SINGLE CELL WESTERN BLOTTING

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ABSTRACT

Understanding protein-mediated cell signaling and differentiation processes requires capturing response heterogeneity for single cells in large populations. We report single-cell western blots on standard microscope slides that achieve 10^3-10^4 molecule detection limits for >6 protein targets per blot in a < 4 hr process. An open microfluidic strategy enables gravity-driven capture of single cells into a polyacrylamide microwell array capable of UV-initiated protein capture. Large-scale lysis of cells within microwells, electrophoretic separation of protein species, blotting, and antibody-mediated detection of specific targets is achieved for ~2,000 single cells per slide.

KEYWORDS

Microfluidic proteomics, western blot, single cell analysis.

INTRODUCTION

Studying biological variation with resolution at the level of a single cell is crucial for the characterization of heterogeneous cell populations [1, 2]. In the proteomics sphere, immunofluorescence methods such as flow cytometry and immunocytochemistry allow comparative, semi-quantitative evaluation of individual cell states [3]. These techniques rely upon the availability of specific antibody probes to targets of interest, and even with careful controls, the task of delineating specific interactions of probes from non-specific (off-target) ones is difficult [4]. Probing multiple epitopes on the same protein target is also challenging since competition or interaction effects are difficult to predict [5]. Such techniques are often applied in concert with cell population-level measurements from assays such as the western blot, the enzyme-linked immunosorbent assay (ELISA), and protein microarrays [6]. The western blot is particularly useful for semi-quantitative analysis of protein expression or post-translational modification (e.g. phosphorylation or glycosylation), since it sieves cell proteins and reports molecular weight information that validates the fidelity of probe-target interaction [7, 8]. On the other hand, ELISA and microarray assays that rely on detection of targets against complex proteomic backgrounds are particularly prone to antibody cross-reactivity [6, 9]. Since cell contents are separated and diluted over a substantial distance (~10 cm) in traditional western blotting through sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), its detection limit is generally not compatible with the analysis of single cells (except for unusually large ones [10]). Also, the intrinsic population-level averaging of protein abundances from single cells cannot evaluate cell-to-cell variation in probe specificity that may bias data from complementary flow cytometry studies.

Here, we describe a microscale western blotting workflow for the parallel analysis of the order of 2,000 individual mammalian cells, in a microscope slide format suited to adoption by a broad life sciences community. Ultra-short protein separation distances of within 500 μm achieve performance adequate for molecular weight estimation and evaluation of antibody specificity, while constraining protein distributions for subsequent immunoprobing with sufficient sensitivity for analysis of targets in the 10^4-10^5 molecule-per-cell range.

EXPERIMENTAL

The single cell western blot assay comprises five integrated stages designed for electrophoretic separations of the protein contents of thousands of individual mammalian cells per assay (Figure 1). Firstly, a 30 μm-thick photoactivatable polyacrylamide gel with tunable porosity (PACTgel [11]) is micropatterned with an array of microwells with dimensions on the order of single mammalian cells by polymerization against a silicon wafer studded with SU-8 microposts. **Stage 1:** A suspension of single cells is settled onto the PACTgel microwell array by gravity, leading to efficient capture of cells typically in the range of 0-4 cells/well and with single cell occupancies of approximately 40-50% depending on the density of the suspension. **Stage 2:** Excess cells are washed from the surface of the hydrophilic PACTgel layer, and cells within the microwells are lysed in an open format by pouring of a modified radio immunoprecipitation assay (RIPA) lysis/electrophoresis buffer over the array. Buffer exchange with the PACTgel layer and microwells occurs within approximately 2 s, leading to rapid lysis and solubilization of cell contents within 10 s. **Stage 3:** An electric field is applied across the submerged slide, driving proteins through the walls of the microwells and into the PACTgel sheet, sieving proteins by size. **Stage 4:** The photoactive protein capture function of the PACTgel is triggered through the application of UV light to the slide for 45 s, covalently attaching protein bands to the gel at their migration distances. Lysis, separation, and blotting steps are complete within 75 s. **Stage 5:** In-gel immunoprobing for protein targets of interest by diffusive antibody probe delivery throughout the thin PACTgel sheet is achieved through sequential 1 hr incubation of the slide with primary and fluorescently labeled secondary antibody solutions, with 45 min buffer wash steps between probe incubations. A standard fluorescence microarray scanner with 3 spectral channels is used to image dried microscale im-

munoblots. The 16 blocks of microwells per slide can be isolated from each other during probing such that a cell sample can be probed for up to 48 targets in a single assay with 3-plex target quantitation.

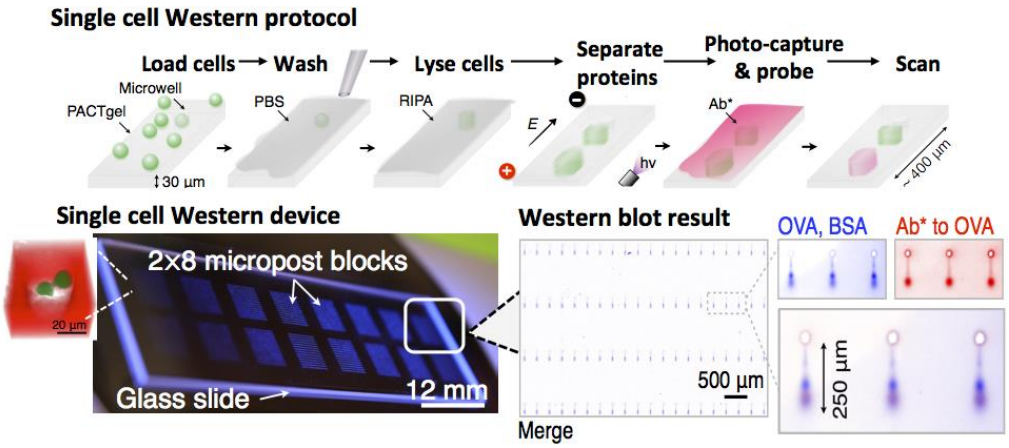

Figure 1: Single cell Western blotting device for analysis of ~3,000 individual cells. (Top) Assay protocol. (Bottom) Microwell (20 µm diameter) arrays house single cells (confocal image) in a 30 µm thick polyacrylamide gel on a glass microscope slide to support full integration of single cell Western blotting with representative Westerns shown bottom right for two model proteins (ovalbumin OVA, bovine serum albumin BSA).

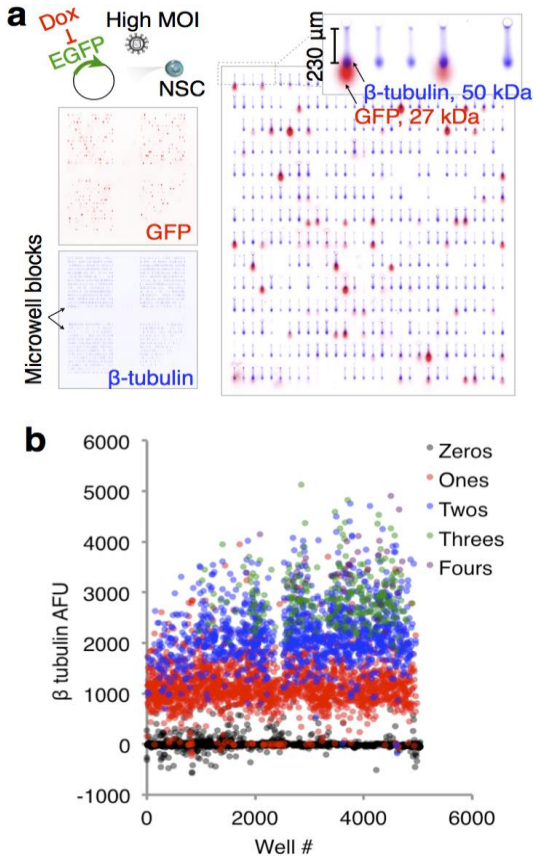

Figure 2: Western blotting of single neural stem cells. (a) Western blotting of ~3,000 EGFP-transfected individual neural stem cells on a single PACTgel microdevice. (b) Quantitation shows β-tubulin fluorescence scales approximately linearly with cell number per well.

RESULTS AND DISCUSSION

In the single cell western blot assay, protein separations begin after cell lysis from a microwell injection point. To study the separation performance of microwell-patterned PACTgels in the standard microscope slide format, we incubated a slide in a mixture of fluorescently labeled chicken egg ovalbumin and bovine serum albumin (Figure 1). Proteins tended to accumulate within the microwell structures, a process that we hypothesize is rooted in a partitioning effect that sets the equilibrium concentrations of proteins in the microwells as a function of the PACTgel. Applying an electric field

across the sandwiched slide stacked proteins against the rim of the microwell and subsequently separated them within the PACTgel. UV-initiated protein capture to the PACTgel and diffusive antibody probing successfully identified targets.

Next, we scaled implementation of the single cell western blotting workflow to the full array of 6,720 PACTgel microwells. Figure 2 presents an assay of EGFP-expressing neural stem cells spanning 4,128 microwell separations in 12 blocks of a single-cell western blot slide (82% of a possible 5,040 devices passed semi-automated gating against the influence of dust particles and similar fluorescence image artifacts). 1,608 (39%) of the 4,128 gated separations originated from single cells; an effective single-cell blot footprint of 0.9 mm^2, an 18-fold increase in separation density over microwestern arrays [8]. Approximately linear fluorescence readouts for β-tubulin were observed as a function of the manually counted number of cells per microwell, and single cell EGFP blots show a wide dynamic range in total probe fluorescence given the retroviral transduction process used to produce them.

CONCLUSION

We present single-cell western blotting with throughput on the order of 2,000 cells per standard microscope slide in a pipeline suited to rapid adoption. Underlying our advances is a photo-activatable polyacrylamide protein blotting gel that marries highly efficient 500 μm protein separations with lithographic capture of protein bands, enabling 10^4-10^5 molecule detection limits. A combination of spectral multiplexing and strip-reprobing capabilities put the number of targets that can be measured in a single blot (6+ in this report) on par with traditional flow cytometry panels.

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