

ON-CHIP MULTI-ANALYTE NATIVE WESTERN BLOTTING IN TWO MINUTES

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ABSTRACT

Western blotting comprises a ubiquitous suite of techniques spanning measurements in clinical and life sciences. Powerful slab-gel Western blotting identifies specific proteins amidst complex biological backgrounds. Unfortunately, bench top blotting is time consuming (hours, days), semi-quantitative, and exhibits low reproducibility - especially when assaying multiple antigens from a single sample. To surmount these shortcomings, we introduce for the first time a multi-analyte protein blotting assay using a 2D microfluidic chamber geometry. The native assay requires just two (2) minutes, is fully automated, and consumes limited sample/antibody (1-10 μ L). We harness the micron-scale dimensions and integrative power of microfluidics to improve and automate all three key immunoblotting mass transfer steps. Separation, transfer and blotting are performed in an assay demonstrating high throughput detection of multiple species.

KEYWORDS: protein separation, polyacrylamide, immunoblotting, microfluidic electrophoresis

INTRODUCTION

Western blotting has become an indispensable piece of the molecular biology toolbox, yet the multiple laborious steps which are required to separate, transfer and detect protein targets can limit experimental throughput and repeatability [1, 2]. These problems are further exacerbated when there is more than one protein of interest - for example, it is impossible to perform quantitative comparison of an analyte against a different control within the same gel due to harsh stripping and reblotting steps which result in significant sample loss. Microfluidic techniques have demonstrated many of the individual component functions required for Western blotting in an efficient and rapid manner when compared against macroscale counterparts. Such "lab on a chip" technologies are uniquely suited for the seamless integration of multiple functions.

Using microscale gel electrophoresis, analyte bands from a complex sample can be resolved in significantly shorter times with more efficient protein transfer as compared to slab gel techniques. In this first report of our on-chip multi-analyte protein blot, we integrate all steps using a 2D microchamber regionally photopatterned with discrete nanostructured separation materials. The microdevice performs automated on-chip separation and detection of multiple antigens in less than two minutes. This differs from previous work [2] in that the technique has been expanded to include multiple capture zones for studying different antigens in a single run.

THEORY

A multi-stage photopolymerization process is key to enabling lossless integration of polyacrylamide (PA) gel electrophoresis (PAGE), post-separation sample transfer and finally, antibody-based affinity blotting in multiple blotting regions. The specificity and flexibility of the multi-analyte blotting assay is made possible through the incorporation of biotinylated antibodies decorating photopatterned streptavidin-acrylamide "blotting regions" [3]. Operation of the device and subsequent interpretation of data is analogous to conventional Western blotting. After separating proteins on the basis of electrophoretic mobility, resulting bands are electrokinetically driven into a second dimension through a series of blotting regions which probe the identity of the species based upon known binding interactions as shown in Figure 1.

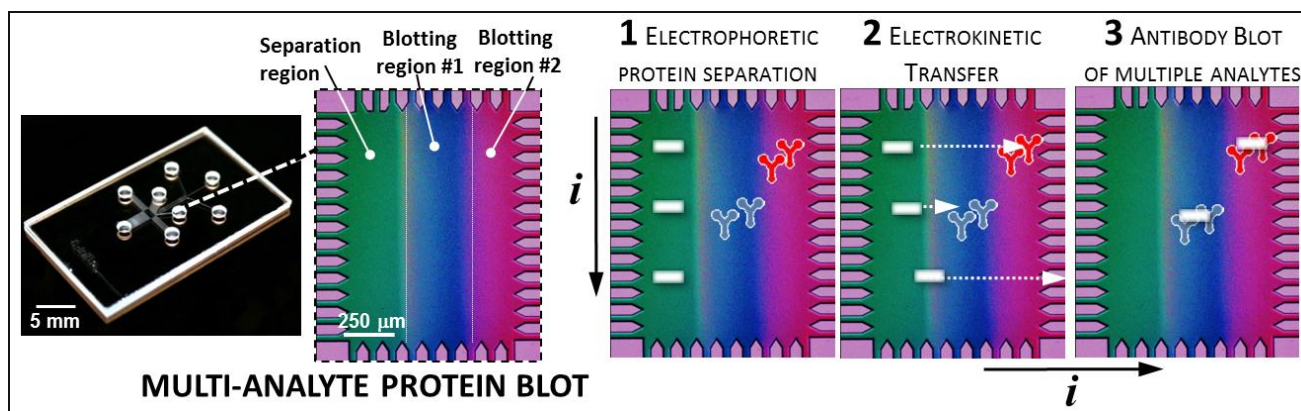


Figure 1. Multi Analyte Native Western Blot Concept: Selective photopatterning enables an integrated microdevice with regions of different physical and functional properties for analyte separation and antibody based identification. "Y" indicates immobilized antibodies. The "i" indicates direction of current flow for 1) separation and 2) transfer steps.

Thus, the laborious blocking and blotting steps (which require long incubation times on the macroscale) can be bypassed. Locations of the resulting immobilized bands within blotting zones reflect the original band positions as ob-

served in the separation gel. Labeled analytes are then detected via optical techniques such as fluorescence microscopy and resulting data can be analyzed using image processing software. PAGE and sample transfer is performed on the microscale, resulting in brief assay times (< 120 s) and short requisite lengths for separation and transfer (< 1 mm).

EXPERIMENTAL

A microfluidic device was designed and wet-etched in glass, producing a shallow central chamber of approximately 1.6 mm x 1.0 mm x 20 μm . This “two dimensional” area contains discretized regions for protein separation and selective capture. Boundaries of the central chamber are lined with microchannel arrays which are used for sample/buffer access and control of interior electrical field distribution. Eight (8) reservoirs on the surface of the chip serve to retain samples or buffer solutions and interface with a set of platinum electrodes to control the voltage at each node (i.e. reservoir).

Using a native Western assay, we demonstrate PAGE analysis (150 V/cm, 6%T PA gel) of C-reactive protein (CRP) and Protein G (PG) on a chip containing antibody functionalized regions against both targets. Use of a native form of Western blotting is key, as we seek to study the pentameric configuration of CRP. Internal standards have been included to calibrate for analyte concentration and electrophoretic mobility. These standards are also used as negative controls to rule out non-specific binding and/or size-based exclusion effects at the functionalized blotting gels. Two separate experiments, demonstrating dual target and single target capture are exhibited in Figures 2 and 3.

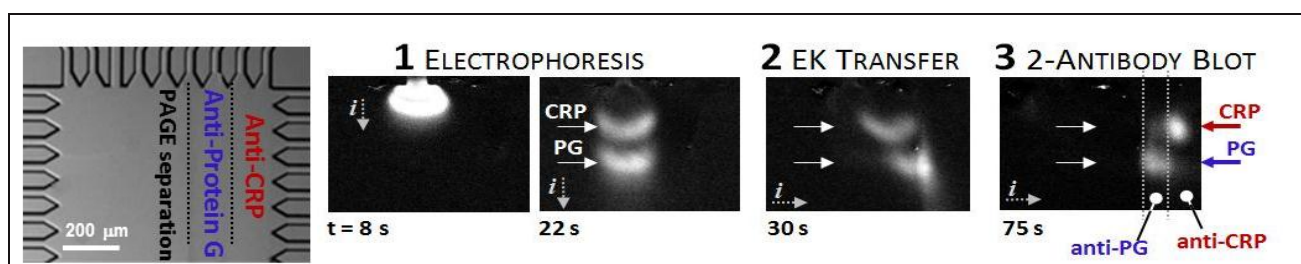


Figure 2. Multiple Species Identified in Two Minutes: Band separation (1), transfer (2) and capture (3) for a 2-Target System in a device containing antibody functionalized regions specific for Protein G and CRP.

Figures 2 and 3 depict the separation, lateral transfer and subsequent capture of various proteins after a sample plug is electrokinetically injected from the loading channel (not pictured) into the separation region. The analytes consist of Protein G (PG, Figure 2) and Bovine Serum Albumin (BSA, internal standard in Figure 3) at 300 nM each and with CRP at 600 nM. All proteins were fluorescently labeled with AlexaFluor 488. Fluorescence measurements were performed with a CCD camera using the 10x objective of an inverted microscope. Both the sample and the electrophoresis run buffers consist of 25 mM Tris and 192 mM Glycine at pH 8.3. A micro-stacking gel of lower density (3%T) is located at the upper interface to facilitate sample loading and separation. This ability to blot multiple separated species represents a major advance over our μTAS 2009 report.

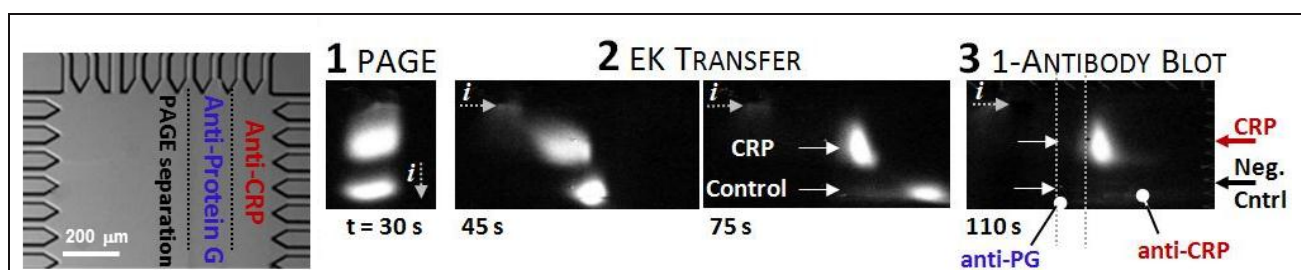


Figure 3. Negative Control Demonstrates No Cross-Reactivity: Band separation (1), transfer (2) and capture (3) for a single target with negative control (CRP and Bovine Serum Albumin) in a device containing antibody functionalized regions specific for Protein G and CRP.

RESULTS AND DISCUSSION

Baseline separation of CRP from PG was completed within 10 s. Dual-analyte blotting demonstrates rapid transfer (<50 s, 50 V/cm) of the resolved species to the two blotting gels with ~80% capture efficiency for each species to its corresponding region (Figure 2). PG and CRP were detected at signal-to-noise ratios of ~65 and 79, respectively. The separation and transfer steps were complete within 90 s.

During separation and transfer, we incorporate electric field control using the surrounding microchannels to conserve the PAGE separation resolution from the separation axis. Maintaining spatial uniformity of the separation and transfer fields is critical as this minimizes the distortion, shifting and dispersion of bands which could compromise the original resolution of individual components from a complex background. The change in post-transfer separation resolution for both experiments was minimal. Separation resolution changed from 2.58 (before transfer) to 2.50 (after transfer) for the CRP/PG separation and from 1.87 (before transfer) to 1.90 (after transfer) for the CRP/BSA separation, representing variations of 3% and 1.6%, respectively. Blotting capture efficiency is robust; Figure 4 demonstrates the high degree of an-

tigen retention that is still visible upon the blotting regions after extended application of the transfer field (>110s). The included negative control yielded no discernable exclusion, cross-reactivity, or non-specific adsorption.

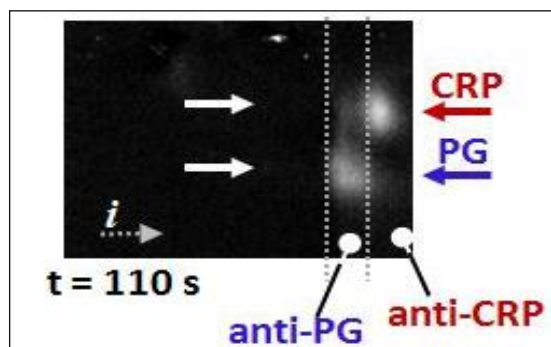


Figure 4. Target Capture is Robust: Fluorescence image of the central blotting region after 110s of extended lateral field application.

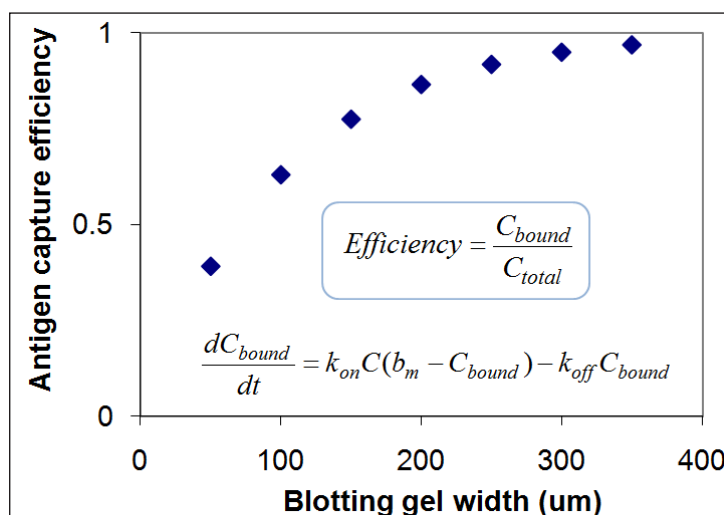


Figure 5. Modeling Informs Multi-analyte Chip Design: Antigen capture efficiency is modeled as a function of design and operational parameters, assuming Langmuir binding kinetics.

Demonstrated here for two (2) analytes, the approach is scalable to tens of analytes in a single sample. However, within a finite blotting space, some physical limits will exist as to the number of blotting regions that can be included in a single device while still providing reliable antigen detection. To optimize capture efficiency in such highly multiplexed systems, a model has been developed to inform chip design and operational parameters in terms of system performance. This computational model simulates Langmuir binding reactions [4] between two sets of differential elements over a series of finite time steps. One set of differential elements represents the protein band, assumed to have a Gaussian distribution with each band element assigned some initial concentration value. Likewise, the gel is divided into elements of equal width, generating a 1D model which corresponds to the lateral axis of the microchamber. Protein migration speed determines a residence time (dt) wherein each target band element is co-localized or “incubated” with a matching gel element. Thus, the protein band is allowed to advance relative to the gel over a series of time steps.

Performance metrics such as capture efficiency and spatial distribution of immobilized target molecules can then be assessed as a function of system parameters such as blotting gel width and lateral field strength. For example, Figure 5 illustrates variations in antigen capture efficiency (# molecules bound/total # of molecules) as a function of blotting gel width. Binding behavior is strongly dependent upon analyte mobility and Langmuir binding variables such as antigen/antibody concentration and binding affinity. This is equally true in experimentation as in simulation, so the flexibility of a multi-parameter model provides a useful tool for designing systems where the affinity of the antigen/antibody pair and the electrophoretic mobility of the target can be highly variable.

CONCLUSION

We have demonstrated seamless microfluidic integration of the separation-transfer-blotting steps required for immunoblotting of multiple proteins in a single, automated assay – an assay that requires 1/1000th of the time needed on the bench top. This work differs significantly from our previous reports [3] by introducing an important and exciting capability for multi-analyte blotting.

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