Published in final edited form as:

Small. 2018 November ; 14(48): e1802865. doi:10.1002/sml.201802865.

Microparticle Delivery of Protein Markers for Single-Cell Western Blotting from Microwells

Dr. John J. Kim,

Department of Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA; UCB-UCSF Graduate Program in Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA

Dr. Peggy P. Y. Chan,

Department of Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA; Faculty of Science Engineering & Technology, Swinburne University of Technology, Melbourne, VIC 3122, Australia

Dr. Julea Vlassakis,

Department of Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA; UCB-UCSF Graduate Program in Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA

Alisha Geldert, and

Department of Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA; UCB-UCSF Graduate Program in Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA

Prof. Amy E. Herr

Department of Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA, aeh@berkeley.edu; UCB-UCSF Graduate Program in Bioengineering, University of California Berkeley, Berkeley, CA 94720, USA; Chan Zuckerberg Biohub, San Francisco, CA 94158, USA

Abstract

Immunoblotting confers protein identification specificity beyond that of immunoassays by prepending protein electrophoresis (sizing) to immunoprobng. To accurately size protein targets, sample analysis includes concurrent analysis of protein markers with known molecular masses. To incorporate protein markers in single-cell western blotting, microwells are used to isolate individual cells and protein marker-coated microparticles. A magnetic field directs protein-coated microparticles to >75% of microwells, so as to 1) deliver a quantum of protein marker to each cell-laden microwell and 2) synchronize protein marker solubilization with cell lysis. Nickel-coated microparticles are designed, fabricated, and characterized, each conjugated with a mixture of

The ORCID identification number(s) for the author(s) of this article can be found under <https://doi.org/10.1002/sml.201802865>.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Conflict of Interest

J.J.K., P.P.Y.C., J.V., and A.E.H. are coinventors on University of California Intellectual Property that, if licensed, would generate financial gain.

histidine-tagged proteins (42.3–100 kDa). Imidazole in the cell lysis buffer solubilizes protein markers during a 30 s cell lysis step, with an observed protein marker release half-life of 4.46 s. Across hundreds of individual microwells and different microdevices, robust log-linear regression fits ($R^2 > 0.97$) of protein molecular mass and electrophoretic mobility are observed. The protein marker and microparticle system is applied to determine the molecular masses of five endogenous proteins in breast cancer cells (GAPDH, β -TUB, CK8, STAT3, ER- α), with <20% mass error. Microparticle-delivered protein standards underpin robust, reproducible electrophoretic cytometry that complements single-cell genomics and transcriptomics.

Keywords

microfluidics; microparticle; microwell; single-cell analysis; western blotting

1. Introduction

Single-cell analysis tools report biomolecular heterogeneity that drives processes from immune-cell response to cancer progression.^[1–4] Molecular standards play an important role in deconvolving biological variation from technical variation arising from a measurement tool.^[5,6] In single-cell sequencing, synthetic spike-ins and unique molecular identifiers (e.g., short-random DNA sequences) directly measure error rates, analytical sensitivity, and biases stemming from sample preparation.^[7–9] In flow cytometry, lasers are calibrated before cell sorting using fluorescent microparticles.^[10,11] In microfluidic protein assays of a single cell lysate (e.g., single-cell barcode assay), estimates of technical variation are inferred from multiple measurements of each lysate.^[12]

Analysis of cell lysate by electrophoretic separations uses protein markers to estimate the molecular mass of a target from the known masses of the markers. In order to accurately size and identify targets, protein markers control for technical variation in the uniformity of the electric field, sieving matrix structure, and buffer composition—all of which influence protein electromigration.^[13–15] Well-validated protein standards are also crucial in identifying and understanding “gel shifting,” a phenomenon in which a protein does not electromigrate proportionally with its molecular mass due to amino acid substitutions, post-translational modifications, or other unknown factors.^[16,17] Consequently, protein markers and ladders find utility in slab-gel, capillary, and microchannel electrophoresis formats.^[18–22] In slab-gel protein polyacrylamide gel electrophoresis (PAGE), the first or last separation lanes of the slab gel are used for analysis of protein markers.^[23–26] In western blotting, protein markers not only estimate the molecular mass of a target, but also indicate the quality of protein transfer from the PAGE slab gel to the immunoblotting membrane.^[27–30] Run-to-run reproducibility in electromigration is monitored using protein markers, providing a quality control indicator.^[22] Such protein markers to assess technical variation in electromigration would be invaluable for quantifying proteoform expression that drives drug resistance in single cancer cells.^[31]

Microwells are widely utilized to isolate intact single cells for subsequent analysis.^[32–35] Microwell arrays have been used for high throughput and automatic analysis of DNA^[35–37]

and protein^[38–39] from hundreds to thousands of cells. Gravity is commonly used to sediment single cells into open microwells, followed by in situ cytometry. To conduct electrophoresis of single-cell lysate, an external electric field is applied across cell-laden microwells (fabricated in agarose or polyacrylamide) to initiate and drive electrophoretic separation. Physically isolated inside microwells, intact individual cells are readily monitored for cell morphology^[40,41] and lysed to measure DNA damage and repair,^[35,42] transcriptional levels,^[36,37] and protein expression.^[43,44]

Our laboratory has developed and utilized an “open” microfluidic device format comprising an array of microwells cast in a thin layer of polyacrylamide gel to perform electrophoretic analyses of isolated mammalian cells, including circulating tumor cells and breast cancer cells.^[5,31,38,45–47] In addition to cell isolation, each microwell acts as a reactor for cell preparation (i.e., imaging, staining, chemical lysis), with each cell lysate then subjected to electrophoretic analysis (i.e., protein PAGE, isoelectric focusing) in the surrounding polyacrylamide gel layer. When photosensitive benzophenone methacrylamide monomer has been cross-linked into the polyacrylamide molecular sieving matrix, protein electrophoresis can be followed by UV-activated protein blotting in the gel (photoblotting, 1 min) and subsequent immunoprobings also in the gel. UV activation of benzophenone changes the polyacrylamide gel from a molecular sieving matrix for electrophoresis into a blotting membrane for the subsequent immunoassay probing steps. The molecular sieving gel rapidly toggles (1 min) to a blotting membrane, thus eliminating the lossy blotting transfer step. To localize known, purified protein markers to each microwell, we report here on design of a vehicle to deliver discrete quanta of protein markers selectively into each microwell. The microparticle-delivery maintains locally high protein marker concentrations by eliminating diffusion of markers into both the gel network and the microwells prior to cell lysis. Single-cell western blotting measures targets that may not be discernable using an immunoassay alone.^[31] For example, protein isoforms are involved in pathological processes including cancer,^[31] autoimmune diseases,^[48] diabetes,^[49] and heart hypertrophy.^[50] However, distinguishing isoforms has been challenging; thus, the exact roles of most isoforms are hence poorly defined.^[51] Protein size markers are needed to make single-cell western blotting more reliable and accurate.

Here, we investigate the performance of a “solid phase” protein marker designed specifically for single-cell western blotting. We design to meet two key performance metrics: quantized delivery to cell-laden microwells and triggered release of fluorescent protein markers in each microwell. In this study, we consider 10 μm diameter magnetic microparticles coated with nickel (Ni^{2+}). To meet the particle-loading metric, we choose magnetic particles so that we might use an applied, external magnetic field for directed loading of the microparticles into cell-laden microwells.^[48,52,53] To meet the more stringent triggered release metric, we utilize microparticles coated with Ni^{2+} for reversible binding of histidine(His)-tagged proteins, which can then be chemically released from the microparticle concurrently with a 30 s cell lysis step.^[52,54]

2. Results and Discussion

2.1. Design of the Rapid-Release Protein Marker Delivery Vehicle

A magnetic microparticle delivery system was selected for delivery of protein markers to each microwell of the multistage, single-cell western blot (Figure 1A). We employed i) an applied magnetic field to actively seat marker-coated microparticles in microwells (i.e., in contrast to gravity-based cell settling; Figure 1B) and ii) a flexible coordination chemistry on the microparticle surface for protein marker immobilization and triggered solubilization/release (Figure 1C). We utilized two key microparticle characteristics to achieve the desired functionality and performance: i) magnetic cores for active microparticle seeding in microwells directed via magnetic field and ii) a Ni^{2+} coating that supports high-efficiency protein immobilization with rapid protein release (<30 s) when exposed to imidazole.

We first sought to assess the efficiency of a two-step microwell loading process. First, we seeded MCF-7 breast cancer cells in each microwell by applying a suspension of the cells (≈ 25 μm diameter) onto the polyacrylamide gel layer stippled with 1300 microwells (Figure S1, Supporting Information). Cells were sedimented via gravity for 10 min (Figure 1A, step 1). Gentle washing removed cells that settled onto the surface of the polyacrylamide gel, with cells that settled into the microwells retained on the chip. Next, to deliver microparticles coated with the 4 protein markers into each cell-laden microwell, we applied a solution of the microparticles (10^6 microparticles mL^{-1}) onto the single-cell western blot device and applied a magnetic field to the device for 2 min to direct microparticles into microwells (Figure 1A, step 2). The circular magnet (4 cm diameter $\times$ 1.53 cm height) was located under the 2.5 cm $\times$ 3.75 cm device and was moved across the device to expose all microwells to the applied magnetic field. Gentle washing was used to remove microparticles from the surface of the polyacrylamide gel, with microparticles that settled into the microwells retained on the chip.

At the completion of the two-stage microwell loading process, we used brightfield microscopy and selected a random region of interest (≈ 300 microwells) from each chip to analyze both microparticle and cell occupancies. We observed $\approx 75\%$ of microwells sampled housed 1 microparticle ($n = 3$ chips, standard deviation $\sigma = 4\%$, Figure 1B). Microwells were designed to house MCF-7 cells (30 μm diameter and 35 μm deep microwells), so we opted for 10 μm diameter microparticles so as not to interfere with cell loading. We observed 38.3% of microwells contained a single MCF-7 cell (Figure 1B), corroborating our previous studies that also utilized gravity for cell settling.^[39] In addition to passive cell sedimentation, we have reported on alternate approaches for cell loading including, for example, precision transfer of rare cells^[51] and two-step centrifugation by utilizing a second layer of polyacrylamide gel as raised and crescent-shaped dam structures on the perimeter of each microwell.^[45] A centrifugal force directed cells to the dams. Upon removal of the centrifugal force, each cell localized to the dam then settles into the proximal microwell.^[45] For the two-step loading process, we observed that 35.3% of microwells contained both a single cell and 1 microparticle ($n = 3$ chips, ≈ 300 microwells sampled per chip, standard deviation $\sigma = 8.1\%$), as desired for concurrent electrophoretic analysis of protein markers and cell lysate from each microwell.

We next sought to quantitatively assess i) the Ni^{2+} chelation for solid phase immobilization of protein markers on the microparticle surface and ii) the subsequent rapid triggered solubilization of protein markers into the PAGE separation stage (Figure 1C,D). We aimed to trigger release of protein markers from the microparticle surface with a <30 s release duration and a “pulse” release profile.^[46] The <30 s duration was sought to match the time frame of the rapid, in-microwell cell lysis, and endogenous protein solubilization step. A pulse profile was sought to minimize injection dispersion (i.e., sample tailing) arising from marker release during electroinjection of microwell contents into the PAGE lane.

To immobilize the protein markers on the microparticle surface, the microparticles were incubated (2 h) in a cocktail of fluorescently labeled His-tagged marker proteins (KDR, $21.9 \text{ ng } \mu\text{L}^{-1}$; ICAM1, $7.40 \text{ ng } \mu\text{L}^{-1}$; Eph B4, $54.5 \text{ ng } \mu\text{L}^{-1}$; CHI3L1, $14.8 \text{ ng } \mu\text{L}^{-1}$). After incubation with proteins, the microparticles were washed three times with a washing buffer containing mild imidazole ($20 \times 10^{-3} \text{ M}$). Via flow cytometry, we observed 98% of microparticles (≈ 2000 particles) were coated with the fluorescent protein markers (Figure 1C; Figure S2, Supporting Information).

After preparing the microparticle-based protein marker delivery system, we examined the release kinetics of His-tagged recombinant Protein A labeled with Alexa Fluor 647 (AF647), as a representative protein marker. To displace the His-tagged proteins from the microparticle surface, imidazole was used as a competitive binding ligand. Imidazole has a similar structure to His and serves as an electron donor.^[55] Release of His-tagged Protein A from the microparticles was modeled according to one-state ligand–receptor kinetics (an exponential decay).

In a negative control, lysis buffer without imidazole resulted in release of Protein A with a half-life of 63.4 s and a dissociation rate constant of 0.011 s^{-1} (Figure S3, Supporting Information). The observed dissociation rate constant was $10\times$ greater than that previously reported,^[56] a difference we attributed to the elevated (55°C) buffer temperature employed here.

Next, we investigated the impact of imidazole concentration on protein marker release from the microparticles using a series of different imidazole concentrations in the cell lysis buffer. During the protein marker release from the Ni–His linkage we assume that the competitive binding of imidazole to the Ni^{2+} is not affected by the molecular size of protein markers conjugated to the His tag, given that the molecular mass of imidazole is 68 Da. The lowest imidazole concentration studied was $15 \text{ mmol imidazole per } 15 \text{ mL}$, which was in excess as compared to the concentration of the His-tagged Protein A ($0.12 \text{ nmol per } 15 \text{ mL}$). Given the orders-of-magnitude excess of imidazole, we assume that the imidazole concentration in the buffer is not significantly reduced over the 30 s time course of protein marker release and cell lysis, even for this lowest imidazole concentration condition. We opt to study Protein A as a model marker protein for the release because Protein A is a low-molecular mass marker (39 kDa) that enables measurement of the protein fraction bound on microparticles using wide-field fluorescence microscopy—the unbound protein diffuses away quickly from the region of interest (estimated diffusion coefficients, $36.1 \text{ } \mu\text{m}^2 \text{ s}^{-1}$ at 4°C to $158 \text{ } \mu\text{m}^2 \text{ s}^{-1}$ at 55°C).^[57,58] The Protein A release kinetics at the 1 M imidazole concentration and at 4°C

yield a protein marker release half-life of 49.2 s (95% confidence interval, CI = 39.0–65.9 s) and a dissociation rate constant of 0.014 s^{-1} ($n = 3$ particles, coefficient of variation, CV = 123.7%; Figure 1D). For the same imidazole concentration, we elevated the temperature of the cell lysis buffer and observed dissociation rate constants of 0.044 s^{-1} (at 23 °C; $n = 3$ particles, CV = 51.5%) and 0.16 s^{-1} (at 55 °C; $n = 3$ particles, CV = 40.6%), with the latter condition yielding a protein release half-life of 4.46 s (95% CI = 4.01–4.97 s). Within 30 s, more than 80% of the His-tagged Protein A was released from the microparticles at 55 °C (Figure 1D). We scrutinized the dissociation rate constant for a range of higher imidazole concentrations at the most elevated temperature condition. We observed the shortest release half-life (4.46 s) with a 1 M imidazole concentration at 55 °C (Figure S3, Supporting Information). Because the estimated time scale of the His-tagged Protein A (39 kDa) diffusing out of a microwell is similar to the protein release half-life in 55 °C 1 M imidazole, the release reaction is rapid compared to diffusion of protein out of the well.^[57,58] Any protein markers with 39 kDa would remain at higher concentrations in the microwell prior to single-cell protein PAGE. As the protein marker delivery would be concurrent with the rapid cell lysis step, we selected the 1 M imidazole at 55 °C as the condition to trigger release of His-tagged protein markers.

2.2. Validating the Protein Marker as a Molecular Mass Standard

After rapid release of the protein markers in the microwell, we scrutinized the protein marker electromigration during single-cell protein PAGE (Figure 1E and 2). At 30 s of cell lysis and protein marker release, protein PAGE was initiated by applying an electric field ($E = 40 \text{ V cm}^{-1}$) for 25 s. Separated protein peaks were immobilized with a 45 s UV exposure of the benzophenone in the polyacrylamide gel. After photoblotting, Ferguson analysis of the protein electromigration was performed.^[21,28] In protein sizing (gel electrophoresis with sodium dodecyl sulfate (SDS)), we expect a log-linear relationship between the molecular mass and observed electrophoretic mobility of each target. Log-linear fitting of the protein markers yielded $R^2 > 0.97$ for >300 PAGE separation lanes (Figure 2A), which is comparable to the log-linearity of many other slab gel and capillary electrophoresis systems.^[59–61] The final protein peak location had CV ranging from 2.2% to 11.0% depending on the protein marker considered ($\mu \pm \sigma$ of CV across 3 chips: ICAM1, 9.41 ± 2.06 ; KDR, 6.29 ± 2.03 ; EpH B4, 5.00 ± 2.30 ; CHI3L1, 5.07 ± 2.53 ; $n_{\text{chip1}} = 147$, $n_{\text{chip2}} = 104$, $n_{\text{chip3}} = 340$; Table S1, Supporting Information).

Across each device, we found no observable spatial dependence on the γ -intercept values of the log-linear regression but did observe a decrease in the slope across the width of the chip (Figure S4, Supporting Information). Recognizing that a $E \approx 1 \text{ V cm}^{-1}$ across the width of the chip (left to right) would result in the observed slope variation, we hypothesized that the apparatus has a slight offset in electrode pair spacing from one end of the chip to the other (Figure S4, Supporting Information).

For each protein marker, we compared the expected molecular mass to the measured molecular mass. An analysis of 168 single-cell PAGE lanes gave a difference between the two values of <5%, with an interquartile range <1% (Figure 2B), for all protein markers that passed quality control (see the Experimental Section). Although peak location CV values are

not significantly different among all four markers (Table S1, Supporting Information, $n = 3$ chips, one-way ANOVA test, $p > 0.5$), the percent mass errors of the smallest ladder component (CHI3L1, 42 kDa) and the largest ladder component (ICAM1, 100 kDa) were significantly higher than those of EpH B4 (58 kDa) and KDR (85 kDa) ($\mu \pm \sigma$ of percent mass error: ICAM1, 3.77 ± 0.49 ; KDR, 1.24 ± 0.53 ; EpH B4, 0.39 ± 0.30 ; CHI3L1, 5.05 ± 0.57 ; Kruskal-Wallis test with Dunn's multiple comparisons test, $***p < 0.0001$, $n = 168$ PAGE separation lanes; Figure 2B). The slightly higher mass errors in CHI3L1 and ICAM1 were ascribable to CHI3L1 and ICAM1 peaks electromigrating less than what the log-linear regression fitting expected, resulting in estimated masses lower than the expected masses. High and low molecular mass proteins should be included in each set of protein marker standards for most accurate estimation of molecular mass, which agrees with previous work demonstrating that the use of a pair of high and low molecular mass protein standards controlled for technical variation in migration rate in a capillary gel electrophoresis platform.^[60]

Next, we examined chip-to-chip variation in molecular mass estimation to assess reproducibility of the protein marker as a size standard (Figure 2C; Figure S5, Supporting Information). Regarding the goodness of the log-linear regression fit, we observed $R^2 > 0.97$ ($n = 3$ chips; Figure 2C). One-way ANOVA tests indicate that the distributions of the R^2 values are significantly different among the chips. However, the magnitude of the chip-to-chip difference in R^2 values is minimal, with a Cohen's $d < 0.2$ (Figure 2C). We further investigated technical variation across the chips by analyzing percent mass errors of the protein markers. Although the slight run-to-run difference in the gel electrophoresis duration might contribute to the fact that distributions of percent mass errors are significantly different across chips (Figure S5, Supporting Information), we observed consistent protein electromigration (CV of peak locations $< 12\%$) and $< 10\%$ mass errors in sizing of the protein markers (42–100 kDa protein masses; Figure S5 and Table S1, Supporting Information).

Given that not all cell-containing microwells were populated with microparticles, we sought to assess the impact of protein marker performance when employing protein markers proximal to single-cell PAGE separations lanes as size standards. We used a semi-variogram analysis of the log-linear fitting coefficients and determined that molecular mass estimates of protein targets are accurate using proximal protein marker electromigration, if the protein markers are released in a microwell that is located ~ 2.5 mm (center-to-center pitch) from the microwell containing the cell of interest (Equation S1 and Figure S6, Supporting information).^[62,63] We observed no significant difference (Kruskal-Wallis test with Dunn's multiple comparisons test, $p > 0.05$) in molecular mass error when protein sizing of endogenous targets was performed either using the protein markers from the same PAGE separation lane or in "proximal" separation lanes (i.e., located ~ 2.5 mm from the PAGE separation lane containing the proteins targets; Figure S7, Supporting Information).

2.3. Imidazole Interferes with Protein Photoblotting

After protein PAGE, efficient photoblotting is critical for protein detection and immunoprobable readout of the single-cell western. During photoblotting, we expected interference between the imidazole (1 M) and the UV-activated benzophenone group, as

benzophenone can abstract hydrogen from a secondary amine group on the imidazole, thus allowing benzophenone to form a covalent bond with the high concentration imidazole (Figure S8, Supporting Information).^[64] In some sense, the imidazole would act to “block” the benzophenone from forming covalent bonds with protein targets. When photoblotting was performed in the presence of 1 M imidazole, we observed just 3.7% of separation lanes with protein marker signals that exceeded the minimum signal to noise ratio (SNR), as described in the quality control protocol (Figure 3A,F, see the Experimental Section).

To enhance the photoblotting efficiency of the protein markers, we modified the standard single-cell western blot workflow to include a buffer-exchange step between the chemical cell lysis step and the protein PAGE step (see the Experimental Section). This buffer exchange step reduces the imidazole concentration in the polyacrylamide gel matrix prior to the UV photoblotting step. A similar approach to buffer exchange has been employed to reduce Joule heating during single-cell electrophoresis.^[65] Comparing mean SNR values for the protein markers, the SNR with the buffer exchange step was >20% greater than the SNR without the buffer exchange step (Figure 3B). For endogenous proteins in each MCF-7 cell lysate, the buffer exchange increased immunoprobable signal from β -tubulin (β -TUB), cytokeratin 8 (CK8), and GAPDH to levels observed with no imidazole included in the chemical lysis step (Figure S9, Supporting Information).

Considering total assay performance, we next asked whether the buffer exchange might affect the goodness of fit in the log-linear regression and separation resolution (SR) of the protein markers (Figure 3C,D; Equation S2, Supporting Information). The goodness of fit distribution of R^2 values with the buffer exchange was not significantly different from that without the buffer exchange (unpaired t -test, $p = 0.20$, $n_{\text{with_exchange}} = 107$, $n_{\text{no_exchange}} = 27$; Figure 3C). Furthermore, no decrease in SR between the protein markers was detectable (unpaired t -test; $\text{SR}_{\text{ICAM1\&KDR}}$, $p = 0.0003$; $\text{SR}_{\text{KDR\&Eph B4}}$, $p = 0.55$; $\text{SR}_{\text{Eph B4\&CHI3L1}}$, $p = 0.19$; Figure 3D). Joule heating was not reduced upon buffer exchange, nor were corresponding improvements in SR observed.^[65] The buffer exchange here utilized room temperature lysis buffer that simply omits imidazole, while still containing high concentrations of conductive ionic detergents that result in Joule heating-induced peak dispersion during electrophoresis.

2.4. Detection Threshold: Minimum Number of Microparticles Per Microwell

We sought to assess the number of microparticles per microwell required to detect protein markers with an SNR ≥ 3 . We used the KDR protein to assess the relationship between SNR of protein peaks ($E = 40 \text{ V cm}^{-1}$; $t = 25 \text{ s}$) and the number of microparticles per microwell. As expected, the SNR increased with an increasing number of microparticles per microwell (Figure 3E). Without buffer exchange, >3 microparticles per microwell were required to achieve an SNR ≥ 3 (Figure 3E). By contrast, the buffer exchange and dilution of imidazole yielded SNR ≥ 5 with a single microparticle in a microwell (Figure 3E). As compared to just 3.7% of separation lanes yielding suitable detection signal without the buffer exchange, under these conditions we observed 55% of lanes yielding an acceptable signal (Figure 3F).

In formulating “design rules” for protein sizing markers based on these findings, one microparticle per microwell was sufficient for detection of the protein markers in single-cell

western blotting. In cases where no microparticles are loaded into a cell-laden microwell, proximal microwells containing protein markers may be used for cellular protein sizing. In cases where protein targets are >100 kDa, microparticle loading of the cell-laden microwell should not exceed 3 microparticles, as we observed nonspecific signal proximal to each microwell—that may interfere with detection of large protein markers—when 4 microparticles were seated in a single microwell (Figure S10, Supporting Information).

2.5. Determining Molecular Mass of Endogenous Protein Targets in MCF-7 Cells

Next, we applied the protein markers to estimate the molecular masses of endogenous proteins from single MCF-7 breast cancer (Figure 4). For microwells containing both individual MCF-7 cells and 1 microparticles, we performed single-cell western blotting and concurrently resolved protein markers and endogenous proteins in all PAGE separation lanes (Figure 4A).

We sought to identify any confounding interactions when running protein PAGE on the protein marker and single-cell lysate from the same microwell. We found no significant difference in the distribution of the peak widths of the protein markers in the presence of MCF-7 cells ($\mu \pm \sigma$ ICAM1: $108.5 \pm 34.5 \mu\text{m}$, KDR: $165.6 \pm 22.8 \mu\text{m}$, EpH B4: $135.8 \pm 12.5 \mu\text{m}$, CHI3L1: $170.4 \pm 24.2 \mu\text{m}$), as compared to the same protein marker analysis without cells present ($\mu \pm \sigma$, ICAM1: $118.1 \pm 27.1 \mu\text{m}$, KDR: $160.3 \pm 25.8 \mu\text{m}$, EpH B4: $137.3 \pm 12.1 \mu\text{m}$, CHI3L1: $176.5 \pm 21.9 \mu\text{m}$). The comparison suggests that negligible interference arises from coloaded, cell lysis concurrent with protein marker solubilization, and concurrent sample injection (Figure 4B; unpaired *t*-test and F-test, $p > 0.05$, $n_{\text{with_cell}} = 141$, $n_{\text{no_cell}} = 127$). The goodness of the log-linear fit to the protein markers remained $R^2 > 0.97$ and was not significantly different from conditions in which no cells were loaded into the microwells (Figure 4C; unpaired *t*-test and F-test, $p > 0.05$, $n_{\text{with_cell}} = 141$, $n_{\text{no_cell}} = 127$).

Reciprocally, for microwells with and without microparticles, we analyzed the immunoprobed endogenous proteins to identify any confounding effects. We probed for endogenous proteins expressed at median or higher copy numbers of the mammalian proteome.^[66] GAPDH, β -TUB, CK8, ER- α , and STAT3 have copy numbers above the limit of detection for the single-cell western blot ($\approx 27\,000$ copies of protein immobilized in the gel).^[5,39,46] We analyzed GAPDH, β -TUB, CK8 and found that the protein peak widths were not notably affected by the presence of protein-loaded microparticles in the same microwells ([median_{1 microparticle}, median_{0 microparticle}]; GAPDH [193.4, 192.5 μm], β -TUB [134.3, 136.9 μm], CK8 [151.3, 151.9 μm]; Mann–Whitney test, $p > 0.05$; Figure 4D).

Next, we applied the protein markers to estimate the molecular masses of endogenous proteins from single MCF-7 cells (Figure 4). For microwells containing both individual MCF-7 cells and 1 microparticles, we performed single-cell western blotting and concurrently resolved protein markers and endogenous proteins in all PAGE separation lanes (Figure 4A).

Here, we utilized the log-linear regression equation developed from the protein markers and the measured peak location for each target (for ladders and single-cell western blots

performed within the 2.5 mm mentioned earlier). We analyzed >100 single-cell western blots with protein markers that passed quality control (see the Experimental Section) and a $R^2 > 0.97$ for the log-linear regression. In these cases, the observed molecular masses of GAPDH (39.6 kDa), β -TUB (47.0 kDa), CK8 (54.3 kDa), and ER- α (66 kDa) and the reported molecular masses (determined by conventional slab-gel western blots) agreed with both median and mean mass errors of <10% (Figure 4E,F). Out of all endogenous protein targets, STAT3 had the highest mean (10.3%) and median (12.2%) mass error values (Figure 4F; Table S2, Supporting Information). Conventional slab-gel western blotting reports three protein peaks within the 70–100 kDa range when probing with the STAT3 antibody (Figure S11, Supporting Information), possibly slightly reducing the accuracy of target sizing in the single-cell western blot, which has lower resolving power than the 4 cm long separation lengths utilized for pooled cell lysate analysis in slab gels. Overall, single-cell western blotting incorporating the protein markers resulted in mean and median mass errors <12% for all endogenous protein targets from the MCF-7 cells (Figure 4F). This observed performance is comparable to the mass sizing performance of both slab-gel PAGE and microfluidic sizing chips (Agilent Bioanalyzer), which reports mass errors $\approx 10\%$.^[22,59] Perhaps not surprisingly, we found lower protein target mass errors estimating mass using the microparticle-delivered protein markers as compared to using endogenous proteins from cell lysate as markers (Figures 2B and 4F). For example, the mean and median mass errors for CK8 were 40% lower when determined by the microparticle-delivered protein markers, as compared to using endogenous cellular protein targets (Figure S12, Supporting Information). We anticipate the microparticle protein marker vehicle will find utility in assigning molecular masses to proteoforms, including truncated isoforms with >20% molecular mass differences from the full-length protein (Figure 4F).^[67–69] In the future, the protein marker microparticles may find application in microfluidic isoelectric focusing,^[70,71] co-immunoprecipitation,^[72] and to the delivery of DNA/RNA markers for single-cell comet assays to accurately measure DNA damage and modifications.^[35,42]

3. Conclusion

As single-cell resolution assays emerge and mature, emphasis on accuracy and reproducibility is increasing. This emphasis is relevant for single-cell resolution genomics and transcriptomics, as well as for complementary single-cell protein assays (flow cytometry, immunofluorescence, immunohistochemistry, mass cytometry/CyTOF). For multistep assays, measuring variation from each assay step isolates biological variation from technical variation imparted by the measurement itself. Standardized tools allow robust quality control and enhanced accuracy to ensure meaningful and reproducible biological insights.

To establish a molecular mass standard for single-cell western blotting, we design, characterize, and apply protein markers delivered to microwells in “solid-phase” as a coating on a magnetic microparticle vehicle. Magnetically directed microparticle delivery and chemical triggering of protein marker release allow concurrent analysis of the protein marker with each electrophoretic separation of single-cell lysate. To assess intraassay and interassay variability, we utilize His-tagged ligands, which allows customization of the protein markers to match the single-cell western target needs. Endogenous protein targets are

identifiable with selectivity greater than immunoassays alone, owing to dual measurements of molecular mass and reactivity with an immunoprobe.

Protein markers promise to aid in identification of unknown protein targets from single cells, as well as very importantly aiding in the identification and subsequent measurement of sources of technical variation in single-cell western blotting. Inclusion of protein markers allows a user to understand the variation arising from each stage of their custom assay—from cell lysis to immunoprobings—which allows adjustment of assay conditions to modulate the dominant contributors to variation (e.g., peak location, area-under-the-curve, dispersion).

Design and development of protein markers that are compatible with other single-cell immunoblotting modalities—including immunoprobed isoelectric focusing and immunoblotting of native species—are underway. With careful design to deliver controlled (and known) quantities of standard proteins to each microwell, protein markers have the potential to aid in absolute protein quantitation of targets from each single-cell lysate and possibly even from sub-cellular compartments.^[5,73]

4. Experimental Section

Chemicals/Reagents:

Acrylamide/bis-acrylamide (40% wt/wt) solution (A7802), N,N,N',N'-tetramethylethylenediamine (TEMED, T9281), ammonium persulfate (APS, A3678), sodium deoxycholate (D6750), β -Mercaptoethanol (M3148), imidazole (792 527), and SDS (L3771), were obtained from Sigma-Aldrich. Triton X-100 was purchased from Fisher Scientific (BP-151). 10 $\times$ Tris/glycine buffer was obtained from Bio-Rad (161-0734). Phosphate buffered saline (PBS), pH 7.4 was obtained from Gibco (10 010-023). Tris-HCl, pH 6.8 was purchased from Teknova (T1568). PureProteome nickel magnetic microparticles with 10 μ m diameter were obtained from Millipore Sigma (LSKMAGH02). A 6-tube magnetic separation rack was obtained from New England BioLabs (S1506S). N-[3-[(3-Benzoylphenyl)-formamido] propyl] methacrylamide (BPMAC) was custom synthesized by PharmAgra Laboratories. SU-8 developer (Y020100) and photoresist SU-8 2025 (Y111069) were obtained from MicroChem. Deionized water (ddH₂O, 18.2 m Ω) was obtained using ultrapure water system (Millipore). Unless stated otherwise, chemicals and reagents were obtained from Sigma-Aldrich.

Proteins:

Recombinant protein A His Tag (Protein A, ab52953) and recombinant human EpH receptor B4 protein His Tag (EpH, ab167746) were obtained from Abcam. Protein A and EpH were fused with 6 $\times$ polyhistidine domains on N- and C-terminus, respectively. VEGFR2 (KDR) recombinant human protein, His Tag (KDR, 10012H08H50), ICAM1 recombinant human protein, hIgG1-Fc. His Tag (ICAM1, 10346H03H5) and CHI3L1 recombinant mouse protein His Tag (CHI3L1, 50929M08H50) were purchased from ThermoFisher Scientific. KDR, ICAM1, and CHI3L1 were expressed from a DNA sequence from the extracellular domains fused to a C-terminal polyhistidine tags. Unless stated otherwise, His Tag proteins

were fluorescently labeled using Alexa Fluor 647 NHS ester succinimidyl ester (Life Technologies, A20006). Fluorescently labeled His Tag proteins were purified using dye removal columns (ThermoFisher Scientific, 22 858) according to the manufacturer protocol. Details of the recombinant proteins used for protein markers are listed in Table S2 (Supporting Information).

Primary antibodies to recognize endogenous proteins include rabbit anti- β -tubulin (ab6046, Abcam), goat anti-GAPDH (SAB2500450, Sigma), mouse anti-cytokeratin 8 (C5301, Sigma), rabbit anti-estrogen receptor α (ab16660, Abcam), rabbit anti-STAT3 (79D7, Cell Signaling). For the primary antibody host species, secondary antibodies with Alexa Fluor were purchased from ThermoFisher Scientific: anti-mouse secondary antibody with Alexa Fluor 555 (A31570), anti-rabbit secondary antibody with Alexa Fluor 488 (A21206), anti-goat secondary antibody with Alexa Fluor 555 (A21432).

Protein Loading on Nickel Microparticles:

Magnetic microparticles (5 μ L) were equilibrated with buffer (50 $\times 10^{-3}$ M sodium phosphate, 300 $\times 10^{-3}$ M sodium chloride, 10 $\times 10^{-3}$ M imidazole, pH 8). A protein solution (500 μ L) containing a mixture of His Tag proteins in (30% v/v) ethanol/PBS was loaded with the microparticles and mixed gently for 2 h at 4 $^{\circ}$ C using a rotator. Unbound proteins were washed 3 times using wash buffer (50 $\times 10^{-3}$ M sodium phosphate, 300 $\times 10^{-3}$ M sodium chloride, 20 $\times 10^{-3}$ M imidazole, pH 8). Residual liquid was separated from the microparticle using a magnetic rack and removed after each of the above steps. The protein bound microparticles were resuspended in 1 $\times$ PBS.

In order to determine protein release kinetics, microparticles (5 μ L) were loaded with Protein A (4.1 μ g). For electrophoretic separations, microparticles (5 μ L) were loaded with either one of two protein mixtures containing: 1) Protein A (2 μ g), or 2) CHI3L1 (14.8 μ g), EpH (54.5 μ g), KDR (21.9 μ g), and HlgG1-F (7.4 μ g). The bound His Tag protein was eluted from the microparticles using lysis/electrophoresis buffer prepared with imidazole (0 or 1 M), SDS (2.5 g), sodium deoxycholate (1.25 g), Triton X-100 (500 μ L), Tris/glycine buffer (25 mL, 10 $\times$), and ddH₂O (474.5 mL).

Measurement of Microparticle Loading Efficiency via Flow Cytometry:

The percentage of microparticles loaded with AF647-labeled proteins was determined by flow cytometry. The fluorescence (RL1-A, with PMT gain 180) of microparticles with and without the protein ladder was measured using an Attune NxT acoustic focusing flow cytometer (ThermoFisher Scientific). Microparticles were drawn from a solution (333 333 microparticles mL⁻¹), and a threshold of FSC-A > 25 000 was applied to avoid detection of debris. Results were analyzed using FlowJo analysis software (FlowJo).

Cell Culture:

The MCF-7 breast cancer cell line was purchased from ATCC and maintained in RPMI 1640 (ThermoFisher Scientific) containing fetal bovine serum (10%) and penicillin/streptomycin (1%) in a humidified incubator held at 37 $^{\circ}$ C under 5% CO₂. MCF-7 was tested mycoplasma

negative and authenticated with short tandem repeat analysis (Figure S13, Supporting Information).

Fabrication of Single-Cell Western Blot Chip:

The master mold comprised a silicon wafer with SU-8 features that was fabricated according to standard photolithography procedure.^[46] Single-cell western blot devices containing an array of microwells (250 μm well-to-well spacing and 1 mm long separation lane) with feature heights and diameters of 35 and 30 μm , respectively, were fabricated by casting a polyacrylamide gel against the mold.^[46] The polyacrylamide gel layer was chemically polymerized (7% T, 3.45% C, 3×10^{-3} M BPMAC, 0.08% APS, and 0.08% TEMED).

Single-Cell Western Blot Buffer Exchange:

After settling cells in microwells by gravity, a chemical lysis buffer (0.5 $\times$ Tris glycine, 0.5% SDS, 0.25% sodium deoxycholate, 0.1% Triton X-100, 1 M Imidazole, pH 9.2, 55 $^{\circ}\text{C}$) was poured into the single-cell western blot for 30 s to release protein markers from microparticles and solubilize endogenous proteins from mammalian cells. Then, a 2 s electrophoresis at 40 V cm^{-1} was applied immediately for protein injection. For protein separation, an electrophoretic buffer (0.5 $\times$ Tris glycine, 0.5% SDS, 0.25% sodium deoxycholate, 0.1% Triton X-100, pH 8.7, 22 $^{\circ}\text{C}$) was introduced, followed by immediate electrophoresis at 40 V cm^{-1} for 23 s.

Fluorescence Imaging:

Protein A bound microparticles were prepared and settled into microwells under a magnetic field. Fluorescence protein release from the microparticles in the microwells was visualized using an Olympus IX71 inverted fluorescence microscope equipped with ASI motorized stage, X-cite mercury lamp light source (Lumen Dynamics), and standard Cy5 filter cube (40 $\times$ objective). Time-lapse images were captured using an iXon+ EMCCD camera (Andor Technology Ltd.) controlled by a MetaMorph software (Molecular Devices) with 60 ms exposure time and 2 s time intervals.

Image Analysis and Quality Control:

For the release kinetics measurement, image processing was performed using a Python script (Anaconda Python 3.5.3). Circular region of interest (ROI) was manually selected via a Graphical User Interface provided using the cv2 Python package (OpenCV 3.1.0). Image pixel statistics were estimated using the numpy Python package (numpy 1.13.1). Mean fluorescence intensity from a ROI covering the whole microparticle with AF647-labeled Protein A was obtained using the widefield fluorescence microscope, which includes the out-of-focus fluorescence along the z -axis. Background signal was designated as the mean fluorescence intensity measured from an adjacent empty microwell. Each mean fluorescence intensity of the microwell was background subtracted and then normalized to the value at the start of the release process ($t = 0$).

For the single-cell western blot analysis, images of proteins were collected by scanning the single-cell western blot devices with a fluorescence microarray scanner (Genepix 4300A, Molecular Devices). The images were processed by applying a median filter with a 2-pixel

radius and a threshold value of 50 (ImageJ). Quantitation of proteins from the images was processed by in-house MATLAB (R2016b) scripts.^[46] Protein peaks were fitted with Gaussian functions. For quality control protocol, the protein peaks with Gaussian fitting R^2 0.7 and SNR ≥ 3 were analyzed.^[46] From the curve fitting, protein peak width and location were extracted to determine protein molecular mass from Ferguson analysis.^[21,28]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

J.J.K. and P.P.Y.C. contributed equally to this work. The authors acknowledge members of the Herr lab for helpful discussions. This work was supported by National Science Foundation Graduate Research Fellowship (J.J.K. and J.V.), National Defense Science and Engineering Graduate Fellowship (A.G.), a US Department of Defense Breast Cancer Research Program Award (BC150439 to A.E.H.), a US National Institutes of Health R01 Award (R01CA203018 to A.E.H.), and Cancer Moonshot R33 Award (R33CA225296 to A.E.H.). J.J.K., P.P.Y.C., J.V., and A.G. performed the experiments and data analysis. All authors designed the experiments and wrote the manuscript.

References

- [1]. Amir EAD, Davis KL, Tadmor MD, Simonds EF, Levine JH, Bendall SC, Shenfeld DK, Krishnaswamy S, Nolan GP, Pe'er D, Nat. Biotechnol 2013, 31, 545. [PubMed: 23685480]
- [2]. Powell AA, Talasz AH, Zhang H, Coram MA, Reddy A, Deng G, Telli ML, Advani RH, Carlson RW, Mollick JA, Sheth S, Kurian AW, Ford JM, Stockdale FE, Quake SR, Pease RF, Mindrinos MN, Bhanot G, Dairkee SH, Davis RW, Jeffrey SS, PLoS One 2012, 7, e33788. [PubMed: 22586443]
- [3]. Werner M, Palankar R, Arm L, Hovius R, Vogel H, Small 2015, 77, 2607.
- [4]. Zhang X, Liu R, Shu Q, Yuan Q, Xing G, Gao X, Small 2018, 14, 1703684.
- [5]. Sinkala E, Sollier-Christen E, Renier C, Rosàs-Canyelles E, Che J, Heirich K, Duncombe TA, Vlassakis J, Yamauchi KA, Huang H, Jeffrey SS, Herr AE, Nat. Commun 2017, 8, 14622. [PubMed: 28332571]
- [6]. Streets AM, Zhang X, Cao C, Pang Y, Wu X, Xiong L, Yang L, Fu Y, Zhao L, Tang F, Huang Y, Proc. Natl. Acad. Sci. USA 2014, 111, 7048.
- [7]. Arzalluz-Luque Á, Devailly G, Mantsoki A, Joshi A, Int. J. Biochem. Cell Biol 2017, 90, 161. [PubMed: 28716546]
- [8]. Jiang L, Schlesinger F, Davis CA, Zhang Y, Li R, Salit M, Gingeras TR, Oliver B, Genome Res 2011, 21, 1543.
- [9]. Islam S, Zeisel A, Joost S, La Manno G, Zajac P, Kasper M, Lönnerberg P, Linnarsson S, Nat. Methods 2014, 11, 163.
- [10]. Perfetto SP, Ambrozak D, Nguyen R, Chattopadhyay PK, Roederer M, Nat. Protoc 2012, 7, 2067. [PubMed: 23138348]
- [11]. Schwartz A, Marti GE, Poon R, Gratama JW, Fernández-Repollet E, Cytometry 1998, 33, 106. [PubMed: 9773870]
- [12]. Shi Q, Qin L, Wei W, Geng F, Fan R, Shin YS, Guo D, Hood L, Mischel PS, Heath JR, Shik Shin Y, Guo D, Hood L, Mischel PS, Heath JR, Proc. Natl. Acad. Sci. USA 2012, 109, 419.
- [13]. Sargiacomo M, Scherer PE, Tang Z, Kubler E, Song KS, Sanders MC, Lisanti MP, Proc. Natl. Acad. Sci. USA 1995, 92, 9407. [PubMed: 7568142]
- [14]. Cleasby ME, Reinten TA, Cooney GJ, James DE, Kraegen EW, Mol. Endocrinol 2007, 21, 215.
- [15]. Bass JJ, Wilkinson DJ, Rankin D, Phillips BE, Szewczyk NJ, Smith K, Atherton PJ, Scand. J. Med. Sci. Sports 2017, 27, 4. [PubMed: 27263489]
- [16]. Rath A, Glibowicka M, Nadeau VG, Chen G, Deber CM, Proc. Natl. Acad. Sci. USA 2009, 106, 1760.

- [17]. Shi Y, Mowery RA, Ashley J, Hentz M, Ramirez AJ, Bilgicer B, Slunt-Brown H, Borchelt DR, Shaw BF, Protein Sci 2012, 27, 1197.
- [18]. Hu S, Zhang L, Cook LM, Dovichi NJ, Electrophoresis 2001, 22, 3677. [PubMed: 11699905]
- [19]. Chen X, Kapil MA, Hughes AJ, Herr AE, Anal. Chem 2011, 83, 6573. [PubMed: 21834519]
- [20]. Hughes AJ, Herr AE, Proc. Natl. Acad. Sci. USA 2012, 709, 21450.
- [21]. Herr AE, Singh AK, Anal. Chem 2004, 76, 4727. [PubMed: 15307783]
- [22]. Goetz H, Kuschel M, Wulff T, Sauber C, Miller C, Fisher S, Woodward C, J. Biochem. Biophys. Methods 2004, 60, 281. [PubMed: 15345296]
- [23]. Gomis DB, Junco S, Expósito Y, Gutiérrez D, Electrophoresis 2003, 24, 1391. [PubMed: 12731024]
- [24]. Kyhse-Andersen J, J. Biochem. Biophys. Methods 1984, 70, 203.
- [25]. Uliana CV, Peverari CR, Afonso AS, Cominetti MR, Faria RC, Biosens. Bioelectron 2018, 99, 156. [PubMed: 28755608]
- [26]. Mahmood T, Yang P-C, North Am. J. Med. Sci 2012, 4, 163.
- [27]. Shapiro AL, Viñuela E, Maizel JV, Jr., Biochem. Biophys. Res. Commun 1967, 28, 815. [PubMed: 4861258]
- [28]. Ferguson KA, Metabolism 1964, 13, 985.
- [29]. Burnette WN, Anal. Biochem 1981, 112, 195. [PubMed: 6266278]
- [30]. Bowen B, Steinberg J, Laemmli UK, Weintraub H, Nucleic Acids Res 1980, 8, 1. [PubMed: 6243775]
- [31]. Kang C-C, Ward TM, Bockhorn J, Schiffman C, Huang H, Pegram MD, Herr AE, npj Precis. Oncol 2018, 2, 10. [PubMed: 29872719]
- [32]. Rettig JR, Folch A, Anal. Chem 2005, 77, 5628. [PubMed: 16131075]
- [33]. Revzin A, Sekine K, Sin A, Tompkins RG, Toner M, Lab Chip 2005, 5, 30. [PubMed: 15616737]
- [34]. Di Carlo D, Aghdam N, Lee LP, Anal. Chem 2006, 78, 4925. [PubMed: 16841912]
- [35]. Wood DK, Weingeist DM, Bhatia SN, Engelward BP, Proc. Natl. Acad. Sci. USA 2010, 107, 10008. [PubMed: 20534572]
- [36]. Gierahn TM, Wadsworth MH, Hughes TK, Bryson BD, Butler A, Satija R, Fortune S, Christopher Love J, Shalek AK, Nat. Methods 2017, 74, 395.
- [37]. Yuan J, Sims PA, Sci. Rep 2016, 6, 1. [PubMed: 28442746]
- [38]. Duncombe TA, Kang CC, Maity S, Ward TM, Pegram MD, Murthy N, Herr AE, Adv. Mater 2016, 28, 327. [PubMed: 26567472]
- [39]. Hughes AJ, Spelke DP, Xu Z, Kang C-C, V Schaffer D, Herr AE, Nat. Methods 2014, 11, 749. [PubMed: 24880876]
- [40]. Rosenthal A, Macdonald A, Voldman J, Biomaterials 2007, 28, 3208. [PubMed: 17434582]
- [41]. Doh J, Kim M, Krummel MF, Biomaterials 2010, 37, 3422.
- [42]. Collins AR, Mol. Biotechnol 2004, 26, 249. [PubMed: 15004294]
- [43]. Varadarajan N, Julg B, Yamanaka YJ, Chen H, Ogunniyi AO, McAndrew E, Porter LC, Piechocka-Trocha A, Hill BJ, Douek DC, Pereyra F, Walker BD, Love JC, J. Clin. Invest 2011, 121, 4322. [PubMed: 21965332]
- [44]. Yang L, Wang Z, Deng Y, Li Y, Wei W, Shi Q, Anal. Chem 2016, 88, 11077. [PubMed: 27644430]
- [45]. Kim JJ, Sinkala E, Herr AE, Lab Chip 2017, 17, 855. [PubMed: 28165521]
- [46]. Kang C-C, Yamauchi KA, Vlassakis J, Sinkala E, Duncombe TA, Herr AE, Nat. Protoc 2016, 11, 1508. [PubMed: 27466711]
- [47]. Kang CC, Lin JMG, Xu Z, Kumar S, Herr AE, Anal. Chem 2014, 86, 10429. [PubMed: 25226230]
- [48]. Ohyama K, Ueki Y, Kawakami A, Kishikawa N, Tamai M, Osaki M, Kamihira S, Nakashima K, Kuroda N, Clin. Chem 2011, 57, 905. [PubMed: 21482748]
- [49]. Gerald P, King GL, Circ. Res 2010, 706, 1319.
- [50]. Yin Z, Ren J, Guo W, Biochim. Biophys. Acta,– Mol. Basis Dis 2015, 7852, 47.

- [51]. Stastna M, Van Eyk JE, Proteomics 2012, 72, 2937.
- [52]. McFadden MJ, Junop MS, Brennan JD, Anal. Chem 2010, 82, 9850. [PubMed: 21067198]
- [53]. Carrick BH, Hao L, Smaldino PJ, Engelke DR, Genetics 2016, 6, 573.
- [54]. Ananth A, Genua M, Aissaoui N, Déaz L, Eisele NB, Frey S, Dekker C, Richter RP, Görlich D, Small 2018, 14, 1703357.
- [55]. Porath J, Protein Expression Purif 1992, 3, 263.
- [56]. Khan F, He M, Taussig MJ, Anal. Chem 2006, 78, 3072. [PubMed: 16642995]
- [57]. Evans R, Dal Poggetto G, Nilsson M, Morris GA, Anal. Chem 2018, 90, 3987. [PubMed: 29481057]
- [58]. Young ME, Carroad PA, Bell RL, Biotechnol. Bioeng 1980, 22, 947.
- [59]. Guttman A, Nolan J, Anal. Biochem 1994, 227, 285.
- [60]. Luo S, Feng J, Pang HM, J. Chromatogr. A 2004, 1051, 131. [PubMed: 15532564]
- [61]. Dolnik V, Gurske WA, Electrophoresis 2011, 32, 2884. [PubMed: 21948216]
- [62]. Matheron G, Econ. Geol 1963, 58, 1246.
- [63]. Cressie N, J. Int. Assoc. Math. Geol 1985, 17, 563.
- [64]. Becker G, Deng Z, Zober M, Wagner M, Lienkamp K, Wurm FR, Polym. Chem 2018, 9, 315.
- [65]. Vlassakis J, Herr AE, Anal. Chem 2017, 89, 12787. [PubMed: 29110464]
- [66]. Li JJ, Bickel PJ, Biggin MD, PeerJ 2014, 2, e270. [PubMed: 24688849]
- [67]. Martegani MP, Del Prete F, Gasbarri A, Natali PG, Bartolazzi A, Am. J. Pathol 1999, 154, 291. [PubMed: 9916943]
- [68]. Figtree GA, McDonald D, Watkins H, Channon KM, Circulation 2003, 707, 120.
- [69]. Inoue K, Fry EA, Genet. Epigenet 2015, 7, GEG.S35500.
- [70]. Tentori AM, Yamauchi KA, Herr AE, Angew. Chem., Int. Ed 2016, 55, 12431.
- [71]. Yamauchi KA, Tentori AM, Herr AE, Electrophoresis 2018, 39, 1040. [PubMed: 29385243]
- [72]. Hillion J, Canals M, Torvinen M, Casado V, Scott R, Terasmaa A, Hansson A, Watson S, Olah ME, Mallol J, Canela EI, Zoli M, Agnati LF, Ibanez CF, Lluís C, Franco R, Ferre S, Fuxe K, J. Biol. Chem 2002, 277, 18091. [PubMed: 11872740]
- [73]. Yamauchi KA, Herr AE, Microsyst. Nanoeng 2017, 3, 16079. [PubMed: 29333327]

Figure 1.

Microparticles deliver protein markers for mass sizing in single-cell western blotting. A) Schematic of the single-cell western blot workflow that incorporates microparticles to deliver protein markers (blue peaks) for molecular mass determination of endogenous proteins from mammalian cells (orange peaks). The single-cell western blot comprises five stages: 1) Cell settling into microwells by gravity ($F_{g,z}$), 2) Microparticle settling into microwells by an applied magnetic field (z -axis) (B_z), 3) In-microwell protein marker release (imidazole) and chemical cell lysis, 4) Concurrent polyacrylamide gel electrophoresis and photo-activated immobilization (photoblotting) of protein markers and single-cell lysate, and 5) In-gel immunoprobing. B) Microwell occupancy of MCF-7 cells and protein-coated microparticles. False-color micrographs of microwells housing a cell (red, nucleus) and/or microparticles (blue). Scale bar is 15 μm . Distributions of microwell occupancy for cells and microparticles. Error bars are standard deviations ($n = 3$ chips). C) Schematic of His-tagged protein marker delivery and release from Ni^{2+} -coated microparticles. D) Time-lapse fluorescence signal during 1 M imidazole-triggered release of a fluorescent protein marker from a single protein-loaded microparticle in a microwell ($n = 3$ microwells, error bars are standard deviation). Colored dashed lines represent exponential

curve fitting. The marker release half-life for each condition is listed. Black: $\gamma = 1.04 \times \exp(-0.16t) + 0.11$, $R^2 = 0.91$. Red: $\gamma = 1.18 \times \exp(-0.04t) + 0.08$, $R^2 = 0.90$; Blue: $\gamma = 1.03 \times \exp(-0.01t) - 0.09$, $R^2 = 0.87$. E) False-color micrograph and intensity profile of four standard proteins (ICAM1, 100 kDa; KDR, 85 kDa; EpH B4, 58 kDa; CHI3L1, 42 kDa) after electrophoresis separation and photoblotting in the polyacrylamide gel. The 30 μm diameter microwell is at the left of the micrograph.

Figure 2.

Ferguson analyses of protein markers support their utility as an estimator of molecular mass for endogenous protein targets. A) Log-linear regression fitting of protein markers (ICAM1, KDR, EpH B4, CHI3L1) in 100 representative single-cell PAGE protein separations. R^2 value for each linear fit is shown in lower left of each plot. Black circles, protein markers; red lines, linear regression fits. B) Box and scatter plots of percent mass errors between expected and estimated (from the log-linear regression fits) molecular masses of protein markers in a single-cell western blot chip. Each black circle represents percent mass error

for each PAGE separation lane. Box extents indicate 25th and 75th quantiles; black line at box midpoint indicates median value; whiskers extend to minimum and maximum values. Kruskal-Wallis test with Dunn's multiple comparisons test, *** $p < 0.0001$, $n = 168$ PAGE separation lanes. C) Scatter plots indicate chip-to-chip R^2 values for linear regression fits. Black circles indicate R^2 values for PAGE separation lanes; red line indicates mean value for each chip ($\mu_1 = 0.991$, $\mu_2 = 0.994$, $\mu_3 = 0.993$); $n_1 = 327$, $n_2 = 204$, $n_3 = 516$; one-way ANOVA test, $p < 0.001$, Cohen's $d < 0.2$.

Figure 3.

Imidazole interferes with protein blotting efficiency thus necessitating buffer exchange prior to UV exposure. A) False-color micrographs of single-cell protein PAGE with 1 M imidazole present during photoblotting (left) and with buffer exchange to dilute imidazole prior to photoblotting (right). Each intensity plot represents the protein markers along one PAGE separation lane. B) Scatter plots of signal-to-noise ratio (SNR) of protein marker peaks with and without buffer exchange ($n_{\text{with_exchange}} = 249$, $n_{\text{no_exchange}} = 149$). C) Scatter plot indicating R^2 values with and without buffer exchange ($n_{\text{with_exchange}} = 107$, $n_{\text{no_exchange}} = 27$). D) Scatter plots of separation resolution between two protein markers with or without

buffer exchange ($n_{\text{with_exchange}} = 107$, $n_{\text{no_exchange}} = 27$). E) Scatter plot of KDR peak SNR as a function of the number of microparticles in the microwell ($n = 4$ microwells, error bar is standard deviation). Each square represents a mean SNR value of KDR with buffer exchange. Each circle represents a mean SNR value of KDR without buffer exchange. With buffer exchange, one microparticle per microwell is sufficient to detect all protein markers with SNR ≥ 3 . F) Bar graph representing fraction of protein markers that passed quality control (SNR ≥ 3 , R^2 for Gaussian curve fitting ≥ 0.7). Error bars represent standard deviations, $n = 3$ chips with exchange and without exchange. Black circles represent data per PAGE separation lane; red lines represent mean values; unpaired t test, *** $p < 0.0001$, ** $p < 0.002$, ns = no significance ($p > 0.05$).

Figure 4.

Protein markers estimate protein target molecular mass in single-cell western blotting. A) Top: false-color micrographs display microwells containing an MCF-7 cell (red) and microparticles (blue). Scale bar, 10 μm . Bottom: intensity profiles and false-color micrographs of four representative PAGE lanes containing both endogenous proteins (STAT3, 80 kDa; ER- α , 66 kDa; CK8, 54 kDa; β -TUB, 50 kDa; GAPDH, 37 kDa) and protein markers (blue). B) Scatter plots indicate no significant difference in peak widths of protein markers with and without cells in microwells. Black circles represent peak width of each protein marker; red lines represent mean values; unpaired t -test and F-test, ns = no significance ($p > 0.05$); $n_{\text{with_cell}} = 141$, $n_{\text{no_cell}} = 127$. C) Scatter plot indicates no significant difference in R^2 values from linear regression with and without a cell in the microwell. Black circles indicate R^2 values per separation lane; red lines represent mean values; unpaired t -test and F-test, ns = no significance ($p > 0.05$); $n_{\text{with_cell}} = 141$, $n_{\text{no_cell}} = 127$. D) Scatter plot representing no significance difference in GAPDH ($n_1 = 55$, $n_0 = 17$), β -TUB ($n_1 = 77$, $n_0 = 28$), and CK8 ($n_1 = 97$, $n_0 = 14$) peak widths in microwells with or without microparticles. Black circles indicate endogenous protein peak width per separation

lane; red lines represent mean values; Mann–Whitney test, ns = no significance ($p > 0.05$). E) Representative log-linear regression plots display estimated (*) and expected (x) molecular masses of endogenous proteins extracted from linear regression of the protein marker. R^2 value for each log-linear fit is in the lower left of each plot. Black circles, protein marker; red lines, log-linear regression fitting. F) Box plots depict % error between estimated and expected molecular masses of STAT3 ($n = 95$ cells), ER- α ($n = 7$ cells), CK8 ($n = 794$ cells), β -TUB ($n = 551$ cells), and GAPDH ($n = 430$ cells) from the Ferguson analysis plots. Box extents indicate 25th and 75th quantiles; black line at box midpoint indicates median value; whiskers extend to minimum and maximum values.