DESIGN AND OPTIMIZATION OF A SINGLE-CELL PROTEIN ASSAY FOR CLINICAL UTILITY

John J. Kim^{1,2}, Elly Sinkala², and Amy E. Herr^{1,2*}

¹UC Berkeley / UC San Francisco Joint Graduate Group in Bioengineering, USA

²University of California, Berkeley, CA 94720, USA

ABSTRACT

We report a novel microdevice for both handling and single-cell protein analysis of rare patient-derived cells. By micropatterning a polyacrylamide gel layer on a glass slide for active cell settling and protein separation, we have created a platform that integrates handling samples of rare cells and analysis of proteins at a single-cell resolution. Centrifugation is used to rapidly settle single cells into microwells, followed by an electrophoretic separation of proteins and immunoprobing. Glioblastoma (brain cancer) and breast cancer cell lines are used as model systems to design and optimize our device. To ensure clinical utility, we design for efficient operation in near-patient settings.

INTRODUCTION

Cell-to-cell variation is a hallmark of life processes (development) and diseases (cancer). In cancer, the development of drug resistance is emerging as a major challenge. Drug resistance emerges from altered protein signaling in subsets of tumor cells (and not changes in DNA) [1]. Therefore, single-cell resolution protein analysis tools are needed. The de facto standard for protein analysis is the immunoassay. Regrettably, subtle chemical changes on proteins implicated in drug resistance are not detectable with available antibody probes [1, 2]. In a paradigm shift, we surmount this gap by introducing a ‘single-cell western blot’ that prepends an electrophoretic separation of proteins to the backend immunoassay [3]. By resolving proteins by both size and antibody binding, the assay can detect key targets not detectable by assay alone (similar size proteins). Precision control of fluid – conferred by microfluidic systems – makes these first-in-kind single-cell protein measurements possible [3-6]. Here we overcome the “macro-to-micro” interface challenge of handling sparingly available cells derived from fine needle aspirates (biopsy) and circulating tumors cells (CTCs) purified from blood [7, 8], by reporting an integrated microdevice for both handling and single-cell protein analysis of sparingly available cells from fine needle aspirates or circulating tumor cells.

To seat single cells in microwells, multiple groups have employed passive gravity-based sedimentation [3, 9, 10]. Using this accepted approach, we have observed successful capture of just 0.05% of cells from millions of starting cells [3]. Unfortunately, with patient-derived biospecimens, starting samples comprise just 50 to 500 of CTCs per an enriched sample [7, 8]. Random passive-gravity settling and sparsely located microwells (only 0.1% slide area is patterned with microwells) result in poor cell settling [3].

Many techniques are developed to actively sediment cells. With magnets and magnetic microbeads coated with antibody, cells of interest can be bound to microbeads and manipulated by magnetic forces [7, 11]. However, this method requires antibody-receptor binding between magnetic beads and target cells. Using an alternative current, non-uniform electric field is created to induce dielectrophoresis (DEP) force to actively settle cells. However, DEP requires a complex circuit fabrication with electrodes, and needs to exchange buffers after settling and

electrophoresis [12-14]. Centrifugation is used as a means to settle samples with a rare number of cells. With a conventional spinning motor, a centrifugal system is robust and suitable for cell handling; centrifugation is independent of buffer properties (i.e. conductivity, detergent, salt concentration, pH), which affect electrophoresis [15, 16]. A centrifugal method has previously reported with a very high capture efficiency over 90% and can be incorporated with electric field and magnetic field [17, 18].

Figure 1. Patterned polyacrylamide gel with microwells integrates centrifugal cell settling and single-cell western blotting technique for sparingly available cells. (A) Two chambers with flanking microwells, fabricated with polyacrylamide gel on a glass slide and a polyester film as a lid, is shown. A bottom diagram shows three apparent forces acting on a cell in a fluid chamber in a rotating reference frame: centrifugal ($F_{centrif}$), Coriolis ($F_{Coriolis}$), drag (F_{drag}). In the right image, three false-colored cells are settled in microwells due to the centrifugal force in a rotating reference frame. (B) A workflow of centrifugal single-cell western blotting is illustrated. After centrifugation, cells that are settled in microwells are chemically lysed, and an electric field is applied to separate proteins. UV is applied to activate benzophenone methacrylate (incorporated in the gel) for protein capture. Fluorescent antibodies are introduced to probe for target proteins.

Consequently, we designed, developed, and demonstrated an efficient and low-loss single-cell western that rapidly guides cells to microwells with centrifugal force in a rotating reference frame (Fig.

1A). Once in a microwell, we performed single cell western blotting on each cell. Initially, fluorescent microbeads were tested to optimize centrifugal velocity. Subsequently, we tested both centrifugal settling and western blotting with glioblastoma (brain cancer, U373) and breast cancer (BT474 and SKBR3) cell lines.

METHODS

Fabrication of centrifugal single-cell western blotting platform

Our polyacrylamide gel platform was molded against a silicon wafer with SU-8 features. The SU-8 3050 (Y311075, MicroChem) mold is composed of two layers by standard soft lithograph methods with the OAI UV light system with a mask aligner (Model 200IR, OAI). Two Mylar masks (CAD/Art Services) were used to make the two-layer molds: the bottom layer consists of fluid chamber sides (80- μ m thickness), and the top layer is composed of microwell posts (40- μ m thickness). Standard guidelines from MicroChem were followed for UV exposure, baking, and developing. The wafer with SU-8 mold was coated via vapor-deposition of 100 μ L of the hydrophobic silane dichlorodimethylsilane (DCMC, 440272, Sigma-Aldrich) for 40 minutes in vacuum. Two fluid chambers and flanking microwells were cast on a standard microscope glass slide with polyacrylamide gel against this hydrophobic wafer with SU-8 features (Fig. 1A, 2A). An 8%T, 2.7%C polyacrylamide gel was diluted from a stock 30%T, 2.7%C acrylamide/bis-acrylamide solution (A3699, Sigma). A 3mM benzophenone methacrylamide co-monomer (in-house) and 75mM Tris HCl pH8.8 (T1588, Teknova) were incorporated into the polyacrylamide gel precursor, and the gel was polymerized chemically with ammonium persulfate (A3678, Sigma) and TEMED (T9281, Sigma). To seal the fluid chambers, a top lid is composed of a hydrophilic polyester film (GelBond[®], Lonza) with inlet and outlet openings. The lid was topped on the wetted polyacrylamide gel slide, and a liquid superglue (Loctite) was applied on edges between the glass slide and the lid. A 1% BSA solution was added into device chambers via an inlet and incubated for 5 min at room temperature.

Cell/microbead injection and centrifugation

FITC-labeled and 15- μ m diameter polystyrene microbeads (F21010, Invitrogen) was diluted in 1x phosphate-buffered saline (PBS, 21040CV, Corning). For glioblastoma (U373-GFP) and breast cancer (BT474, SKBR3) cell lines, cells were cultured in a tissue-culture flask with ATCC recommended cell culture media. U373-GFP cells were dissociated with trypsin:EDTA (0.055:0.53mM, 400-150, Gemini), while BT373 and SKBR3 cells were dissociated with 0.5 mM EDTA (AM9260G, Ambion). Dissociated cells were counted with a hemocytometer and diluted in an ice-cold 1x PBS. Microbead or cell solution with a desired volume (1 ~ 20 μ L) was pipetted into an inlet of the device chamber. Then, a Kimwipe wiper (Kimtech) was applied on an outlet for 1 sec to inject the microbead/cell solution inside the device chamber. Then, a conventional upright spinner was used to spin the device at a desired rotational duration and velocity.

SDS-PAGE and photocapture of proteins

After centrifugation of the device, the top lid was cut and slowly slid off from the polyacrylamide gel slide. To lyse cells, a 10 mL of a RIPA-like lysis/electrophoresis buffer consisting of 1% SDS (L3771, Sigma), 0.1% v/v Triton X-100 (X100, Sigma), 0.25% sodium deoxycholate (D6750, SigmaAldrich) in 12.5 mM Tris, 96 mM glycine, pH 8.3, (161-0734, Bio-Rad) was poured over the slide for 15 seconds. A 200V (40V/cm) was applied across the slide to electrophorese proteins for 20 seconds. Immediately after the protein electrophoresis, we shone the slide with UV for 45 seconds

to activate benzophenone moieties, which immobilize proteins in the polyacrylamide gel. The slide was washed with 1x TBS with tween (TBST, 77500, Affymetrix) for 10 min before probing with antibodies.

Figure 2. Fluid chamber design and rotational speed is optimized by numerical simulations. (A) A top view of the centrifugal single-cell western blot device (upper image) and a schematic diagram of a left fluid chamber (bottom image) are shown. During centrifugation, drift velocity (U_{drift}) determines time and settling efficiency for moving cells to microwells in chamber. On the other hand, deflection velocity ($U_{deflection}$) affects distribution of cells across the flanking microwells. (B) In a rotating reference frame, force body diagram and equations to calculate drift and deflection velocities of a cell inside a fluid chamber. Drag force is created against centrifugal ($F_{centrif}$) and Coriolis ($F_{Coriolis}$) forces. By balancing these forces, numerical estimations of drift and deflection velocities are calculated. (C) Drift and deflection velocities with various rotational speeds. Drift velocity is over 100 μ m/s at 2000 rpm, which generates a low deflection velocity (0.02% of drift velocity) and ensures every cell in a fluid chamber to travel to microwells within 2 minutes. (D) The settling efficiency is over 80% for both beads and cells after spinning at 2000 rpm for 2 minutes.

Immunoprobing

Primary and secondary antibodies were diluted with 1xTBST with 2% BSA. The slide area, where microwells and immobilized proteins are present, was soaked in a 30 μ L of 0.1 g/L primary antibodies for 2 hours at a room temperature. Afterwards, we washed the slide with 1x TBST for 30 min. Subsequently, the slide area was incubated with a 30 μ L of 0.05 g/L secondary antibodies with fluorescent tags for 1 hour at a room temperature, followed by a 1xTBST wash for 1 hour. Finally, the gel in the slide was dried off completely by a nitrogen stream and imaged with a fluorescent microarray scanner (GenePix 4300A). GAPDH primary antibody was purchased from Sigma (SAB2500450), β -Tubulin from Abcam (ab6046), HER2/ErbB2 from Thermo Fisher (MA5-13105), and PR from Cell Signaling (3513).

RESULTS AND DISCUSSION

Optimization of centrifugal cell settling by numerical simulation and empirical iterations with microbeads and cells

We first investigated how fast cells are moving towards microwells during centrifugation inside a fluid chamber of the device (Fig. 2). In order to settle into microwells, cells were injected via an inlet and had to be moved about less than or equal to a 10 mm distance to reach microwells (Fig. 2A). During centrifugation, a

fluid (i.e. 1x PBS) inside the fluid chamber was stagnant. However, in a rotating reference frame, three apparent forces are present on particles inside the chamber: centrifugal, Coriolis, and drag forces (Fig. 2B). Because Reynold's number was far less than 0.01 at below 5000 rpm inside a fluid chamber, Stokes' law could be used to calculate a drag force acting on the bead. A force balance between centrifugal and drag forces resulted in a drift velocity (U_{drift}), which represents how fast cells are moving to microwells. Similarly, Coriolis and drag forces determine a deflection velocity. The deflection velocity, perpendicular to the drift velocity and the row of flanking microwells, affected distribution of cells and had to be minimized to prevent multiple cells from piling on microwells in a corner of the fluid chamber (Fig. 2C).

Figure 3. Centrifugal force is effective and efficient for rapid seating of glioblastoma cells into microwells in preparation for subsequent single-cell protein analysis in a rotating reference frame. (A) False-color fluorescence micrograph of before (left) and after (right) centrifugation for 2 min at 2,000 RPM. Green signal indicates fluorescing cells in microwells. No cells are detectable at microwells prior to centrifugation, as cells are deposited outside the field of view. (B) Cell occupancy distribution from the centrifugal seating approach shows most microwells contain 1 cell, as desired. (C) Comparative performance metrics for passive gravity-based cell settling (previous method) and active, centrifugation based cell settling (new method introduced in this work). Note that across all metrics, active centrifugal settling outperforms passive settling. These high-performance metrics indicate the new technique is appropriately designed for analysis of single cells in limited samples, including fine needle aspirates as is underway.

To investigate an efficiency of centrifugal settling in our system, homogeneous and fluorescent polystyrene microbeads ($14.8 \pm 0.1332 \mu\text{m}$, 1.06 g/cm^3) were first used to test settling in microwells by centrifugation. Assuming that the bead acceleration was instantaneous, drift velocities of microbeads towards traps at 3000 rpm were calculated as $50 \mu\text{m/s}$, respectively. Over 80% of

polystyrene beads were settled into microwells in 2 minutes at 2000 rpm (Fig. 2D). Subsequently, we tested with glioblastoma cancer cell line with GFP fluorescence (U373-GFP) to test the centrifugal cell settling. At 2000 rpm for 2 min, we successfully settled 80% of cells in microwells (Fig. 2D). Sample seating times were 10% of the time required for gravity-based, passive seating (Fig. 3C), as corroborated with numerical simulation estimates. Deflection velocities and trajectories (due to Coriolis force) inform chamber geometry and size so as to minimize cell accumulation along just one wall of each sample chamber (i.e., $\sim 0.05 \mu\text{m/s}$ at 2000 rpm, Fig. 2C). Fluorescence imaging of glioblastoma cells revealed >70% of microwells contain single cells (Fig. 3A), even when starting with just 100 cells. Importantly, centrifugation results in settling efficiencies increased by > 5-fold while shortening settling time by > 10-fold (Fig. 3C).

Figure 4. The integrated centrifugation settling and single-cell western blot microdevice successfully analyzes multiple proteins in single cells from two types of cancer. (A) False-color fluorescence images of glioblastoma cells (U373-GFP) seated in microwells (left image) and subsequent western blot of 3 protein targets in each cell (β -tubulin, GAPDH, and GFP). Fluorescence signal along the separation axis is also shown. Multiple cells are handled and assayed simultaneously. (B) False-color fluorescence images of two different types of breast cancer cells, with Case #1 having cells express the protein HER2 but not the protein PR; whereas Case #2 sees all cells express HER2 (as expected) but cell-to-cell variation in expression of the protein PR (unexpected, see peak marked with a *). Measuring cell-to-cell variation is critical to understanding cancer progression and response to treatment.

Single-cell western blotting after centrifugal cell settling

After validating centrifugal cell handling, we integrated with single-cell western analysis. We successfully completed the full western by detaching the polymer film lid, then subjecting each cell to 10-sec in-microwell chemical lysis, 15-sec protein electrophoresis separation, UV-activated immobilization of proteins to the polyacrylamide gel layer (incorporating light-sensitive

benzophenone), and an in-gel immunoassay for each of our three target proteins. In the integrated device, we observed the protein targets at the appropriate molecular masses (β -tubulin, 51 kDa; GAPDH, 35 kDa; and GFP, 27 kDa; Fig. 4A). In a striking result, the integrated assay fully resolved proteins with just an 8 kDa mass difference (Fig. 4A).

After validating protein separations and probing in U373-GFP cells in our single-cell western blotting device, we have started to investigate cell-to-cell heterogeneity of receptor proteins in breast cancer cell lines, BT474 and SKBR3 [19]. Currently, a breast cancer diagnosis consists of three protein markers: estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) [20]. Based on qualitative analysis of these proteins from biopsies, breast cancer patients would receive various treatments for their cancer. Quantitative measurement of these receptor proteins at a single-cell resolutions would provide valuable proteomic-information from sparingly available cells derived from fine needle aspirate biopsy to understand correlations among these receptors and elucidate drug resistance from monoclonal antibodies for effective breast cancer treatments.

We tested our centrifugal single-cell western blot to measure HER2 and PR protein expressions from BT474 and SKBR3 single cells. Our preliminary data also showed presence of HER2 in both BT474 and SKBR3 cells, while SKBR3 cells did not express PR (Fig. 4B). Interestingly, we found cell-to-cell variation of PR in BT474 cells. We are currently performing more cells on this centrifugal single-cell western blot and probing western blot data with other breast cancer markers.

Our integrated rare cell handling and protein analysis platform enables direct measurement of aberrant protein signaling during development of drug resistance in brain and breast cancer cells. We are measuring cell-to-cell variation in fine-needle aspirate specimens and will report on our findings during development of cancer drug resistance.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge members of the Herr Laboratory at UC Berkeley for assistance and helpful discussions. Research reported in this publication was supported by the National Institute of Biomedical Imaging and Bioengineering (NIBIB) of the National Institutes of Health (NIH) under Award Number U54EB015403. This work is supported by a National Science Foundation (NSF) CAREER award (CBET-1056035 to A.E.H). J.J.K. is supported by a US NSF Graduate Research Fellowship. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH or NSF. Travel support has been generously provided by the Transducer Research Foundation.

REFERENCES

- [1] Hoferlin LA, C EC, Park MA. Challenges in the Treatment of Triple Negative and HER2-Overexpressing Breast Cancer. *Journal of surgery and science*. 2013;1:3-7.
- [2] Ward TM, Iorns E, Liu X, Hoe N, Kim P, Singh S, et al. Truncated p110 ERBB2 induces mammary epithelial cell migration, invasion and orthotopic xenograft formation, and is associated with loss of phosphorylated STAT5. *Oncogene*. 2013;32:2463-74.
- [3] Hughes AJ, Spelke DP, Xu Z, Kang CC, Schaffer DV, Herr AE. Single-cell western blotting. *Nature methods*. 2014;11:749-55.
- [4] White AK, VanInsberghe M, Petriv OI, Hamidi M, Sikorski D, Marra MA, et al. High-throughput microfluidic single-cell RT-qPCR. *Proceedings of the National Academy of Sciences of the United States of America*. 2011;108:13999-4004.

- [5] Eastburn DJ, Sciambi A, Abate AR. Ultrahigh-throughput Mammalian single-cell reverse-transcriptase polymerase chain reaction in microfluidic drops. *Analytical chemistry*. 2013;85:8016-21.
- [6] Wu AR, Neff NF, Kalisky T, Dalerba P, Treutlein B, Rothenberg ME, et al. Quantitative assessment of single-cell RNA-sequencing methods. *Nature methods*. 2014;11:41-6.
- [7] Stott SL, Lee RJ, Nagrath S, Yu M, Miyamoto DT, Ulkus L, et al. Isolation and characterization of circulating tumor cells from patients with localized and metastatic prostate cancer. *Science translational medicine*. 2010;2:25ra3.
- [8] Armstrong AJ, Marengo MS, Oltean S, Kemeny G, Bittling RL, Turnbull JD, et al. Circulating tumor cells from patients with advanced prostate and breast cancer display both epithelial and mesenchymal markers. *Mol Cancer Res*. 2011;9:997-1007.
- [9] Warrick J, Casavant B, Frisk M, Beebe D. A microfluidic cell concentrator. *Analytical chemistry*. 2010;82:8320-6.
- [10] Deutsch M, Deutsch A, Shiriha O, Hurevich I, Afrimzon E, Shafran Y, et al. A novel miniature cell retainer for correlative high-content analysis of individual untethered non-adherent cells. *Lab on a chip*. 2006;6:995-1000.
- [11] Kang JH, Krause S, Tobin H, Mammoto A, Kanapathipillai M, Ingber DE. A combined micromagnetic-microfluidic device for rapid capture and culture of rare circulating tumor cells. *Lab on a chip*. 2012;12:2175-81.
- [12] Voldman J, Gray ML, Toner M, Schmidt MA. A microfabrication-based dynamic array cytometer. *Analytical chemistry*. 2002;74:3984-90.
- [13] Taff BM, Voldman J. A scalable addressable positive-dielectrophoretic cell-sorting array. *Analytical chemistry*. 2005;77:7976-83.
- [14] Hsiao AP, Barbee KD, Huang X. Microfluidic Device for Capture and Isolation of Single Cells. *Proceedings of SPIE--the International Society for Optical Engineering*. 2010;7759.
- [15] Burger R, Kirby D, Glynn M, Nwankire C, O'Sullivan M, Siegrist J, et al. Centrifugal microfluidics for cell analysis. *Current opinion in chemical biology*. 2012;16:409-14.
- [16] Madou M, Zoval J, Jia G, Kido H, Kim J, Kim N. *Lab on a CD*. Annual review of biomedical engineering. 2006;8:601-28.
- [17] Burger R, Reith P, Kijanka G, Akujobi V, Abgrall P, Duce J. Array-based capture, distribution, counting and multiplexed assaying of beads on a centrifugal microfluidic platform. *Lab on a chip*. 2012;12:1289-95.
- [18] Kirby D, Siegrist J, Kijanka G, Zavattoni L, Sheils O, O'Leary J, et al. Centrifugo-magnetophoretic particle separation. *Microfluid Nanofluid*. 2012;13:899-908.
- [19] Holliday DL, Speirs V. Choosing the right cell line for breast cancer research. *Breast Cancer Res*. 2011;13:215.
- [20] Boyle P. Triple-negative breast cancer: epidemiological considerations and recommendations. *Annals of oncology : official journal of the European Society for Medical Oncology / ESMO*. 2012;23 Suppl 6:vi7-12.