutes is due to the fast antibody diffusion. Adding vacuum actively pulls the antibodies through the membrane for maximum interaction with the antigens without a residual high background.

In our hands, this method while faster produced more than 10-fold lower signal, suggesting a low antibody binding efficiency. One way to improve signal may be to reapply antibody multiple times to the membrane. For example, Figure 5-1 shows 10 fold S/N increase when applying both primary and secondary antibody solutions 3 times to the membrane. Therefore, this approach in principle would take 30 min but achieve the same sensitivity as 4-10 h incubations.

One potential problem with this modification is the cost of antibodies. It is recommended to use 3-5 folds higher concentration than a standard immunoassay for both primary and secondary antibodies in a normal SNAP i.d. system.⁴ Antibody consumption will be higher when reapplying fresh antibodies multiple times. One

solution is to collect the antibody from the end of vacuum line. Although the recovery is about 60-70%, antibody cost is vastly reduced.

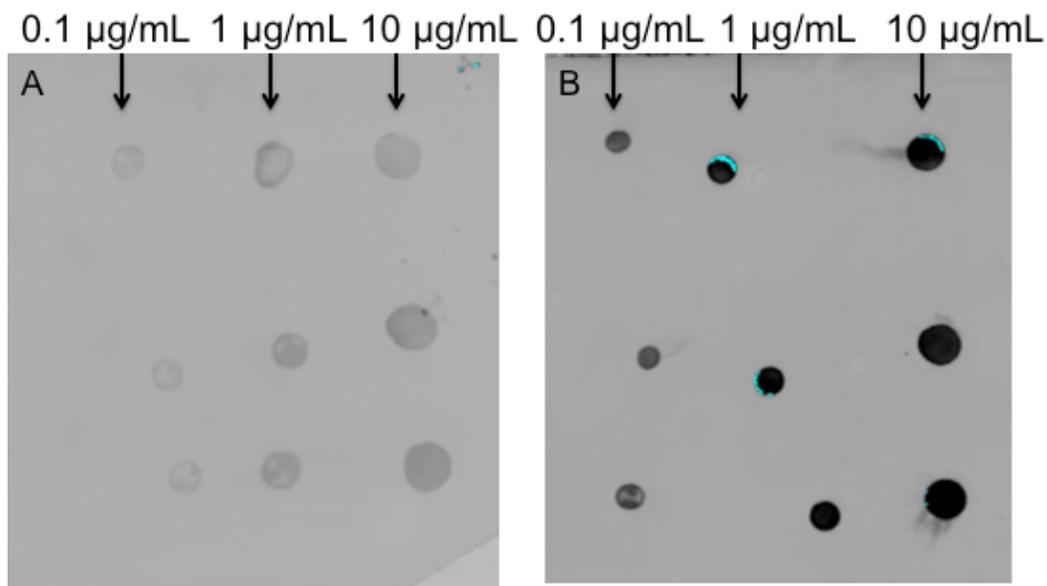


Figure 5-1. Compare actin dot blotting using A) traditional SNAP i.d. and B) modified SNAP i.d. with 3 rounds of antibody incubations. Each row shows protein dots with 3 different concentrations and each column shows 3 replicates.

Another problem associated with SNAP i.d. system is that it is incompatible with multiplexing assays that are possible with the microchip Western blotting system (see Chapter 3). The SNAP i.d. has only one well (8.5 cm x 13.5 cm) to fit a normal size membrane for Western blot and only one analyte can be tested each time. Due to the limited space, setting up multiple wells is not pursued in SNAP i.d. This design is also not good for miniaturization. Microchip based Western blotting system described in this thesis captures proteins on membrane strips about 3-4 mm wide. It is a waste to use large amount of antibody solution (1-3 mL) to cover a single membrane strip. A potential way to address these issues is to use a miniaturized, multi-well system with vacuum to facilitate the flow. In such a system, a grid of channels (custom-made) will be clamped over the membrane, which is placed on a frit, for example a glass fiber spacer. The

channels will be the size of a protein track on the membrane and different antibody solutions are used in different channels to target multiple proteins.

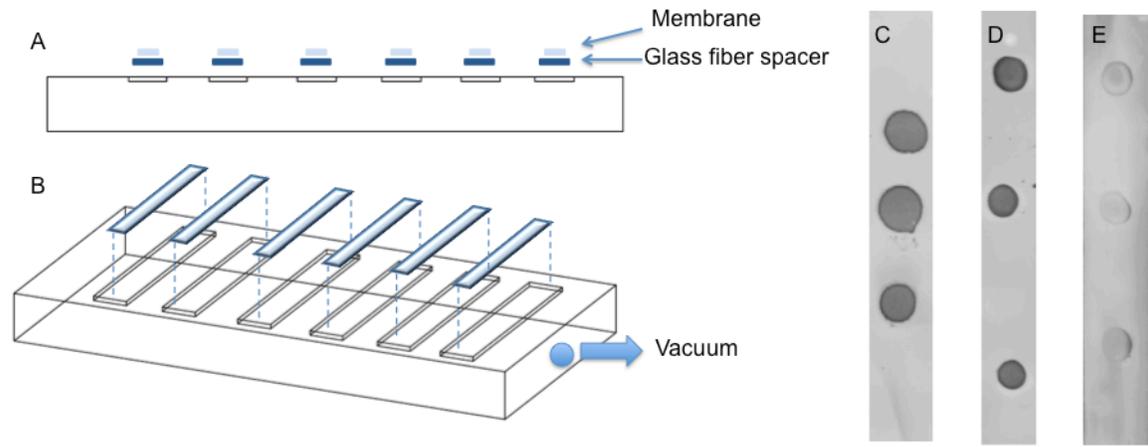


Figure 5-2. Miniaturized SNAP i.d. apparatus overview. Drawing is not to scale. A) Top view of the modified SNAP i.d. system. B) 3D view of the modified SNAP i.d. Membranes and glass fiber spacers sit in each well. C) 1 $\mu\text{g}/\text{mL}$ actin standards dot blotting using normal SNAP i.d. method. D) 1 $\mu\text{g}/\text{mL}$ actin standards dot blotting using miniaturized SNAP i.d. E) Actin standards dot blotting using miniaturized SNAP i.d. without the glass fiber spacer underneath the membrane.

Figure 5-2A and 2B shows a prototype of the miniaturized immunodetection system. 4 mm x 6 cm wells were milled on a 10 cm x 8 cm polypropylene piece. The depth of the wells was 3 mm. A hole was drilled in the center of each well to allow reagent flow when applying vacuum. A 3 mm x 6 cm blotting membrane (typical size for microchip Western blotting) was placed on top of a glass fiber spacer and then put into each well. A bulk piece of polypropylene was machined to make a good seal with the other piece. When two pieces were put together, it created a cavity for antibody collection. This system was preliminarily tested by dot-blot immunoassay and compared results with using normal SNAP i.d. Results in Figure 5-2C and 5-2D indicate similar signal to noise ratio when using miniaturized SNAP i.d. compared to normal SNAP i.d. system. It is important to add the glass fiber spacer. Figure 5-2E shows very weak signal when there was no glass fiber spacer underneath the blotting membrane. My hypothesis is

that the glass fiber spacer helps to hold the antibody solution during incubation. Without the glass fiber spacer, majority of the antibody solution will leak from the drilled hole during incubation.

Using this system, 0.2 mL antibody solution was enough to cover membranes in each well while normal SNAP i.d. used 1-3 mL solution. It is worth mentioning that although 3 mm wide membrane is tested, the actual width of protein track is only 1.4 mm. Using a grid of channels proposed before instead of cutting membranes into individual pieces, volume could be further reduced to less than 0.1 mL.

Another work reported before also focused on immunoassay miniaturization.⁵ In that regard, a microfluidic network (5-10 channels) was placed on a conventional blotted membrane with the channels perpendicular to the protein bands. Multiple proteins were detected by incubating different antibodies in different channels. However, the number of antibody channels that can be accommodated is limited by the width of the well on slab gel when doing SDS-PAGE. Using the miniaturized immunodetection system proposed here, an array of at least 30 channels could be fabricated and placed on a 10 cm x 8 cm size membrane with multiple lanes of separations. Therefore 30 different immunoassays could be finished simultaneously using less than 0.1 mL antibody solution in each assay.

Fast immunoassay by direct depositing antibodies on blots. The success of using vacuum to facilitate diffusion and binding kinetics between antibody and antigen has been proved and compatibility with microchip-Western blotting could be easily achieved by miniaturization. However it is still labor intensive and automation is not addressed. One benefit of applying microfluidic technology is that multi-step, complex operations could be integrated on a small scale device with precision. Instead of doing

immunoassay in a traditional way or using miniaturized SNAP i.d., we propose to accommodate all the steps on a single chip, including blocking, primary antibody incubation, secondary antibody incubation, and wash. The concept is illustrated in Figure 5-3. Separation is finished on chip as described in Chapter 2, 3, and 4. After proteins are blotted on the membrane, immunoassay buffer will be deposited sequentially on same track on the stage instead of processing the membrane ‘off stage’. Coupling multiple primary antibodies also enables multiple targets detection.

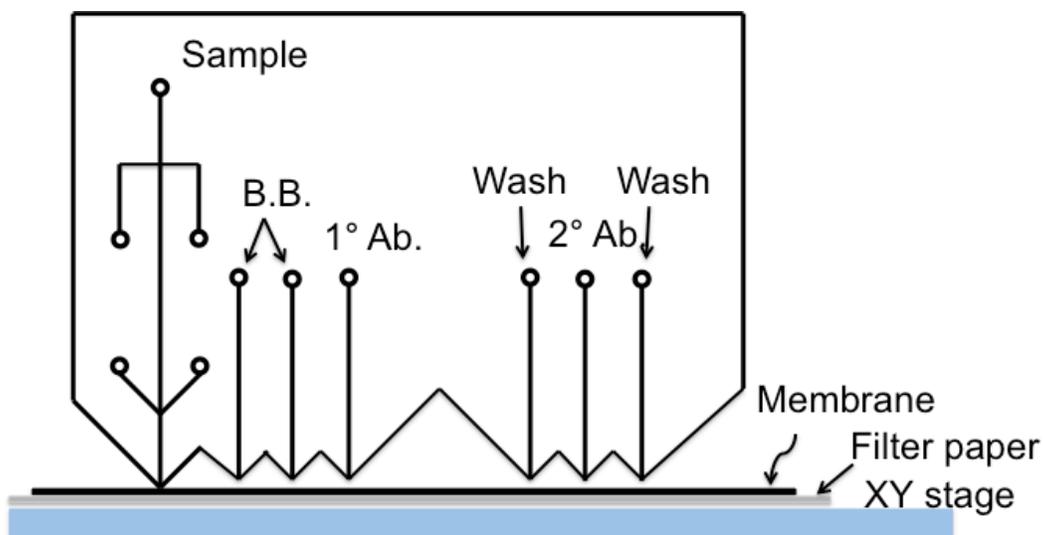


Figure 5-3. Performing separation and fast immunoassay on microchips. Drawing is not to scale. Sample is loaded in sample reservoir and injected using gated injection method. Blocking buffer (B.B.) is pumped through two channels after separation channel to ensure good blocking. Primary antibody (1° Ab) and secondary antibody (2° Ab) are pumped onto membrane afterwards. Between primary antibody and secondary antibody, wash buffer (20 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20) is added to wash away unbound antibodies.

The idea was first tested by flowing assay solutions through Teflon tubes and directly depositing onto the protein trail (Figure 5-4).

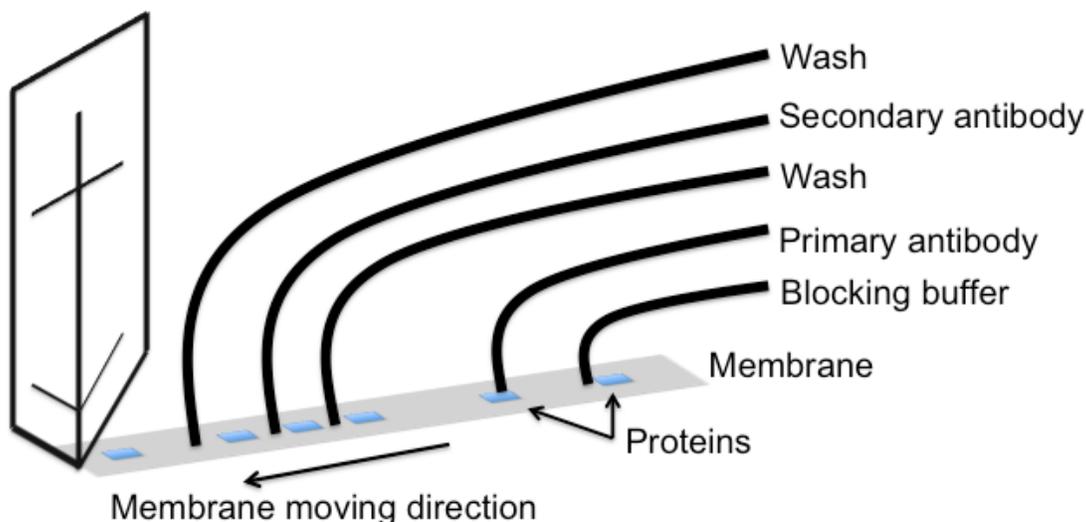


Figure 5-4. Coupling microchip gel electrophoresis to a fast immunoassay method. Drawing is not to scale. Protein sizing happens on a microchip with a 4 cm long separation channel. Electric field applied for separation is 400 V/cm. After separation is captured on membrane, a set of 5 Teflon tubes (each with 250 μm i.d.) is positioned at the top of the membrane. Solutions are pumped at 5 $\mu\text{L}/\text{min}$, except the blocking buffer is pumped at 7 $\mu\text{L}/\text{min}$. Distance between two adjacent tubes is 5 mm, except the primary antibody tube and the wash tube are 20 mm apart.

To operate, a chip with 4 cm long separation channel was used for protein sizing. The electric field applied was 400 V/cm and stage was moving at 4 mm/min. Sequential injections of lysozyme standards were made every 30 s. After the separation was captured on the membrane, the chip was replaced by a holder with 5 Teflon tubes (250 μm i.d.). The tubes were connected to 5 different syringes containing blocking buffer, primary antibody, wash buffer, secondary antibody, and wash buffer. Distance between two adjacent tubes was 5 mm, except that primary antibody tube and first wash buffer tube were 20 mm apart. During experiments, the tubes were aligned together and overlaid on top of the protein trail. The tubes were 100-150 μm away from the membrane surface to prevent any damage to the membrane during movement. As the stage moved, blocking buffer, primary antibody solution, wash buffer, secondary antibody solution, and another wash buffer deposited onto the separation track sequentially. Flow rate was set at 5

$\mu\text{L}/\text{min}$, at which protein track was covered. To get sufficient blocking and washing, we increased the flow rate for these steps to $7 \mu\text{L}/\text{min}$. The membrane was then dried and imaged using a fluorescent scanner.

Figure 5-5 shows lysozyme blotting results using different immunoassay methods. Using SNAP i.d., the whole immunoassay was finished in 30 min and average peak area was 34 ± 5 gray scale value ($n = 3$). Traditional immunoassay was done after 12 hours primary antibody incubation and 1 hour secondary antibody incubation. Peak area was 970 ± 70 gray scale value ($n = 3$). Direct depositing solutions as the stage moved only took 20 min to finish and the average peak area was 430 ± 50 gray scale value ($n = 3$). Coupling more antibodies tubes may enhance the signal. These results have demonstrated good sensitivity and fast immunoassay using the sequential antibody deposition method. Furthermore, the antibody consumption is 100 folds less than required in a traditional method.

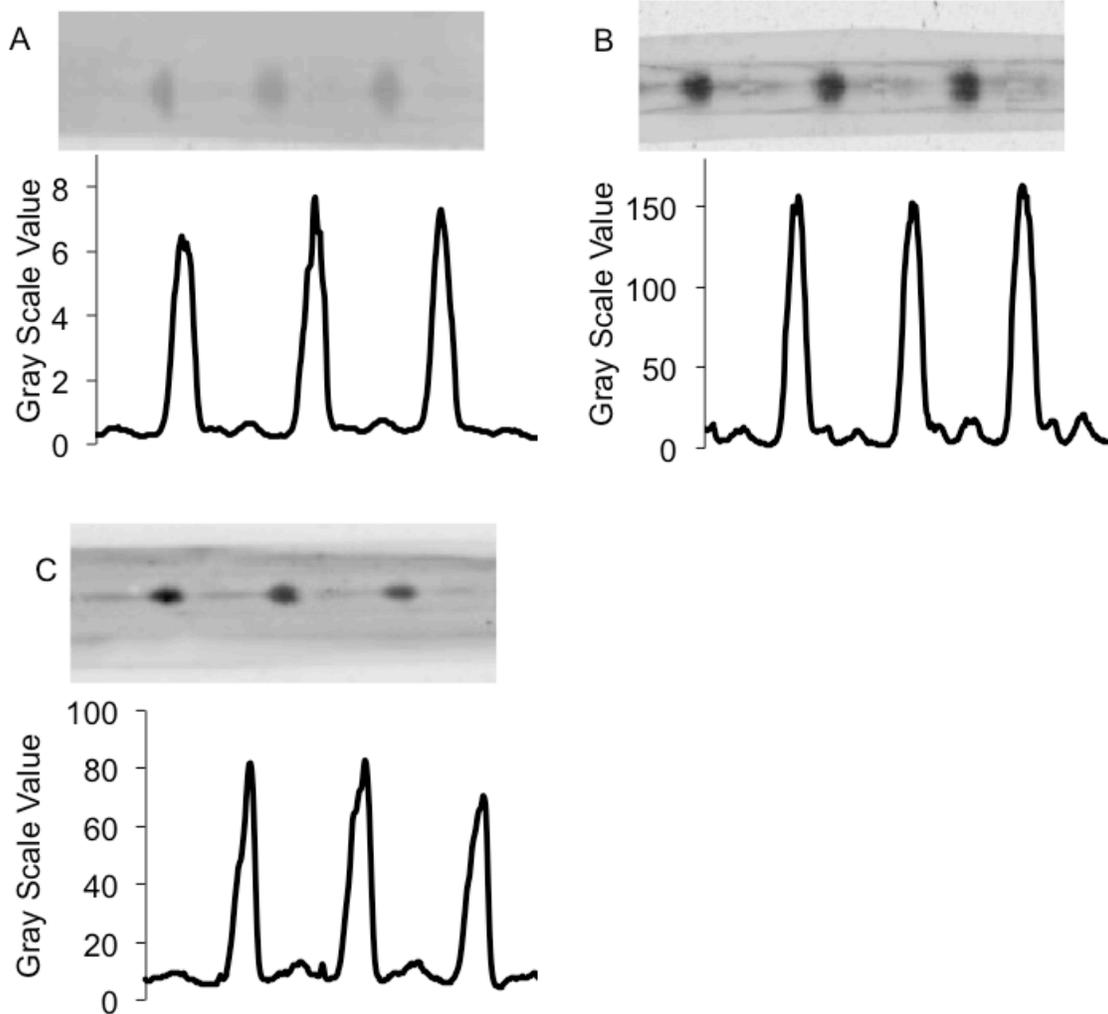


Figure 5-5. Compare lysozyme Western blotting signals using A) SNAP i.d., B) a traditional immunoassay method and C) a fast immunoassay method. Proteins were injected and separated on a microchip, and transferred to a membrane. Line scans show that peak areas are 34 ± 5 ($n = 3$), 970 ± 70 ($n = 3$), and 430 ± 50 ($n = 3$) using SNAP i.d., traditional immunoassay, and fast immunoassay methods.

The principle behind fast immunoassay method is still under investigation. One possible explanation is that the strong capillary force, which drives the reagents onto membrane surface, facilitates antibody diffusion into the pore structure and speeds the antibody-antigen binding. We envision better sensitivity when applying additional force to distribute the antibodies faster, such as applying vacuum underneath the membrane.

This novel method could be coupled with the miniaturized SNAP i.d. system for fast multiplexing.

Another way to deliver antibodies would be applying voltage across the antibody delivery channel and use EOF to drive the solution. It is suitable with microchip-Western blotting system because the membrane underneath the chip is already electric ground. A stable connection will be established once voltage is applied in the antibody reservoir. Other work using the electrophoretic force to drive the antibodies through gel and capture target proteins has been reported. OVA was recognized by fluorescent labeled primary antibody with 10-min total assay time. The rapid probing kinetics was ascribed as the electrokinetic through-pore delivery strategy that minimized surface boundary layer diffusion resistances.⁶

Minimize sample consumption using droplets. In the microchip Western blotting system, typically at least 20 μL solution is needed in a sample reservoir to cover the access hole in order to build a stable connection. However, the actual sample consumption for each injection is much lower. Although multiple injections could be made, a good portion of 20 μL sample is wasted. In chapter 3 we reduced the size of the sample reservoir so less sample was needed. Multiple analytes detection was accomplished by processing membranes with different antibodies. In that regard, a minimum of 2 μL sample (0.4 μg total protein) was used to finish 11 western blotting assays. Considering each injection is about 1 nL (equivalent to 0.2 ng total protein), there is still potential to further reduce the total sample consumption.

Droplet-based microfluidics has recently emerged as a valuable instrumental platform for performing high throughput chemical and biological analyses.^{7,8} Aqueous

samples are encapsulated in immiscible oil or air to form subnanoliter sample droplets. A significant advantage of coupling droplet or segmented flow to microchip CE is that it is compatible with low sample volume (pL to nL) analysis. The challenge is to extract aqueous from the oil carrier phase and achieve precise and controllable injections. There are a few methods published on coupling droplets with gel electrophoresis. One is using slipchip devices, which consist of two plates with small wells fabricated in each.^{9,10} And by sliding one layer relative to the other, sample held in between the two plates could be slid into droplets and led to other analysis. Other methods include use of oleophilic films to remove the carrier oil.^{11,12}

A hybrid PDMS-glass device was developed previously in our group⁸ to extract and analyze the droplets (Figure 5-6). Briefly, after making droplets with assistant of an XYZ translational stage on a traditional well plate platform, the droplets will be transferred from a tube onto a PDMS device and be extracted onto a glass chip, where the sample injection and separation happen. Chips could be diced so the separated proteins are captured on membrane for immunoassay. In the previous work, EOF was used to rapidly drive the sample toward injection cross after droplet extraction. However in gel electrophoresis, all the channels will be dynamically coated and EOF is suppressed. Therefore samples will be introduced by electrophoretic force, which could be slower than EOF. One question remaining is whether new sample will reach the injection cross before the next injection begins.

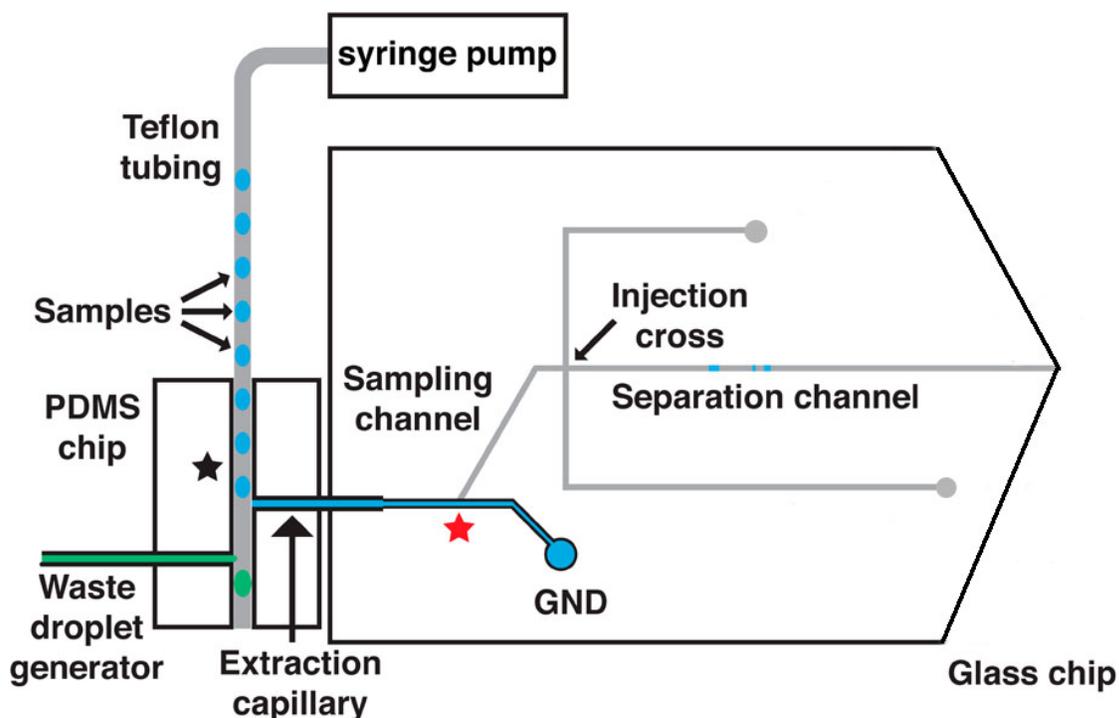


Figure 5-6. Schematic of PDMS–glass hybrid microfluidic device for analysis of segmented flow samples. Figure is adapted from Guetschow et al., *Anal. Chem.* 86, 2014, 10373–10379. The chip will be diced so proteins can be captured on membrane.

We reported that a full separation of protein ladder (11 kDa to 155 kDa) took 2 min on chip.¹³ Monitoring SDS-FITC protein movement under a fluorescent microscope, sample reaches injection cross from sample reservoir within 30 s. Assuming the size of the droplets is 10 nL, oil gap is also 10 nL, and droplets are pumped at 20 nL/min, we can achieve one separation using two droplets (only 4 ng total protein). By doing overlapping injections, more replicates could be analyzed. Coupling droplets to microchip gel electrophoresis significantly reduces the sample consumption and addresses the inconvenience when pipetting small volumes into reservoirs.

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Appendix

Use polymethyl methacrylate (PMMA) chips for multiplex operation. In Chapter 4 parallelization operation on glass chips was discussed. However dicing the chip to make multiple outlets was challenging. Milling the glass to make outlets was also not a good option because it tended to shatter the glass. An alternative is to use materials that are easier to be machined, such as PMMA.

To make PMMA devices, different fabrication methods have been tried. The simplest way to make channels on PMMA surface is direct milling. We have tried to mill the channels using a CNC machining system. However, the small channel dimension (15 μm deep 50 μm wide) made the milling process very difficult. The milling bit was also easy to break. The cost of the time and materials defeated the purpose of using polymeric devices.

Another method is to engrave the channels using laser-based technologies. In this laser ablation process, the energy of a laser pulse is used to break the bonds in a polymer molecule and to remove the decomposed polymer fragments from the ablation regions. We used a CO₂ laser to make channels on PMMA. The process was ultra-fast with a cost of channel surface smoothness. Methylene chloride vapor polishing gave smooth channels. In practice, simply warmed the methylene chloride (boiling point 39.6 °C) and put the engraved side of the PMMA against the vapor. When the opaque material turned

transparent (in about 30 s), the piece can be moved away from methylene chloride vapor and cleaned with ethanol and water.

When bonding the slide with channel features to a blank substrate to make a complete microchip, solvent assisted method was used. Organic solvents were applied to the surface of both pieces to soften the material before bonding. We found that 20% 1,2-dichloroethane and 80% ethanol worked best. Hot embosser was employed for bonding purpose. In practice, surface was first cleaned with water, ethanol and then treated with dichloroethane. The two pieces were then mounted on the embossing system, facing each other. Hot embosser was operated at 200 psi for 20 min at 107 °C. Sometimes vacuum was applied to prevent the formation of air bubbles due to the entrapment of air in small cavities.

To test gel electrophoresis of proteins on PMMA chips, we made a PMMA chip with a simple cross design. All channels were filled with commercial gel (AB Sciex, Framingham, MA, part number 390953). FITC-BSA was injected using gated injection method and separation was recorded at end of the channel. Peak height and peak area decreased significantly in 2 min after the experiment started (Figure A-1). When looking at the injection cross under a fluorescent microscope, sample was first introduced to the injection cross by electrophoretic force. But then in 2 min sample moved away from the injection cross and back into the sample reservoir. As a result, less and less sample was injected and peak height decreased. My hypothesis was that the channel surface was not coated well so EOF still existed.

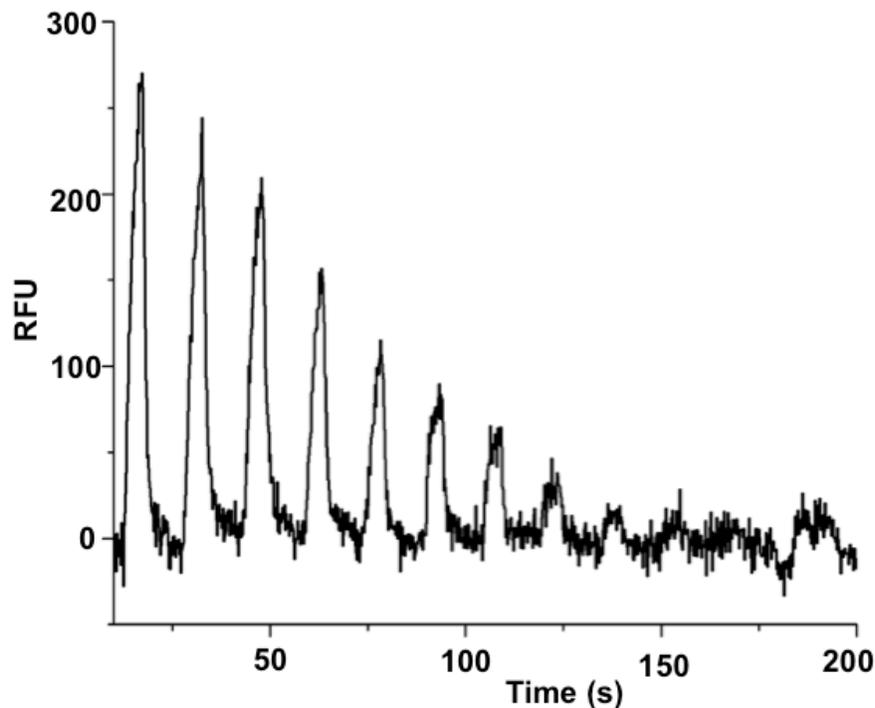


Figure A-1. Electropherograms recorded from repetitive injections of FITC-BSA on a PMMA microchip. RFU is the relative fluorescent unit.

In summary, PMMA chips have the potential for mass production. However current results with PMMA were not promising. To make it suitable for gel electrophoresis, channel surface should be treated to prevent protein adsorption and eliminate electroosmotic flow.

Reduce sample injection bias. In Chapter 4, we briefly talked about difference between gated injection and pinched injection method. In gated injection, the sample flow is electrokinetically pumped through the cross region, and is gated by the buffer flow at the cross after injection.¹ Sample volume is controllable and the injection is automated. The amount of sample being injected depends on the velocity of the analytes and the injection duration. However, when the protein mixture is injected at the same electric field for same time, the difference in mobility favors the smaller proteins. In other words, smaller proteins will be injected more relative to larger proteins. To solve this injection

bias issue, pinched injection scheme is introduced.² In this case, sample reaches equilibrium before injection. The whole sample plug defined by the physical dimensions of the channel intersection is injected in the separation channel. Therefore the injection bias is significantly eliminated.

Figure A-2 shows the voltage applied in different reservoirs when doing pinched injection. Figure A-3 shows protein ladder separations using gated injection and pinched injection method. It is worth mentioning that due to the well-defined sample plug in pinched injection, separation efficiency ranged from 1.7×10^4 to 1.0×10^5 theoretical plates, while using gated injection method efficiency was 3×10^3 to 1×10^4 theoretical plates.

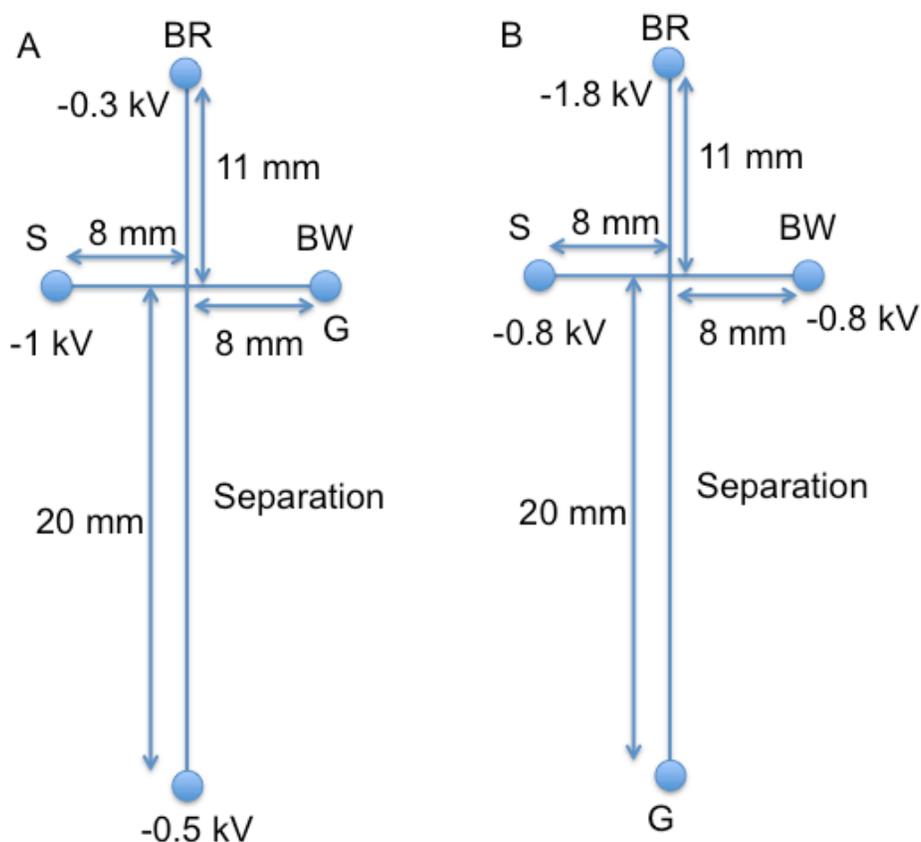


Figure A-2. Voltage program for pinched injection during A) sample loading and B) separation. Reservoirs containing sample, buffer, and buffer waste are labeled S, BR, and

BW. Channel lengths and voltage applied are labeled in the figure. Drawing is not to scale.

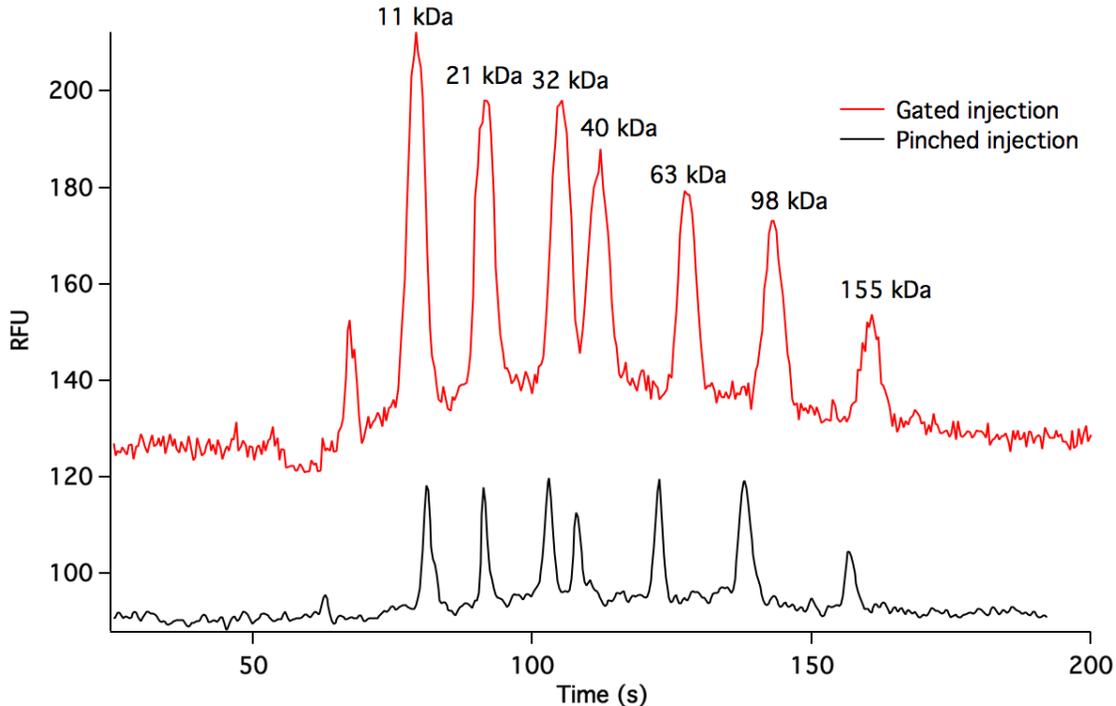


Figure A-3. Protein ladder separation using pinched (black) and gated (red) injection methods. Separation field was 400 V/cm. Separation was recorded at 2 cm from the injection cross.

To evaluate the injection bias in gated injection, we compared the peak areas of all 7 proteins. All peaks were normalized to the 11 kDa peak. The same protein ladder was also separated on capillary using same sieving polymer. Electrokinetic injection was used to introduce sample into the capillary. Figure A-4 shows that the peak area of 21 kDa, 32 kDa, 40 kDa, 63 kDa, 98 kDa, and 155 kDa peaks relative to 11 kDa peak were $57 \pm 1\%$, $64 \pm 1\%$, $41 \pm 2\%$, $53 \pm 3\%$, $41 \pm 1\%$, $19 \pm 2\%$ ($n = 3$) when using gated injection. We got similar results when doing electrokinetic injections on capillary, which was also subject to injection bias. In contrast, peak area of 21 kDa, 32 kDa, 40 kDa, 63 kDa, 98 kDa, and 155 kDa peaks relative to 11 kDa peak were $77 \pm 2\%$, $93 \pm 1\%$, $76 \pm 1\%$, $90 \pm 1\%$, $85 \pm 1\%$, $57 \pm 1\%$ ($n = 3$) when doing pinched injections on chip.

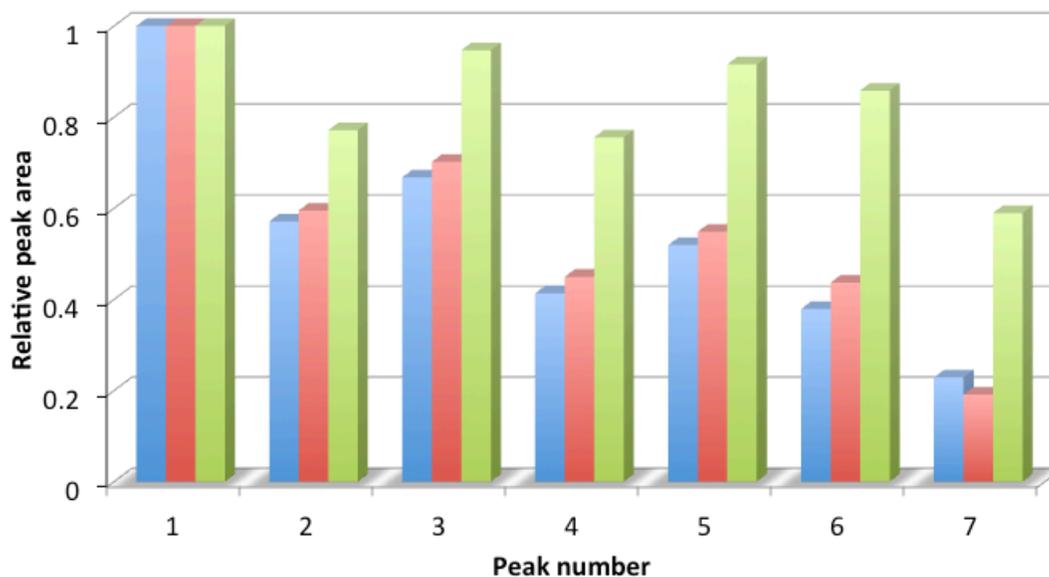


Figure A-4. Compare peak areas using electrokinetic injection on capillary (blue columns), gated injection (red) and pinched injection (green) on chips. Peak areas of all the other 6 peaks are normalized to the first peak in each separation. Peak number 1 to 7 correspond to 11 kDa, 21 kDa, 32 kDa, 40 kDa, 63 kDa, 98 kDa, and 155 kDa peaks in Figure A-3.

In conclusion, pinched injection method introduces less injection bias compared to gated injection. Another advantage of this approach is a narrow injection zone for high efficiency separation, but the disadvantage is limited sample volume. Using a double T geometry³ for sample loading will also produce nearly bias-free sample injection and allow better sensitivity.

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