10 MINUTE WESTERN BLOTTING WITH 54-PLEX THROUGHPUT FOR CLINICAL CONFIRMATORY HIV DIAGNOSIS IN HUMAN SERUM

Alex J. Hughes[1] and Amy E. Herr[1,2*]

[1]UC Berkeley – UCSF Graduate Program in Bioengineering, USA and
[2]UC Berkeley Bioengineering, USA

ABSTRACT

We translate western blotting to a microchip workflow that cuts conventional assay times from 3-18 hours to 10-60 min with up to 5-plex analyte detection and 54-plex channel throughput. An engineered photoactive protein capture matrix with tunable porosity (PACTgel) constitutes 1) a spatially addressable denaturing polyacrylamide gel electrophoresis (SDS-PAGE) separation matrix compatible with mask-based visible light photopatterning and 2) a UV light-toggled protein capture matrix with unprecedented capture efficiency (>75%). We report the first 10 minute western blot consisting of protein sizing and immunoaffinity detection in a single microchannel, and validate a rapid confirmatory HIV diagnostic using human serum. Our capability for rapid confirmatory HIV diagnosis at the point of care has the potential to circumvent the western blotting bottleneck in the current diagnostic pipeline.

KEYWORDS

Microfluidic proteomics, HIV, western blot, biomarker immunoblotting.

INTRODUCTION

Western blotting is perhaps the most widely used targeted proteomic method in the life and medical sciences [1-3]. The combined power of weight-based electrophoretic separations and immunological characterization make western blotting central to modern biological and diagnostic inquiry [3, 4]. The western blot is likely to remain a bench-top go-to technique for the life scientist, and as such, requires continued innovation to advance throughput, minimize resource usage, and reduce manual intervention [5].

Major performance limitations stem from the immunoblotting process, which physically transfers proteins from the protein-sieving polyacrylamide gel separation stage (SDS-PAGE) to the immunoprobing stage. The electrotransfer of proteins from gel to a polymer or paper-based "blotting" material prior to antibody probing leads to incomplete and biased protein capture (60-100% depending on the physicochemical properties of the protein [6]), and demands trained manual intervention. In recent advances, Ciaccio *et al*. achieved remarkable miniaturization and scale-up of the western blotting workflow, but retained the membrane electrotransfer step and added a liquid handling front-end for picoliter droplet aliquoting [4]. Further, the study conceded a loss in SDS-PAGE separation resolution owing to elimination of electrophoretic sample "stacking", standard in conventional slab gels. In another recent approach, a capillary electrophoresis technology commercialized by ProteinSimple replaced membrane electrotransfer with photoactivated capture of proteins onto a capillary inner wall, after charge or size based separations [7, 8]. The capillary platform streamlines and automates western blotting, but suffers from low protein capture efficiencies of 0.01% (~10^4-fold lower than membrane electrotransfer) and 3-5 hour run times.

EXPERIMENT

To advance performance and simplify the western blotting workflow, we introduce a microfluidic approach. Microchannels filled with PACTgel separation and blotting polymer are the core of our new integration strategy (Figure 1). Using microfluidic integration and the functional polymer, all steps from isotachophoretic (ITP) stacking during sample injection to weight-based separation of denatured protein analytes (SDS-PAGE) to immunoblotting with fluorescently labeled primary and secondary antibodies are achieved in one microfluidic channel in 10-80 min (depending on the signal detection strategy). Channel architectures are streamlined, with one access well pair per blot and channel widths on the order of the diameter of a human hair.

A transient-ITP buffer arrangement inspired by the tris-glycine SDS-PAGE system popularized by Laemmli [1, 9] is implemented as the first assay stage. During the stacking phase, a diffuse plug of protein injected at the microchannel entrance is electrophoretically compacted into a ~200 μm zone prior to electromigration across a sharp sieving gel interface (commonly a 7.5%T sieving PACTgel at ~400 μm into the 6 nl microchannel). Protein electromigration through the sharp sieving gel interface causes a transition from transient-ITP to SDS-PAGE, as the trailing glycine electrolyte overspeeds the stacked protein zones. Stacking achieves >2-fold sample pre-concentration and minimizes injection dispersion, boosting analyte resolution during the sieving phase. Minimized separation distances translate to appreciable time savings during SDS-PAGE, which typically requires 60 s to complete on-chip, a 40- to 90-fold improvement over gold-standard techniques.

Directly following SDS-PAGE, brief (20 s) exposure to UV light activates protein capture on the channel-filling PACTgel for subsequent antibody-probing. Pendant benzophenone groups built into the polyacrylamide gel scaffold via a novel methacrylamide monomer (BPMAC) undergo hydrogen abstraction and covalent coupling to nearby biomolecules via

978-0-9798064-5-2/μTAS 2012/$20©12CBMS-0001

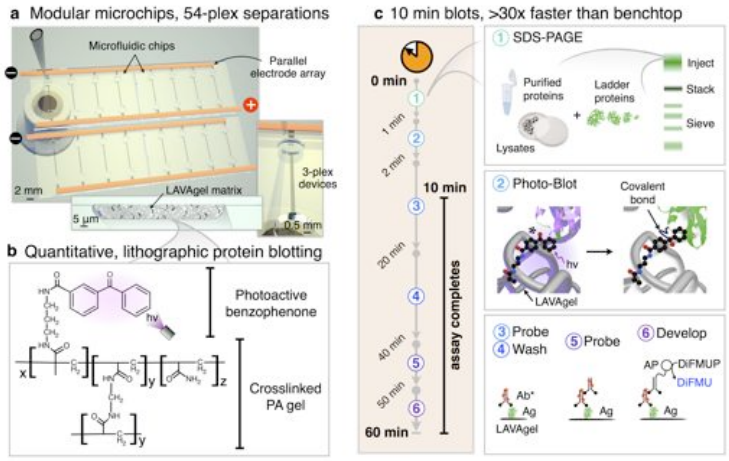

Figure 1: Micro-Western workflow in a parallelized glass chip with functional PACTgel protein capture matrix.

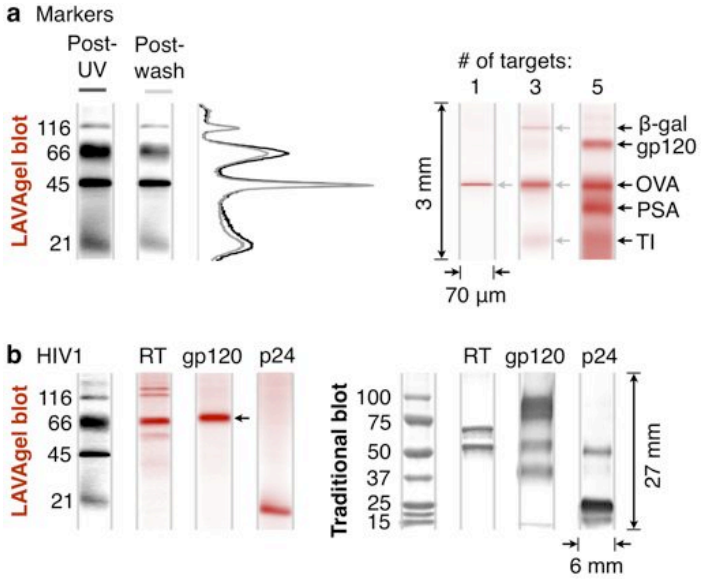

Figure 2: Fluorescent antibody probe readouts for (a) marker cocktail, and (b) HIV antigens.

a radical mechanism [10]. Capture efficiencies for fluorescently-labeled marker proteins assayed simultaneously with unlabeled target proteins were 75-100% (respective within- and between-device coefficients of variation of <5% and <20% for each band, Figure 2a). The PACTgel capture efficiency range rivals conventional electrotransfer blotting efficiencies on polymer membranes [6] and is a 10,000-fold improvement over capillary surface-capture reported by ProteinSimple (~0.01% for green fluorescent protein [7]).

Following photocapture, analyte bands are probed *in situ* with fluorescently-labeled primary and secondary antibodies *via* active electrophoretic introduction and washout from the nanoporous PACTgel. Antibody probe signal was specific to the target of interest in a single-probe assay for ovalbumin (OVA, Figure 2a). To demonstrate multiplexed analyte detection, we used cocktails of detection antibodies to probe three, and then five immobilized proteins in a single channel (Figure 2a).

RESULTS AND DISCUSSION

With a view towards performing rapid confirmatory diagnostic western blotting for infectious diseases, we first performed assays for several purified HIV proteins (Figure 2b). Comparison of the Micro-Western readout with slab-gel SDS-PAGE yielded molecular weight estimates for the major bands of reverse transcriptase and the viral envelope glycoprotein gp120 that agree to within ~12%. Sizing nonlinearity at the 20 kDa edge of the molecular weight range for both the Micro-Western and traditional western blots likely contribute to a 25% error in weight measured for the capsid protein p24. Nevertheless, precision in molecular weight prediction on-chip was adequate; within-device CVs for all bands were <2% (n = 3 for each). Minor bands for both gp120 (56, 40 kDa) and p24 (49 kDa) observed only on slab-gel western blots may have arisen from factors that differ between the macro- and microscale workflows, including differences in blotting efficiency, differences in the SDS-PAGE and probing buffer systems, and the degree of analyte renaturation prior to immunoprobing in each format.

Quantitative analyte blotting is achieved in the Micro-Western assay over a linear dynamic range of ~2.1 logs with limits of detection (LOD) on par with enzyme-amplified chromogenic signal development in traditional western blots.

Moving from validation to a pressing analytical need in the biomedical sciences, we demonstrate the Micro-Western as a proof-of-principle HIV diagnostic assay on human sera. Currently, HIV diagnosis employs a traditional western blot as the final (confirmatory) assay, following a positive ELISA-based screening diagnostic result [11]. We translated the currently accepted HIV diagnostic to our microfluidic platform by assaying human sera against purified gp120 and p24 HIV proteins. Specific serum cross-reactivity to each "bait" protein was determined following fluorescently labeled secondary antibody detection of human IgG on the PACTgel. The resulting dose-response is consistent with the expected antibody titer in each of three serum samples (strongly reactive, weakly reactive, non-reactive); meeting the US Centers for Disease Control and Prevention (CDC) guidelines for determining HIV infection in humans [11].

CONCLUSION

Our efforts towards modernizing and automating conventional western blotting have achieved state-of-the-art analytical performance in key areas: protein blotting efficiency (near complete analyte capture), run time (all steps from sample injection to probing in 10-80 min), device footprint (800-fold smaller device area compared to traditional gel lane) and reagent economy (10^3-fold reduction in antibody and buffer requirements over slab-gel western blot). Micro-Western assays yield quantitative readouts with the potential for >5-plex probing in a single microchannel. The advances reported here present an exciting opportunity for "western blot microarrays" that achieve the excellent throughput capacity of protein microarrays while providing the crucial aspects of sample deconvolution and molecular weight assignment that western blotting affords. The result is the prospect of micropatterned next-generation immunoblots for challenging analytical applications in cell signaling, cancer biology and infectious disease diagnostics.

ACKNOWLEDGEMENTS

A.J.H. is a U.S. Department of Defense NDSEG research fellow. A.E.H. is an Alfred P. Sloan research fellow in chemistry. This project was supported by New Innovator Award (DP2OD007294 to A.E.H.) from the NIH.

REFERENCES

[1] U. K. Laemmli, "Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4," *Nature,* vol. 227, pp. 680-685, 1970.

[2] H. Towbin, T. Staehelin, and J. Gordon, "Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications," *Proc Natl Acad Sci USA,* vol. 76, pp. 4350-4354, 1979.

[3] B. T. Kurien and R. H. Scofield, *Protein blotting and detection: Methods and protocols*. New York: Springer, 2009.

[4] M. F. Ciaccio, J. P. Wagner, C. P. Chuu, D. A. Lauffenburger, and R. B. Jones, "Systems analysis of EGF receptor signaling dynamics with microwestern arrays," *Nat Methods,* vol. 7, pp. 148-155, 2010.

[5] M. He and A. E. Herr, "Automated microfluidic protein immunoblotting," *Nat Protoc,* vol. 5, pp. 1844-1856, 2010.

[6] P. Jungblut, C. Eckerskorn, F. Lottspeich, and J. Klose, "Blotting efficiency investigated by using two-dimensional electrophoresis, hydrophobic membranes and proteins from different sources," *Electrophoresis,* vol. 11, pp. 581-588, 1990.

[7] R. A. O'Neill, A. Bhamidipati, X. Bi, D. Deb-Basu, L. Cahill, J. Ferrante, E. Gentalen, M. Glazer, J. Gossett, K. Hacker, C. Kirby, J. Knittle, R. Loder, C. Matroieni, M. MacLaren, T. Mills, U. Nguyen, N. Parker, A. Rice, D. Roach, D. Suich, D. Voehringer, K. Voss, J. Yang, T. Yang, and P. B. Vander Horn, "Isoelectric focusing technology quantifies protein signaling in 25 cells," *Proc Natl Acad Sci USA,* vol. 103, pp. 16153-16158, 2006.

[8] A. C. Fan, D. Deb-Basu, M. W. Orban, J. R. Gotlib, Y. Natkunam, R. A. O'Neill, R. A. Padua, L. Xu, D. Taketa, A. E. Shirer, S. Beer, A. X. Yee, D. Voehringer, and D. W. Felsher, "Nanofluidic proteomic assay for serial analysis of oncoprotein activation in clinical specimens," *Nat Med,* vol. 15, pp. 566-571, 2009.

[9] L. Ornstein, "Disc electrophoresis - I Background and theory," *Ann NY Acad Sci,* vol. 121, pp. 321-349, 1964.

[10] G. Dorman and G. D. Prestwich, "Benzophenone photophores in biochemistry," *Biochemistry,* vol. 33, pp. 5661-5673, 1994.

[11] CDC, "Interpretation and Use of the Western Blot Assay for Serodiagnosis of Human Immunodeficiency Virus Type 1 Infections," *MMWR,* vol. 38(S-7), pp. 1-7, 1989.

CONTACT

*Amy E. Herr, tel: +1-510-666-3396; aeh@berkeley.edu