3D projection electrophoresis for single-cell immunoblotting

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Immunassays and mass spectrometry are powerful single-cell protein analysis tools; however, interfacing and throughput bottlenecks remain. Here, we introduce three-dimensional single-cell immunoblots to detect both cytosolic and nuclear proteins. The 3D microfluidic device is a photoactive polyacrylamide gel with a microwell array-patterned face (xy) for cell isolation and lysis. Single-cell lysate in each microwell is “electrophoretically projected” into the 3rd dimension (z-axis), separated by size, and photo-captured in the gel for immunoprobing and confocal/light-sheet imaging. Design and analysis are informed by the physics of 3D diffusion. Electrophoresis throughput is > 2.5 cells/s (70× faster than published serial sampling), with 25 immunoblots/mm² device area (>10× increase over previous immunoblots). The 3D microdevice design synchronizes analyses of hundreds of cells, compared to status quo serial analyses that impart hours-long delay between the first and last cells. Here, we introduce projection electrophoresis to augment the heavily genomic and transcriptomic single-cell atlases with protein-level profiling.
Proteins are biomolecules that play a direct role in nearly all cellular processes. As such, protein expression is a primary metric for quantifying cell state. Although genomics and transcriptomics analysis of gene and RNA expression are powerful and complementary measurement techniques, they often do not predict protein expression at the single-cell level. Proteoforms, or different forms of proteins arising from the same gene, are also critical to understanding cell state: cellular heterogeneity at the proteoform level plays critical roles in cellular processes including regulating tumor growth and resistance to treatment in cancer. Many single-cell proteomic technologies are limited to protein detection by antibodies with limited protein specificity, which may not be proteoform-specific and thus cannot distinguish proteoform-level heterogeneity. Protein separations based on electrophoresis can overcome these antibody specificity limitations through dual measurements of the physicochemical properties of protein targets (i.e., molecular mass, isoelectric point) and primary antibody reactivity for protein detection.

To offer a comprehensive understanding of complex cellular systems, selective detection of protein targets in individual cells is required; yet, key single-cell analysis challenges persist in throughput, sample preparation, and interfacing. Measurement throughput is crucial to detect rare but important subpopulations of cells (e.g., metastasis, resistance to treatment), but—to attain suitable statistical power—the minimum sample size increases as subpopulation prevalence decreases. Thus, the ability to assay hundreds or even thousands of cells is valuable. Furthermore, the challenge of measuring a large cell population with single-cell resolution is synchronously assaying all of the single cells. Proteomic cell state is dynamic, so differences in analysis time across cells can add artefactual heterogeneity—particularly when assessing cell response to drugs or environmental stimuli. Furthermore, single-cell lysate preparation and interfacing for interrogation must also preserve detectable protein concentrations from low single-cell copy numbers. Dilution into larger-than-a-cell volumes, sample losses, and lysate changes during transfer to an analysis platform can further challenge single-cell proteoform measurement.

Since the early 1990s, capillary electrophoresis (CE) has been applied to surmount challenges in the analysis of lysate from individual cells. To maintain protein target concentrations during analysis, CE uses fused silica capillaries with micron-scale diameters, facilitating efficient dissipation of Joule heating and ultra-rapid electrophoresis. For lysate transfer, the CE systems use the physical alignment of the capillary to reagent baths or isolated single cells. Early single-cell CE technologies were capable of assaying 10s of cells per day, making measurement of single-cell heterogeneity among large cell populations out of reach. CE improvements use capillary arrays for simultaneous analyses of samples and bundled 168-plex microstructured silica fibers (demonstrated to improve heat transfer rather than throughput). Analyte loss via adsorption to the capillary surface (due to large surface-area-to-volume ratios) challenges single-cell CE measurements.

To advance single-cell resolution analyses, researchers brought both automation and microfluidic design to bear to efficiently integrate disparate sample preparation and electrophoretic analysis. In an excellent example, the Allbritton group introduced an automated, serial single-cell electrophoresis system by integrating a single glass capillary with a microwell array for cell isolation, using a motorized stage for microwell alignment to the capillary for serial sampling and analysis. For a population of 219 mammalian cells, throughput was clocked at 2.1 cells/min (cell lysis, electrophoresis, and real-time fluorescence detection of sphingosine fluorescent and sphingosine-1-phosphate fluorescent). Also exemplar of integrated approaches, planar microfluidic devices support analysis of up to 12 cells/min. While useful, serial analysis of large cell populations is inherently asynchronous and, as mentioned, can introduce artefactual cell-to-cell heterogeneity, particularly critical to assessing response to stimuli.

Mass spectrometry is a powerful, complementary separations technology that is promising for single-cell protein detection. Aside from mass cytometry, other forms of mass spectrometry do not rely on antibody specificity for selective protein detection. Highly multiplexed protein detection from single cells (1000s of targets per cell) is possible with bottom-up mass spectrometry, although throughput has not yet exceeded 100 individual cells. Mass spectrometry is limited to highly abundant proteins (>10^4 copies per cell) and small-to-intermediate molecular masses for top-down proteomics (e.g., typically 25 kDa for MALDI-TOF, although specialized detectors facilitate detection of proteins up to 110 kDa). Furthermore, the vast majority of single-cell mass spectrometry approaches are bottom-up, wherein the requirement for protein digestion to peptides confounds proteoform stoichiometry. While top-down mass spectrometry does measure intact proteins—making the approaches relevant to proteoforms—and imaging mass spectrometry can assay larger numbers of cells (e.g., >1000 dorsal root ganglia), larger proteins present a measurement challenge even with optimization for wider-range mass measurement (m/z 400 to 20,000).

Immunoblotting is a class of separations with a powerful capacity for targeted proteomics. To detect protein targets a priori identified through hypotheses or discovery tools, this suite of separations approaches integrate two analytical modalities to yield enhanced target specificity over either alone: separation of proteins by electrophoresis and probing of specific targets by immunoreagents. While only recently developed for single-cell and sub-cellular resolution by our group, immunoblotting (and its most popular form, western blotting) has been a workhorse in biological and clinical laboratories for decades. Using automation in a different way to advance towards both single-cell resolution and multiplexed detection, the Kennedy group designed an automated system that integrates microchip electrophoresis with immunoprobing on an off-chip PVDF membrane. Integration between the microchip and membrane uses a mobile membrane, with the separations effluent deposited (blotted) onto that moving membrane. While not demonstrated for single-cell analysis, multiplexing was boosted to 11 separated protein targets from 9 serial separations in 8 min, with each separation from the same aliquot of bulk cell lysate with a total protein content similar to that of a single cell (400 ng total protein). Taking a different approach inspired by single-cell DNA electrophoresis (COMET assays), we introduced single-cell western blotting with a throughput of ~200 cells/min. The planar 2D device uses a thin layer of polyacrylamide gel stipped with microwells for parallel cell isolation and lysis. Protein lysate is subjected to immunoblotting in the photoactive polyacrylamide gel abutting each microwell. We have applied the single-cell western blotting technology to studying heterogeneity of circulating tumor cells, smooth muscle cells, and HER2 isofrom expression in clinical specimens. Furthermore, using similar design principles we have introduced single-cell immunoblotting tools based upon other physicochemical protein properties (e.g., subcellular localization and isoelectric point) and adapted the single-cell western blot to assess adherent cells (without detachment) and study invasive motility. These tools have emerged as powerful technologies for single-cell analysis; however, their throughput and sample consumption remain limited by the large spacing between microwells required for the protein separation axis.

Here, we introduce a high-density parallelized single-cell immunoblotting device that uses the third dimension (z-axis), to enhance the microwell array density and, hence, throughput.
and sample consumption. In projection electrophoresis, we sought to leverage the full gel volume to map protein size and originating cell position from the 3D location of separated protein bands. The 3D design is inspired by multifunctional 3D hydrogel materials like Expansion Microscopy\(^{38}\) and CLARITY\(^{39}\), as well as the efficient electrotransfer of protein from gel to PVDF membrane in conventional western blotting\(^{27,31}\) and bulk separations in 3D and layered systems\(^{42-44}\). Using a 3D volume improves microwell array density to 25 microwells/mm\(^2\) from 2 microwells/mm\(^2\) in 2D (planar) devices. Owing to this density improvement, the assay consumes an order of magnitude less volume of a cell suspension (25 μL vs. 300 μL) to assay ~300 cells (similar to planar systems), resulting in a 10-fold improvement in sample consumption. In this work, we describe the separation performance and use the physics of 3D diffusion to design the projection electrophoresis system, and perform near-simultaneous immunoblotting of both cytoplasmic (GAPDH, actin, β-tubulin) and nuclear (PTBP1) protein targets from hundreds of single mammalian breast and brain tumor cells.

**Results**

**Establishing projection electrophoresis as an analytical tool.** In lieu of serial interrogation and electrophoretic analysis of individual mammalian cells, projection electrophoresis (Fig. 1a) yields synchronous, concurrent analyses of hundreds of single cells. Compared to serial-cell measurements, the parallel approach reduces assay-induced protein expression heterogeneity (Fig. 1b). After in-gel immunoprobing for the protein targets glyceraldehyde 3-phosphate dehydrogenase (GAPDH, involved in glycolysis, transcription, and apoptosis) and poly pyrimidine tract-binding protein 1 (PTBP1, RNA-binding protein involved in cellular processes including splicing), projection electrophoresis yields 3D data with \(xy\) position describing the originating microwell, \(z\)-position describing protein size, Fig. 1c–g). Mean- \(z\)-direction migration distances were 199 ± 9 μm for PTBP1 (57 kDa) and 346 ± 7 μm for GAPDH (37 kDa). Furthermore, the multifunctional, micropatterned polyacrylamide gel (PAG) serves as the cell isolation device, lysis vessel, separation matrix, and protein capture scaffold. This multifunctional gel thus facilitates in situ lysis, electrophoresis, and blotting, mitigating losses incurred in sample transfer steps, while synchronous analysis and fast assay times yield high assay throughput and drastically reduced sampling delays between cells in a population.

We first sought to verify the separation mechanisms governing protein electrophoresis in the concurrent analyses (Fig. 2a, b). To understand the protein separation mechanism, we assessed electromigration of a ladder of well-characterized protein standards (donkey immunoglobulin anti-mouse IgG, IgG: 150 kDa; bovine serum albumin, BSA: 66.5 kDa; ovalbumin, OVA: 42.7 kDa, each labeled with AlexaFluor® dyes). When the purified protein solution is pipetted on top of the gel block (gel block face stippled with microwells), the protein solution preferentially partitions into the microwells (versus the hydrogel), thus providing a convenient, well-controlled means for sample loading into each microwell sample injector. To minimize 3D diffusional spreading during PAGE, we designed an ultra-short separation axis (1 mm) and rapid (<1 min) protein PAGE duration. Upon completion of PAGE, the multifunctional gel was toggled from the separation matrix to a protein capture scaffold using a 45-second exposure to UV illumination\(^{45}\).

We observed (Fig. 2c) a log-linear relationship of electromigration with molecular mass, as expected in size-sieving gel electrophoresis\(^{46}\). Further, in this unique format, we observed constant-velocity and size-dependent electromigration for the ladder and additional protein assayed (Fig. 2d); electrophoretic mobilities of OVA: 3.3–3.7 × 10\(^{-5}\) cm\(^2\)/Vs; BSA: 2.5–2.9 × 10\(^{-5}\) cm\(^2\)/Vs; transferrin: 1.3–1.7 × 10\(^{-5}\) cm\(^2\)/Vs, lectin: 0.96–1.14 × 10\(^{-5}\) cm\(^2\)/Vs; IgG: 7.4–8.0 × 10\(^{-6}\) cm\(^2\)/Vs). Constant-velocity migration required mitigation of deleterious effects of electrolysis (i.e., buffer pH changes, bubble formation at the electrodes), which increased the \(R^2\) of linear fits to the protein migration data from 0.87 ± 0.06 to 0.97 ± 0.03 for the three ladder protein species (Supplementary Figure 1). For BSA and OVA ladder species, both a protein monomer and dimer are resolvable, as expected in high-performance protein PAGE\(^{47-49}\). Having established the separation mechanism, we next estimated the PAGE performance by assessing the separation resolution (\(R_z\)). For two ladder proteins (OVA, BSA) in a 1 mm-thick 10%T PAGE volume (Fig. 2e), the \(R_z\) reached 1.0 within 20 s of PAGE, yielding fully resolved species.

Based on the dominant separation mechanism and rapid protein separation, analysis of the purified protein ladder solution suggests that projection electrophoresis is suitable for analytical-quality protein analysis. The high performance of the rapid microfluidic protein analysis described here is in contrast to another 3D system, designed for preparatory \(z\)-direction separations and performance, as previously demonstrated for bulk samples using a multilayered gel to coarsely fractionate small proteins (14–77 kDa) from large proteins (20–343 kDa)\(^{44}\). In terms of throughput, each purified protein projection electrophoresis gel (100 μm microwell pitch) contains >4000 microwells, facilitating >4000 parallel (replicate) purified protein separations for a total active assay throughput of 44 separations per second (not including readout time). For comparison, capillary array electrophoresis of a single sample yields a throughput of 5 separations per second\(^{15}\).

**Device design is informed by 3D diffusion of target proteins.** Given the open microfluidic design of the projection electrophoresis device that uses a microwell array to perform sample isolation and preparation, with an abutting gel volume that performs the analytical functions (protein PAGE, immunoblotting), we sought to understand physics-based factors that set the minimum acceptable microwell-to-microwell spacing (microwell pitch, \(\Delta_{\text{well}}\)). The \(\Delta_{\text{well}}\) in turn, sets the maximum number of concurrent protein PAGE separations per projection electrophoresis device. As illustrated schematically in Fig. 3a, the \(\Delta_{\text{well}}\) spacing is influenced by the length scale of diffusional band broadening (\(\sigma_{\text{xy}}\), in the \(xy\) plane) during protein PAGE along the \(z\)-axis. As design guidelines, the throughput of each single-cell projection electrophoresis device (number of single cells assayed per device) will be a function of \(\Delta_{\text{well}}\) (sets separation lane density) and overall usable device dimensions (Fig. 3b). As \(\Delta_{\text{well}}\) depends linearly on \(\sigma_{\text{xy}}\), the maximum lane density is inversely proportional to \(\sigma_{\text{xy}}^2\), as computed in Fig. 3c. Two design rules are plotted: at \(\Delta_{\text{well}} > 4\sigma_{\text{xy}}\), we would estimate <5% protein overlap between neighboring lanes, while at the more conservative \(\Delta_{\text{well}} > 6\sigma_{\text{xy}}\) we would estimate <0.3% protein overlap assuming Gaussian protein distributions.

During electromigration, protein peaks will diffuse in three dimensions, with diffusion along the \(z\)-axis determining separation resolution (\(R_z\)) and diffusion in \(xy\) defining the minimum \(\Delta_{\text{well}}\). Diffusional spreading of protein bands in all three dimensions depends on protein molecular mass, temperature, time, and gel density (pore size)\(^{30,31}\). To assess the impact of protein diffusion on setting \(\Delta_{\text{well}}\), we assessed the well-characterized fluorescently labeled OVA/BSA/IgG protein ladder during protein PAGE in a 7%T gel projection electrophoresis device. For each time point analyzed by confocal imaging, we determined the \(z\) position of the maximum of the summed fluorescence intensity, for an \(xy\) region of interest surrounding
**Fig. 1** Projection electrophoresis simultaneously lyases and separates both nuclear and cytosolic proteins from hundreds of single cells. (a) Projection electrophoresis device photograph and workflow schematic. A 9 × 9 mm projection electrophoresis PAG contains >1000 microwells that each serves as a separation lane. Cells in PAG microwells are lysed in situ using a lysis buffer-soaked hydrogel delivery matrix, and the lysate is then electrophoretically injected into the polyacrylamide and proteins are separated by size through the depth of the gel. After separation, proteins are covalently linked to the gel matrix using a UV-initiated capture. All cells are lysed and analyzed simultaneously, the active assay time (from cell lysis to photocapture) is less than 90 s, and in situ analysis reduces potential losses from transfer steps. (b) In contrast, capillary electrophoresis analyzes cells in series, with each cell lysed and proteins separated at a different time. (c–f) Different visualizations of z-directional protein separation in a single separation lane. (c) xz (y-summed) contour plots of background-subtracted protein signal within the separation lane for nuclear and cytosolic example proteins. Protein signal is visualized as peak height and false-colored. (d) z-directional intensity profiles (summed fluorescence intensity in arbitrary fluorescence units, AFU, vs. z) for PTBP1 (nuclear, blue) and GAPDH (cytosolic, green) in the same lane, revolved around the z-axis to generate a 3D rendering of fluorescence distribution. (e, f) Representative confocal xy slice images (representative of 9 confocal stacks acquired of different regions of 2 independent separation gels) for separated PTBP1 (blue) and GAPDH (green) at two z-depths into the gel ((e) at the 185 µm depth of the PTBP1 band; (f) at the 335 µm depth of the GAPDH band). Each slice image shows 4 separation lanes, 3 of which appear to have been occupied by BT474 cells prior to analysis. Overlaid squares depict xy regions of interest for the lane plotted in (c) and (d), over which fluorescence intensities were summed to yield z-directional intensity profiles. Scale bar represents 50 μm. (g) Quantified z-migration distances for PTBP1 (57 kDa) and GAPDH (37 kDa) from n = 15 (PTBP1) and n = 17 (GAPDH) separation lanes. Source data are provided as a Source Data file.
each microwell injector. At this z position, we assessed xy resolution by Gaussian fitting x and y intensity profiles and extracting the mean fitted peak width \( \sigma_{xy} \). For duplicate gels of 5 electrophoresis times, we plotted the squared peak width vs. time in gel, fitting to the expected diffusional peak spreading \( \sigma_{xy}^2 \):

\[
\sigma_{xy}^2 = \sigma_{xy,0}^2 + 2Dt_{\text{diff}}.
\]

For each protein target, \( \sigma_0 \) is related to the injected peak width (dictated by microwell diameter), \( D \) is the in-gel diffusion coefficient, and \( t \) is the total elapsed time since protein injection. Figure 3a shows an example confocal fluorescence data xy slice image for OVA and associated \( \Delta \text{well} \) design rule. Applying the analysis to the full protein ladder (Fig. 3d), yields estimates of \( \Delta \text{well} \text{b} \) across a range of protein targets and diffusion coefficients (\( D_{\text{OVA}} \approx 16 \mu m^2/s \), \( D_{\text{BSA}} \approx 7 \mu m^2/s \), \( D_{\text{IgG}} \approx 2.7 \mu m^2/s \) calculated from linear fits to the plot of \( \sigma_{xy}^2 \) vs. diffusion time. Under the described conditions, the protein target with the largest \( D \) (OVA) suggests that a \( \Delta \text{well} \) of 200 \( \mu m \) will satisfy the trade-off of maximizing separation lane density while minimizing separation lane overlap (7%T gels, 10 s protein PAGE). For comparison, top-down MALDI imaging mass spectrometry utilizes a protein spot pitch of 20–200 \( \mu m^2 \).

To further understand the diffusion-driven interdependency of the microwell spacing and separation performance, for the highest diffusivity ladder protein (OVA) we modeled the maximum assay time (the time at which protein signal is expected to bleed into the neighboring lane, from the diffusional peak spreading function above) for a range of microwell spacings (Fig. 3e), as well as the \( R_x \) as a function of electrophoresis time and electric field strength (Fig. 3f). The separation resolution \( R_x \) is
Fig. 3 The physics of 3D diffusion dictate projection electrophoresis device design and inform image analyses. (a) Lane density (limited by minimum spacing between separation lanes \( \Delta_{\text{well}} \)) is dependent on separated xy bandwidth \( \sigma_{xy} \) to avoid microwell-microwell crosstalk. \( \sigma_{xy} \) in turn depends on 3D diffusion of the injected protein. Simulated data shown in the left schematic; measured OVA data shown in micrograph and intensity profile (representative of two independent separation gels). Scale bar represents 100 \( \mu \)m. (b) Throughput is a function of both lane density and usable gel area; separated protein bands parallel to the gel edges in a cross-sectional image of the gel show uniform migration across the gel. Scale bar represents 1 mm. (c) Theoretical maximum lane density at a spacing of 4\( \sigma_{xy} \) (~5% overlap between lanes) and 6\( \sigma_{xy} \) (~0.3% overlap between lanes). Maximum lane density is inversely proportional to \( \sigma_{xy}^2 \). (d) Measured diffusional \( xy \) band broadening (Gaussian fit peak width \( \sigma_{xy} \)) from purified proteins initially partitioned into 32 \( \mu \)m microwells, as a function of in-gel diffusion time \( t_{\text{diff}} \). Left: \( \sigma_{xy} \) vs. \( t_{\text{diff}} \). Right: \( \sigma_{xy}^2 \) vs. \( t_{\text{diff}} \) with linear fits \( \sigma_{xy}^2 = \sigma_{xy,0}^2 + 2D_{\text{diff}}t \). After 10 s EP, we measure \( \sigma_{xy} \) < 30 \( \mu \)m for all proteins, suggesting that 200 \( \mu \)m microwell spacing is sufficient. Linear fitting yields OVA \( \sigma_{xy}^2 = 32.5t_{\text{diff}}-198 \) (\( R^2 = 0.75 \)); BSA \( \sigma_{xy}^2 = 14.3t_{\text{diff}} + 29.8 \) (\( R^2 = 0.88 \)); IgG \( \sigma_{xy}^2 = 5.41t_{\text{diff}} + 153 \) (\( R^2 = 0.42 \)). (e) Modeled time for ovalbumin bands to diffuse into the neighboring lane, as a function of microwell spacing \( \Delta_{\text{well}} \). Modeled separation resolution for BSA and OVA, as a function of EP time and electric field strength. (g) Modeled Péclet number (defined as the ratio of the time to reach a BSA-OVA separation resolution of 1 to the time at which the OVA band is expected to diffuse into the neighboring separation lane). (h) Physics-driven postprocessing. For each confocal slice (BSA, 7%T gel), the original image, that after physics-driven postprocessing (deconvolution of a point spread function modeling 3D diffusion), and summed pre- and post-deconvolution intensity profiles for a 100 \( \mu \)m region surrounding each row of protein bands are shown. Each image pair is scaled to the maximum of the (higher-intensity) deconvolved image. Scale bar represents 50 \( \mu \)m. Source data are provided as a Source Data file.

modeled as\(^{52}\):

\[
R_t = \frac{\Delta z}{\frac{1}{2}(4\sigma_1 + 4\sigma_2)} = \frac{\Delta \mu_{\text{EP}}Et}{\frac{1}{2}(4\sqrt{\sigma_0^2 + 2D_1t} + 4\sqrt{\sigma_0^2 + 2D_2t})},
\]

where \( \Delta z \) is the difference in migration distance between the two protein targets, \( \sigma_1 \) and \( \sigma_2 \) are the \( z \)-direction peak widths, \( \Delta \mu_{\text{EP}} \) is the difference in electrophoretic mobility between the two targets, \( E \) is the electric field, and \( D_1 \) and \( D_2 \) are the diffusion coefficients for the two targets. The diffusion coefficients for BSA and OVA (\( D_{\text{BSA}} = 13.1 \mu \text{m}^2\text{s}^{-1} \), \( D_{\text{OVA}} = 16.8 \mu \text{m}^2\text{s}^{-1} \)) were estimated at 25°C from the Stokes-Einstein equation\(^{48} \) and adjusted for in-gel diffusion\(^{51} \) (7%T gel) as described in the Methods. The electrophoretic mobilities were empirically determined from the results of Fig. 2d (\( \mu_{\text{BSA}} = 2500 \mu \text{m}^2\text{V}^{-1}\text{s}^{-1} \), \( \mu_{\text{OVA}} = 3300 \mu \text{m}^2\text{V}^{-1}\text{s}^{-1} \)). We next defined a Péclet number as the ratio of the maximum assay
time to the time required to reach $R = 1$, with results presented in Fig. 3g and the Péclet number given by:

$$N\text{P}_c = \frac{t_{\text{band,overlap}}}{t_{R,1}} = \frac{t_{\text{band}}}{t_{R,1}} = \frac{6\sigma_{xy}}{\nu t_{R,1}}.$$

At $\Delta_{\text{well}} = 200 \, \mu m$ (Fig. 3d), $N\text{P}_c \sim 1$ for an applied electric field strength of 60 V/cm. As protein band diffusion measurements (Fig. 3d) suggest measured diffusion coefficients smaller than predicted values, our Péclet analysis is a conservative estimate of the trade-off between separation performance and achievable microwell density. Separation resolution is proportional to electric field $E$, thus, increasing $E$ increases the Péclet number. $E$ is equal to the voltage drop divided by the distance over which the voltage is dissipated. If the distance between electrodes is increased (e.g., by placing separation lanes in series between the electrodes, as configured for 2D immunoblotting$^{30}$), higher driving voltages are required to reach the same $E$ which can exceed power supply voltage limits. While we demonstrate electric fields of 43-68 V/cm in this work, future research regarding even higher $E$ operating regimes (e.g., 100 V/cm using 30 V across 0.3 cm) would shed light on limitations arising from regime-relevant physics and chemistry (i.e., Joule heating, electrolysis). Joule heating and electrolysis both increase with electric current. Joule heating reduces separation resolution and introduces nonuniformities (via nonuniform heat dissipation)$^{32}$. Electrolysis generates acid and base ions and creates bubbles that can modulate the electrophoretic mobility and disrupt the electric field$^{33}$ (e.g., pre-optimization projection electrophoresis system depicted in Supplementary Figure 1).

Building on an understanding of the dominant physics, namely diffusion, we next sought to investigate computational approaches to recover starting concentration distributions (here, the microwell array) from endpoint confocal fluorescence images of the protein PAGE (Fig. 3h). Recovery of starting protein concentration distributions will both enhance separation lane density, and distributions in the 3D gel volume, all based on the endpoint fluorescence readout of protein PAGE.

Sample preparation design for single-cell projection electrophoresis. Having considered the design of the projection electrophoresis device and assay using a well-characterized protein ladder, we next sought to identify factors important to high-performance protein PAGE of single cells (Fig. 4). We first assessed the setting of single BT474 cells in 25 μm diameter microwells within the projection electrophoresis gels. Figure 4a depicts settled Calcein-stained BT474 breast tumor cells in microwells after gravitational settling and convective wash-off of cells settled outside of microwells. A corresponding full-gel-widefield microscopy image of immunoprobed GAPDH fluorescence after the projection electrophoresis assay is also shown; labeled cells bands correlate with settled cell positions. Cell settling efficiencies (populated microwells) were at 43 ± 8% with the number of settled single cells $356 \pm 86$ per 9 × 9 mm projection electrophoresis device ($n = 5$ devices). The fraction of microwells occupied by more than one cell was 10 ± 3%. Further optimization of cell settling densities, microwell geometries, settling times, and wash parameters would likely improve these values and thus assay throughput.

After protein solubilization, diffusion-driven dilution of single-cell lysate occurs rapidly in the open microwell geometries. To
determine how the concentration of the single-cell protein lysate changes during the lysis and electrophoresis stages of the assay, we used a combination of finite-element modeling (geometries are shown in Fig. 4b) and experimental monitoring of TurboGFP-expressing U251 cells during cell lysis (Fig. 4c) and finite-element modeling during electrophoresis (Fig. 4d). To compare simulation and experiment, we integrated the 3D protein concentrations over the full z range of the model to mimic detected wide-field fluorescence intensities (Fig. 4e, f). Using this metric, after a typical 25 s lysis time, we measure 17% ± 11% of the initial protein intensity. Our simulated profiles overestimate the diffusional dilution of protein, predicting only 2.2% of the initial intensity for in-well lysis after 25 s. We attribute the ~15% discrepancy to delayed solubilization of cellular protein (the models assumed complete solubilization at t = 0), overestimates of the predicted diffusion coefficient used in the simulation, or a possible diffusion barrier on the microwell wall surface arising from either the presence of Rhinohide® in the gel matrix or the presence of residual GelSlick® or dichlorodimethylsilane used during gel fabrication. One important consideration for projection electrophoresis is...
Immunoprobing of model proteins tries to minimize lysis and solubilization times and used diffusive pore size) separation gels. Analytes accommodated by moving to higher-density (smaller migration, and dispersion of the model protein buffer that utilizes the anionic detergents sodium dodecyl methods. We selected a dual-function lysis and solubilization, which were dependent on buffer composition and delivery zation ef

Immunoglobulin fragments (F(ab) fragments) to assess solubilization of size-excluded species, we explored whether lower density as PAG density negatively correlates with the in-gel concentration of protein is dependent on both analyte size and gel density. The relatively large-pore-size gels used in this work (7%T) are optimal for large analytes (80–200 kDa), with adaptation for smaller analytes accommodated by moving to higher-density (smaller pore size) separation gels.

In optimizing the projection assay, we sought buffer chemistries to minimize lysis and solubilization times and used diffusive immunoprobing of model proteins β-tubulin and GAPDH using immunoglobulin fragments (Fab fragments) to assess solubilization efficacy. Here, we assessed a range of cell lysis and protein solubilization chemistries (Fig. 4h). Across a range of chemistries, we observed differences in protein electromigration and dispersion, which were dependent on buffer composition and delivery methods. We selected a dual-function lysis and solubilization buffer that utilizes the anionic detergents sodium dodecyl sulphate (SDS) and sodium deoxycholate, augmented with a strong chaotrope (8 M urea). Comparing solubilization, electromigration, and dispersion of the model protein β-tubulin from U251 glioblastoma cells lysed both without (i) and with (ii) 8 M urea in the lysis buffer, we observed rapid electromigration into the 3D gel from the microwell and lower protein peak dispersion with urea present. Without urea, the 3D protein bands exhibited a hollow, bowl-like shape (conceivably towards the microwell), rather than the 3D Gaussian distribution which would be expected from diffusion theory. In a sub-set of separation lanes, two β-tubulin peaks were detectable after solubilization with urea lysis buffer (Fig. 4h), suggesting delayed solubilization for a subset of the β-tubulin molecules, as might be expected depending on the intracellular state of the β-tubulin.

In formulating design guidelines for the dual-function lysis-electrophoresis buffer, we consider two additional points. First, detergents such as SDS and Triton X-100 form micelles of size on the order of nanometers. Consequently, we explored the corollary hypothesis that size-exclusion partitioning, solutes hinders delivery of lysis reagents from PAG matrices. As PAG density negatively correlates with the in-gel concentration of size-excluded species, we explored whether lower density (6%T vs. 20%T) polyacrylamide lysis gels may facilitate improved protein solubilization. By moving to 6%T lysis gels, we observed lower apparent GAPDH mobility (1.08 ± 0.03 × 10⁻⁴ cm²/V s using 6%T lysis gel, compared with 0.83 ± 0.08 × 10⁻⁴ cm²/V s using 20%T lysis gel, n = 12–14 separation lanes) and potential reduction in protein band dispersion (Supplementary Figure 3). Second, strong chaotropes like urea solubilizes proteins by disrupting hydrogen bonds as well as electrostatic and hydrophobic interactions to unfold hydrophobic protein regions. Urea-based lysis buffers can solubilize different subsets of the proteome, as compared to RIPA-like buffers. High concentrations of urea (e.g., 8 M) can break down detergent micelles and disturb detergent-protein complexes. Urea, as a small molecule, is less susceptible to size-exclusion partitioning from hydrogels. Just as in other protein separations, the ideal lysis buffer depends on the system and target of interest. Analysis of another endogenous target protein, GAPDH, using the 8 M urea lysis buffer that better-solubilized β-tubulin also yielded protein peaks with low dispersion (Fig. 4i).

Lastly, we verified the device design suggested by analysis of the well-characterized protein ladder now for the analysis of mammalian cells using the optimized cell preparation protocol (Fig. 4). We anticipated that the cell preparation may increase lysis diffusion from that assessed using the idealized protein ladder system in Fig. 3, both because diffusion of protein targets in each single-cell lysis occurs during the time required for lysis and solubilization and because single-cell PAGE is run at higher temperature (~37 °C vs. ~4 °C) to improve protein solubilization. As discussed above, microwell spacing dictates the achievable cell throughput on one device. After 10 s EP, we measured σ_r = 32 ± 13 µm for GAPDH (36 kDa) in the xz plane. At Δ_w = 192 µm (6σ_0), we estimate that <0.3% of the fluorescence signal from the GAPDH in each cell lysate should bleed into the neighboring lane. Thus, Δ_w = 200 µm is also sufficient to limit cross-contamination between adjacent separation lanes for GAPDH under these assay conditions.

Immunoblotting of protein targets from hundreds of single mammalian cells. We applied projection electrophoresis to immunoblotting analyses of well-characterized endogenous proteins GAPDH and actin across populations of individual human BT474 breast cancer cells. As depicted in Fig. 5, projection electrophoresis concurrently analyzes hundreds of single cells by parallelized separation after near-simultaneous lysis. To expedite full-gel volumetric fluorescence readout of protein immunoblots, we employed light-sheet microscopy in Fig. 5. Comparison measurements using scanning laser confocal microscopy are presented in Supplementary Figure 4. Data processing allows us to visualize immunoblot readouts as maximum intensity projection 3D renderings (Fig. 5a), 2D contour plots showing xz fluorescence peaks for each fluorescence color channel (corresponding to a target/antibody pair) (Fig. 5b), and revolved 1D z-directional fluorescence intensity plots (Fig. 5c).

Cells lyse nearly simultaneously (Fig. 5d). Across 4 replicate experiments, 31/36 monitored cells (86%) lysed within 5 s of placing the lysis gel on top of the microwell gel. Of the 5 remaining cells (all within the same replicate), 2 lysed during the 80 s monitoring period while 3 did not, potentially due to a bubble between the two gels. This near-simultaneous lysis, combined with parallelized electrophoretic separation over the full gel (>1000 separation lanes), facilitates concurrent analysis of hundreds of single cells. Figure 5e depicts revolved 1D intensity profiles for the 159 separation lanes within a single projection electrophoresis gel that passed R² (>0.7 for Gaussian fit to 1D z-intensity profile) and SNR (>3) quality control in both protein channels (222 lanes passed these quality controls in the GAPDH channel; 204 lanes passed in the actinin channel). The intensity profiles show a GAPDH peak at a depth of 552 ± 54 µm and an actinin peak at a depth of 165 ± 42 µm (median ± one standard deviation). The profiles also show another peak in the actinin channel near the depth of the GAPDH peak, potentially due to off-target antibody binding and/or spectral bleed-through.
between the channels (optical filter sets) of the light-sheet microscope.

Projection electrophoresis is compatible with multi-modal imaging of the intact cells before separation, as well as the separated protein bands after the assay. Pre-separation live-cell imaging of intact BT474 cells (Fig. 5f, g) correlated well with detected probed bands. The in situ separations facilitated by projection electrophoresis allow comparison of live-cell fluorescence – prior to projection immunoblotting (via wide-field fluorescence microscopy in Fig. 5f, g) – and endpoint probed GAPDH signal (via wide-field fluorescence microscopy in Fig. 5h and light-sheet microscopy in Fig. 5i). The analysis revealed appreciable spatial correlation between live cell imaging prior to separation and wide-field fluorescence images of separated GAPDH (Fig. 5j–k). Comparison shows 63–74% of detected live cells are correlated with GAPDH detection. In two duplicate separations, 76 and 63% of detected live cells corresponded to probed GAPDH bands, 24 and 37% of detected live cells did not correspond to a probed GAPDH band, and 17 and 22% of probed bands did not visibly correspond to a live cell. This correlation (and potentially cell settling efficiencies and analysis throughput) could potentially be further improved in future work by encapsulating settled cells in hydrogel to mitigate cell loss/movement during manual gel transfer and electrophoresis stack setup.

We observe the expected differential in electrophoretic mobility and nearly equivalent peak widths for the GAPDH (37 kDa) and actinin (100 kDa) targets for a total of 507 (GAPDH) and 303
Fig. 5 Projection electrophoresis permits the simultaneous analysis of hundreds of single cells by concurrent separation during simultaneous lysis. 
(a) Maximum intensity projection 3D renderings of example separation lanes read out by tiled light-sheet microscopy. (b) z(y-summed) contour plots of background-subtracted actin and GAPDH protein signal within the lanes depicted in (a). (c) Revolved z-intensity profiles (arbitrary fluorescence units, AFU, vs. z) for the four separation lanes depicted in (a, b). Each plot depicts z-directional intensity profiles (summed fluorescence within each x/y ROI vs. z) for GAPDH (magenta) and actin (cyan), revolved around the z-axis to generate 3D rendering of fluorescence distribution. (d) Histogram quantification shows 86% of U251 cells lyse within 5 s of initiating lysis. (e) Quantified fluorescence intensity data for n = 159 separation lanes passing quality control for both the GAPDH and actin channels. Each plot depicts revolved z-intensity profiles. (f) Full-gel wide-field fluorescence image of calcein-stained live BT474 breast tumor cells before analysis. (g) Subset of the live cells from (f), within a 1.75 × 1.75 mm light-sheet microscopy field of view (scale bar depicts 200 μm). (h) Post-separation wide-field fluorescence image of probed GAPDH signal within the same field of view as (g). (i–k) Representative of n = 3 separation gels. (i) Maximum intensity projection 3D rendering (representative of duplicate separation gels) of a light-sheet microscopy image (same field of view as (g, h)), showing 3D separations of GAPDH and actin from tens of separation lanes, each corresponding to signal from the settled cells in microwells depicted in (f, g). (j) Overlay image of segmented spots corresponding to live BT474 cells in microwells (green) and probed GAPDH bands after separation (magenta), for the same separation gel depicted in (f, i). (k) Quantification of correspondence between the segmented live cells and bands (via intensity thresholding) within the same separation lanes as (j). (l) Quantified migration distances from a total of n = 507 (GAPDH) and n = 303 (actinin) lanes passing quality control in two projection electrophoresis gels. (m) Quantified z-direction peak widths for the same bands analyzed in (i). 

(n–o) Map of the variation in GAPDH (n) and actinin (o) electromigration distances across the xy gel area. Source data are provided as a Source Data file.

(actinin) single-cell separations across duplicate separation gels (Fig. 5l, m). In considering variation in electromigration, the coefficients of variation in electromigration are for GAPDH ~8.5% and for actinin ~27%. Qualitatively, we observe modestly higher electromigration on one side of the gel, on each of two gels (Fig. 5n, o, Supplementary Figure 5), which is inconsistent with Joule heating-induced electromigration nonuniformity (e.g., higher mobility in the gel center, as observed in bulk separations42). Consequently, we attribute the modest, observed electromigration variation to nonuniformities in protein solubilization or electrophoresis (e.g., E and/or temperature), or to inaccuracies in gel surface detection (z = 0) during light-sheet image analysis. Including a protein-sizing ladder in each separation lane enhances size-based protein identification, as our group has reported in similar 2D single-cell immunoblot devices (either as a ladder of well-defined, cell-endogenous proteins26 or as ladder protein-conjugated beads26). Although from diffusion theory we would expect a larger peak width for the smaller protein target, differences in peak dispersion between targets can result in wider measured peak widths.

We compared scanning laser confocal microscopy (Supplementary Figure 4) and light-sheet microscopy (Fig. 5) imaging of the same gel devices to acquire volumetric protein immunoblot readouts from the protein PAGE separation lanes. Each imaging modality presents a trade-off in field of view and z-axis resolution, but the imaging throughput of light-sheet microscopy was >10× higher than scanning laser confocal, moving from ~120 s/lane readout time down to ~8 s/lane, while retaining sufficient z-axis resolution to localize protein peaks. The laser scanning confocal imaging with 20× NA = 1.0 water immersion objective (required for high-resolution optical sectioning) supported a 425 × 425 μm field of view, while light-sheet microscopy with 5× detection objective (NA = 0.16) provided a much larger 1.75 × 1.75 mm field of view. Because its optical sectioning is facilitated by the light-sheet objectives forming a thin illumination sheet, light-sheet microscopy allows the imaging optical section thickness to be decoupled from the detection objective NA, facilitating the use of lower NA detection objectives while maintaining optical sectioning59. The light-sheet images acquired here had optical section thicknesses on the order of 10 μm, which should be sufficient to assess the diffusion-limited z-directional peak widths of tens of microns for our separated protein bands. Further, light-sheet microscopy detected both protein targets with similar expected differential electrophoretic velocity and comparable peak widths of the immunoprobed targets to those measured with confocal (Supplementary Figure 4). Differences may be partly attributed to the impact of a slight refractive index mismatch between the hydrogel and water immersion media on apparent confocal z-depths (Supplementary Note 1). With both readout modalities, we also observe the log-linear relationship between migration distance and molecular mass for endogenous targets that would be expected for a size-sieving separation (Supplementary Figure 6). Given similar results in detection, migration location, and peak width for the model endogenous protein targets, the substantially larger field of view of light-sheet microscopy proved beneficial, allowing endpoint imaging and analysis of 10× larger number of immunoblots (imaged separation lanes passing quality control: n = 22 with confocal; n = 303 actinin and n = 507 GAPDH with light-sheet microscopy, over two separation gels).

The parallel cell analysis approach described here overcomes shortcomings of serial analysis of cells. Although synchronous cell lysis is also not instantaneous across cells (with biological variation in lysis time on the order of seconds70), serial interrogation of individual cells leads to asynchronous analysis with longer time delays between analysis of the first cell and last cell in a population. Considering one published example single-cell enzyme analysis separation, where individual cells are interrogated by a capillary sampler after cell lysis via a UV light pulse71, we estimate a 104 min delay between interrogation of the first cell and interrogation of the last cell (219 cells analyzed with an analysis throughput of 2.1 cells/min). In contrast, concurrent analysis of ~300 measurable separation lanes is completed with <10 s delay between the first and last cell, assuming a small delay in cell lysis arising during the application of the lysis and solubilization buffer. Furthermore, projection electrophoresis uses a single SDS-PAGE sieving gel for hundreds of concurrent single-cell protein-sizing separations. In contrast, serial electrophoresis separations performed in capillaries or microchannels require periodic renewal of the sieving matrix between separations to mitigate residual sample and matrix degradation13. Consequently, higher-throughput electrophoresis systems often use free solution17 or sieving polymers28. By introducing a new, rapid, and parallelized electrophoresis approach, we demonstrate simultaneous single-cell separations of hundreds of single cells with an active assay time of 2.5 cells/s (from lysis through photocapture), depending on settling efficiency — this represents a >70-fold improvement in assay throughput over serial capillary systems.

Projection electrophoresis addresses key bottlenecks in single-cell protein analysis by achieving rapid (<1.5 min active assay time), synchronous size-based protein separation for hundreds of
unfixed cells in parallel, without sample transfer steps that can result in sample losses and changes in sample composition. Detection specificity combines antibody recognition with size-separation to confer proteoform-level specificity even when specific probes do not exist, and avoids the need for pre-separation tagging of proteins for detection. In situ cell lysis, separation, and photocapture of protein to the gel matrix mitigates deleterious effects of sample transfer between systems, including losses from adsorption to glassware, potential sample contamination, and sample changes between lysis and separation. Effectively synchronous analysis of hundreds of cells in parallel mitigates artificial changes in cell population heterogeneity induced by heterogeneous lysis times, while also facilitating high assay throughput. Using rapid whole-cell lysis of unfixed cells, we demonstrate the detection of both nuclear and cytosolic proteins. We design and characterize the system by both modeling and measuring the results of microscale physics. Compared with planar (2D) single-cell western blotting, we demonstrate a 10-fold reduction in the volume of cell suspension settled (and thus the number of cells used) to assay the same number of cells, and further through simple improvement it may be possible by optimizing parameters for settling efficiency. While 2D devices are conducive to immunopanning, imaging as readout, and efficient data analysis, projection electrophoresis increases the density of single-cell analyses for the same device footprint, by shifting the separation dimension to the z-axis, while maintaining similar protein losses and outputting rich 3D information about protein band morphology and dispersion. Important beyond enhancing data density, 3D projection electrophoresis holds promise for future profiling of cellular “connectomes” by supporting analyses of complex cellular networks such as intact tissue slices and adherent cells cultured on planar hydrogel surfaces.

Looking forward, the performance of projection electrophoresis can be improved in future work by moving to larger-area gels to further parallelize analysis, and by using our understanding of the driving small-scale physics to optimize gel materials for targets of interest and thus enable the use of even higher microwell densities (or adherent cells) and improved separation performance. With a straightforward, open microfluidic format and advantages complementary to existing protein analysis tools, we anticipate that projection electrophoresis will assist in the development of proteoform-level atlases of single-cell diversity.

**Methods**

**Wafers and microfabrication and silanization.** Wafers with 40 μm-high features (32 μm diameter microwells for purified protein experiments and lysis monitoring, or 25 μm diameter microwells for BT474 and U251 single-cell separations) were fabricated using SU-8 3050 (Microchem, now Kayaku Advanced Materials, Inc.) according to the manufacturer’s instructions and then silanetreated. 

Silicon wafers (WaferPro C04009) were cleaned by 5-minute agitated immersion in acetone followed by rinsing in acetonitrile, isopropanol, and deionized water. Wafers were then dried under a nitrogen stream and dehydrated by a 10-minute bake on a hotplate maintained at 65 °C and cooled slowly by convection. Once cooled to room temperature, the wafers were prepared for use in the microfluidic devices by soft baking for 2 min on a 65 °C hotplate followed by 15 min on a 95 °C hotplate and finally 3 min on a 65 °C hotplate. The resist was exposed to UV light within a mask aligner for 10.8 s at an optical power of 29.26 mW/cm² (for an exposure dose of 316 mJ/cm²), through a transparency mask (CAD/Art Services, Inc.) and a longpass optical filter (Omega PL-360LP) (both of which attenuate the dose applied to the wafer below the 316 mJ/cm² stated above). After exposure, each wafer was post-exposure baked for 1 min at 65 °C, 5 min at 95 °C, and 1 min at 65 °C and cooled slowly by convection. Once cooled to room temperature, the wafers were trimmed to size using a razor blade and released from the glass substrate by carefully sliding the razor blade under the gel, applying firm pressure with the blade onto the glass and gently adding water between the razor blade and gel to lubricate and prevent tearing. Separation and shield/lysis gels were stored in the appropriate buffer solution for a minimum of 12 h and up to 4 days prior to running separations. Buffer storage conditions are presented in Supplementary Table 1 and were either (1) dual function lysis-electrophoresis modified RIPA buffer (1x or 2x RIPA as described in Supplementary Table 2), (2) 1x tris-glycine buffer for lysis and protein solubilization or 3 mm (for cell experiments) removable polymer spacer (C.B.S Scientific Gel Wrap) at the top and bottom of the electrodes. The planar electrodes were commercial platinum-coated electrotransfer anodes (Bio-Rad Criterion anode plates) with plastic housings modified to permit close proximity of the electrode surfaces. Electric fields were provided by a Bio-Rad PowerPac Basic (BP) connected to each pair of the standard banana plug interfacing. Cold packs on the back side of each electrode maintained gel temperature at −4 °C to help to mitigate deleterious effects of Joule heating during purified protein separations. To aid in lysis and protein solubilization, the cold packs were heated in a 55 °C water bath for single-cell separations or held at −37 °C incubators for cell settling in the 55 °C water bath for single-cell separations. After polymerization, gels were trimmed to size using a razor blade and released from the glass substrate by carefully sliding the razor blade under the gel, applying firm pressure with the blade onto the glass and gently adding water between the razor blade and gel to lubricate and prevent tearing. Separation and shield/lysis gels were stored in the appropriate buffer solution for a minimum of 12 h and up to 4 days prior to running separations. Buffer storage conditions are presented in Supplementary Table 1 and were either (1) dual function lysis-electrophoresis modified RIPA buffer (1x or 2x RIPA as described in Supplementary Table 2), (2) 1x tris-glycine buffer for lysis and protein solubilization or 3 mm (for cell experiments) removable polymer spacer (C.B.S Scientific Gel Wrap) at the top and bottom of the electrodes.

The z-directional electrode system consists of planar electrodes integrated into a custom laser-fabricated acrylic -directional electrode system. The z-axis, while maintaining similar protein losses and outputting rich 3D information about protein band morphology and dispersion. Important beyond enhancing data density, 3D projection electrophoresis holds promise for future profiling of cellular “connectomes” by supporting analyses of complex cellular networks such as intact tissue slices and adherent cells cultured on planar hydrogel surfaces. Looking forward, the performance of projection electrophoresis can be improved in future work by moving to larger-area gels to further parallelize analysis, and by using our understanding of the driving small-scale physics to optimize gel materials for targets of interest and thus enable the use of even higher microwell densities (or adherent cells) and improved separation performance. With a straightforward, open microfluidic format and advantages complementary to existing protein analysis tools, we anticipate that projection electrophoresis will assist in the development of proteoform-level atlases of single-cell diversity.
ultrapurp water dilution of 10× stock, Bio-Rad 1610734) containing 0.5% (v/v) Triton X-100 (Sigma). High molecular mass purified protein solutions were prepared by using the Bio-Formats libraries provided by Prof. Daniel Blair and Prof. Eric Defresne79 was used to track the positions of protein originating from each microwell through the z depth of the image. The tracked centroids were then subject to a quality control step to remove protein spots that were only found in small portions of the full z volume. After this was complete, the tracking code output a set of ‘spots’ – protein spots originating from a single microwell, each with a list containing the xy location of the centroid of each protein spot for every z location in the image. To create the intensity profiles, the intensities in a 300 pixel (102 μm2) square ROI were analyzed around each protein spot after the data were background-subtracted by subtracting from each pixel the average measured intensity 15 μm below the bottom of the microwells, in regions at least 100 pixels (34 μm) from any segmented protein spots. After Gaussian fits to find the migration distance, z-direction peak width, and xy peak width for each protein peak, the data from all of the ROIs from multiple gels (multiple electrophoresis times in duplicate) were plotted and fit to the expected linear physical relationship (migration distance vs. electrophoresis time, square peak width and squared xy peak width vs. total in-gel time).

Cell culture. U251 human glioblastoma cells stably transduced with TurboGFP by lentiviral infection were kindly provided by Prof. Sanjay Kumar’s laboratory at UC Berkeley (the naive, pre-modification U251 cell line was obtained from the UC Berkeley Tissue Culture Facility, originally sourced from the American Type Culture Collection). Cells were cultured in tissue culture flasks in a standard cell culture incubator (Herculab 150s) at 5% CO2 and 37 °C, in DMEM (Invitrogen 10566016) supplemented with 10% FBS (Gemini Bio-Products Benchmark 100–106), 1% penicillin/streptomycin (Life Technologies 15140–122), 1x sodium pyruvate (Thermo Fisher 11360070), and 1x non-essential amino acids (Life Technologies 11140–050 1× stock). The cells were passaged at 1:40 after reaching ~80% confluence by detaching with 0.05% Trypsin–EDTA (Life Technologies 25300120), centrifuging for 3 min at 1000 RPM, and resuspending in complete media to resed. BT474 human breast cancer cells were purchased from the UC Berkeley Bioengineering Divisional Services Cell Culture Facility. Cells were maintained in tissue culture flasks in a standard cell culture incubator (Herculab 150s) at 5% CO2 and 37 °C, in DMEM (Invitrogen 10566016) supplemented with 10% FBS (Gemini Bio-Products Benchmark 100–106) and 1% penicillin/streptomycin (Life Technologies 15140–122). The cells were passaged at a density of 1:2 to 1:8 after reaching ~80% confluence by detaching with 5× EmCyte Collagenase U251 (647-labeled transferrin, Bio-Formats libraries provided by Prof. Daniel Blair and Prof. Eric Defresne79 was used to track the positions of protein originating from each microwell through the z depth of the image. The tracked centroids were then subject to a quality control step to remove protein spots that were only found in small portions of the full z volume. After this was complete, the tracking code output a set of ‘spots’ – protein spots originating from a single microwell, each with a list containing the xy location of the centroid of each protein spot for every z location in the image. To create the intensity profiles, the intensities in a 300 pixel (102 μm2) square ROI were analyzed around each protein spot after the data were background-subtracted by subtracting from each pixel the average measured intensity 15 μm below the bottom of the microwells, in regions at least 100 pixels (34 μm) from any segmented protein spots. After Gaussian fits to find the migration distance, z-direction peak width, and xy peak width for each protein peak, the data from all of the ROIs from multiple gels (multiple electrophoresis times in duplicate) were plotted and fit to the expected linear physical relationship (migration distance vs. electrophoresis time, square peak width and squared xy peak width vs. total in-gel time).

Cell lysis monitoring experiments. We compared diffusion profiles of cells lysed after settling in 32 μm diameter, 40 μm high microwells within 1-mm-thick gels. Microwell gels and lysis gels (18 × 18 mm in area) were prepared and equilibrated in PBS for ~12 h, as described above. To settle cells in microwells, we followed a procedure similar to that used for single-cell western blotting27: U251 TurboGFP cells were detached using 0.05% trypsin–EDTA (Life Technologies 25300120), resuspended in PBS (Life Technologies 10010049) at a concentration of 100 000 cells/mL and filtered using a cell strainer (Corning 352235). The 18 × 18 mm microwell gels were placed microwell side up in a 60 mm tissue culture dish. 18 mm diameter petri dish gels were pipetted onto the surface of each microwell gel (to cover but avoid spillage over the edge of the gel) and spread to cover the full array of microwells. The cells were allowed to settle for 5 min on ice, gently agitating every ~2 min, before rinsing with 1–3 mL PBS by tilting the tissue culture dish at a ~40° angle, pipetting PBS at the top surface of the microwell gel, and allowing it to flow over the solution of the microwell gel into the bottom of the petri dish. After rinsing was complete, the excess PBS/cell solution was aspirated for biohazard disposal.

To facilitate monitoring, each microwell gel was immobilized within a 60 mm petri dish by pipetting 200 μL of a warmed solution of 5% agarose (Invitrogen 16500044) in PBS until the agarose gel reached 4 mm in thickness. The agarose gel was placed microwell side up in a 60 mm tissue culture dish. 18 mm diameter petri dish gels were pipetted onto the surface of each microwell gel (to cover but avoid spillage over the edge of the gel) and spread to cover the full array of microwells. The cells were allowed to settle for 5 min on ice, gently agitating every ~2 min, before rinsing with 1–3 mL PBS by tilting the tissue culture dish at a ~40° angle, pipetting PBS at the top surface of the microwell gel, and allowing it to flow over the solution of the microwell gel into the bottom of the petri dish. After rinsing was complete, the excess PBS/cell solution was aspirated for biohazard disposal.

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thresholding of the median-filtered (3 × 3 neighborhood) image. The segmentation was improved using morphologic open and close operations, and the centroids of the segmented cells were computed and stored. The MATLAB® particle tracking library (based on a previous IDL implementation) was made publicly accessible by Prof. Daniel Blair and Prof. Eric Defares 28 was used to track the centroids of each cell over the course of the experiment, although minimal drift was observed. The maximum fluorescence intensity and total fluorescence intensity in a 100-μm diameter circle surrounding the centroid of each segmented cell was tracked for each time point. All pixel intensities were background-subtracted using the average fluorescence intensity of the background region at each time point, taken to be the image regions greater than 30 μm away from all segmented regions.

Finite-element modelling of protein diffusion. We used finite-element modelling of dilute species transport in COMSOL®. Multiphysics to predict protein concentrations during lysis and electrophoresis. The simulation geometries for the projection electrophoresis simulations are presented in the cross-sectional view shown in Fig. 4b. 2D axisymmetric models were used since the inherent symmetry of the geometry. Diffusion coefficients in free solution portions of the model were estimated from the Stokes-Einstein equation 29, while in-cell diffusion was estimated from the free-solution diffusivity using the methods presented by Park, et al. 31. Protein hydrodynamic radii were estimated from the number of amino acids 30. Thermodynamic partitioning of protein was simulated using flux boundary conditions relating in-cell concentration to in-solution concentration using a partition coefficient k = Cgel/Csolution. Partition coefficients were estimated using the Ogsten model 33 (assuming size-exclusion partitioning), using estimates of fiber radius from Tong and Anderson 25 and hydrogel volume fraction from the data presented by Baserga, et al. 33. A temperature of 4 °C was assumed for all simulations except the comparison with the single-cell western blot platform, which assumed 10 °C for both platforms. The diffusion and partition coefficients for each protein at 4 °C are presented in Table 1.

In each model, gel-solution boundaries were modeled as flux boundary conditions taking size-exclusion into account. The edges of each model were modeled as flux boundary conditions permitting protein to freely leave the model; however, the simulation region was sufficiently large (500 × 500 μm) that changing these boundary conditions to no flux resulted in negligible change to the modeled concentration profiles. The initial protein concentration in the cell region was 2 μM and the particle concentration was zero. In models of the single-cell western blotting platform, the bottom surface of the gel was modeled as no flux to model the presence of the glass slide present in that system. The model was meshed with a physics-controlled mesh calibrated for fluid dynamics, and a user-controlled override with maximum element size of 0.5 μm was used. The cell solution was well mixed.

TABLE 1 Protein hydrodynamic radii (rH), diffusion coefficients (D) and partition coefficients (k) used in finite-element modeling of diffusion during lysis and electrophoresis.

| Protein | rH [nm] | Dfree [m²/s] | Dgel [m²/s] | D2Dgel [m²/s] | kgel/k | k2Dgel/k2Dgel |
|---------|---------|--------------|-------------|---------------|--------|---------------|
| TurboGFP | 2.3     | 6.61 × 10⁻¹¹ | 1.36 × 10⁻¹¹ | 9.49 × 10⁻¹³ | 0.501  | 0.0415        |
| BSA     | 3.01    | 5.05 × 10⁻¹² | 7.90 × 10⁻¹² | 3.49 × 10⁻¹³ | 0.344  | 0.00744       |
| HER2    | 3.76    | 4.04 × 10⁻¹¹ | 4.88 × 10⁻¹¹ | 1.39 × 10⁻¹³ | 0.213  | 8.16 × 10⁻⁴   |

After 20 min of settling, gels were rinsed by holding the petri dish at a ~40° angle and pipetting 0.5 mL of DEP buffer onto the top corner of the gel, allowing the fluid stream to wash over the full gel into the tissue culture dish. The wash fluid was aspirated for biohazardous waste disposal, and the wash was repeated with an additional 0.5 mL of DEP buffer after pipetting ~40 μL DEP buffer on top of the gel to prevent drying during imaging. Tiled and stitched images of the settled cell fluorescence were captured using the ScanSlide plugin for the MetaMorph® imaging software (Molecular Devices), using an Olympus IX51 inverted wide-field fluorescence microscope fitted with an X-Cite® illumination source (Excitex Technologies), GFP filter set (Chroma 49011 ET), 4× objective, and CoolSNAP HQ2 CCD camera (Teledyne Photometrics). Cell occupancy was calculated from a MATLAB® script that analyzed tiled live-cell brightfield and fluorescence images, determined whether there was a fluorescent object in a region surrounding each microwell (via thresholding segmentation) and, if found, displayed the region and prompted user input of number of cells in the microwell region.

Aliquots of lysis buffer (4 mL) were prepared by dissolving urea to a final concentration of 6 M in 2x RIPA buffer in a 13×13 cm gel set to 55 °C. Lysis gels (14 × 14 × 1 mm) were stored in 2× RIPA-like lysis buffer and transferred to aliquots of urea lysis buffer as soon as the urea had dissolved (10–60 min prior to running the separations). The lysis gels in buffer aliquots were heated to 55 °C in a water bath until immediately prior to use, agitating periodically to ensure the solution was in the well mixed.

After cell settling and live-cell imaging, 4 mL 2× tris-glycine (diluted from Bio-Rad 1610734 10× stock) was pipetted into the tissue culture dish containing the separation gel and incubated for 1 min to reduce the concentrations of potentially unwanted ions. A 10 × 10 × 1 mm filter paper (cut to size from Thermofisher Scientific® Whatman® blotting paper) was placed on top of the gel, and 45 s of photobleaching illumination was placed and anode on the placed. The separation gel was placed (microwell side up) on top of the filter paper immediately after tris-glycine incubation, placing slowly from one corner to reduce bubble entrapment between the layers. The lysis gel was removed from the buffer aliquot and placed on top of the separation gel, again placing gradually so as to not introduce bubbles between the gels. The lysis timer was started as soon as the lysis gel was placed, and the electrode system was closed by placing the cathode on top of the lysis gel. After 25 s lysis, the separation was initiated by supplying 80 mA of constant current (typically yielding 13–16 V internal voltage for an average electric field of 43–53 V/cm across the gel stack) using a DC power supply (Bio-Rad PowerPac Basic) and recording the voltage at 5 s intervals during electrophoresis. After electrophoresis was complete, the power supply was stopped, electrode system opened, and 45 s photocapture was completed using a Hamamatsu Lightningcure LC5 UV source. The gel was then rinsed briefly in deionized water before equilibrating in tris-buffered saline with Tween® (1× TBST, from Cell Signaling Technology 9997 S 10× stock) overnight to remove any residual lysis buffer (exchanging the buffer after 2 h). Projection electrophoresis gels probed with Fab fragments were blocked overnight in 5% BSA in TBST at 4 °C or for between 2-4 h on a shaker at room temperature prior to immunoblotting.

Immunopanning and imaging. Single-cell projection electrophoresis immunoblotting gels were probed either diffusively (for initial characterization experiments using labeled Fab fragments in Fig. 4) or electrophoretically (using standard primary and fluorescently-labeled secondary antibodies in Figs. 1 and 5). All probing wash steps were performed using an electrophoretic wash platform. In-gel probing requires high concentrations of immunoprobes to mitigate size-exclusion partitioning effects 27,28. To minimize reagent consumption, we probe...
Table 2 Probe delivery gel fabrication parameters for electrophoretic and diffusive probing.

| Gel type                          | Final agarose concentration after probe dilution | Antibody probes                                                                 | Gelation time | Fabrication setup |
|----------------------------------|-------------------------------------------------|----------------------------------------------------------------------------------|---------------|-------------------|
| Primary antibody electrophoretic probe delivery agarose gel | 1.5% (w/v) (dissolved in 1× tris-glycine) UltraPure LMP Agarose, (Thermo-Fisher: 16520050) | For the studies reported in Fig. 5 and Supplementary Figures 4–6, dilutions from each stock were: (1) Rb α-actinin (1:15, 6.67% (v/v)) & (2) Gt α-GAPDH (1:10, 10% (v/v)). For the studies reported in Fig. 1 and Supplementary Figure 8, dilutions from each stock were: (1) Ms α-PTBP1 (1:10, 10% (v/v)) & (2) Gt α-GAPDH (1:10, 10% (v/v)). | 5 mins on ice pack | Glass plate and glass slide |
| Secondary antibody electrophoretic probe delivery agarose gel | 1.5% (w/v) (dissolved in 1× tris-glycine) UltraPure LMP Agarose, (Thermo-Fisher: 16520050) | For the studies reported in Fig. 5 and Supplementary Figures 4–6, dilutions from each stock were: (1) Dk α-Rb AF555 (1:10, 10% (v/v)) & (2) Dk α-Gt AF488 (1:10, 10% (v/v)). For the studies reported in Fig. 1 and Supplementary Figure 8, dilutions from each stock were: (1) Dk α-Ms AF555 (1:20, 5% (v/v)) & (2) Dk α-Gt AF488 (1:20, 5% (v/v)) | 5 mins on ice pack | Glass plate and glass slide |
| hfAB diffusive probe delivery agarose gel | 1.5% (w/v) - dissolved in 1× tris-buffered saline solution with Tween® (1× TBST, from C.B.S. Scientific) UltraPure LMP Agarose, (Thermo-Fisher: 16520050) | For the studies reported in Fig. 5 and Supplementary Figure 4–6, dilutions from each stock were: (1) Dk α-GAPDH or (2) hfAB Rhodamine α-tubulin | 1:1 (10% (v/v)) dilution from stock of either: (1) Bio-Rad hfAB Rhodamine α-GAPDH or (2) hfAB Rhodamine α-tubulin | 5 mins on ice pack | Glass plate and glass slide |

For full-gel imaging, gels were imaged using a Zeiss LSM 880 laser-scanning confocal microscope fitted with a 2× water dipping objective (NA 0.75, Zeiss W Plan Neofluar 20×/0.75 DICM). A confocal z-slice spacing of 0.5 μm was chosen, and volumes extending ~100 μm past visible fluorophore bands were imaged. As we were not quantifying or comparing protein abundance, excitation laser powers were adjusted to permit fluorescence visibility depending on the sample brightness, as fluorescence intensities were not compared between cell separations. Similarly, images were brightness and contrast-adjusted in Fiji (based on ImageJ, National Institutes of Health) to ensure visibility of protein bands.

For gel stacking, gels were fabricated using a gel stacking system (Thermo Fisher Scientific 48783). Gel stacking was performed with a 5× detection objective (Zeiss EC Plan-Neofluar 20×/0.75 DICM) and 4× light-sheet objectives (Zeiss LSM 510 C-Apochromat 20×/0.75 DICM). The gel stack was imaged using a custom laser-scanning confocal microscope fitted with a 1× water dipping objective (NA 0.75, Zeiss W Plan-Neofluar 20×/0.75 DICM). A confocal z-slice spacing of 0.5 μm was chosen, and volumes extending ~100 μm past visible fluorophore bands were imaged. As we were not quantifying or comparing protein abundance, excitation laser powers were adjusted to permit fluorescence visibility depending on the sample brightness, as fluorescence intensities were not compared between cell separations. Similarly, images were brightness and contrast-adjusted in Fiji (based on ImageJ, National Institutes of Health) to ensure visibility of protein bands.

For confocal and light-sheet microscopy datasets were analyzed using custom analysis scripts in MATLAB®, similar to those described above for purified protein datasets. Zeiss C2I confocal z-stacks and associated metadata were imported into MATLAB® (MathWorks®, Inc.), a commercial software system for data analysis and visualization. The analysis workflow is described in Supplementary Figure 8, and 3D datasets are made up of 2D (xy) slice images that are each processed to assess the 3D positional data for each separated protein peak. Intensity profiles were background-subtracted by subtracting the average intensity of a 5-pixel (light-sheet) or 20-pixel (confocal) border surrounding each xy ROI from each ROX pixel at each z location prior to summing the intensities to generate z-intensity profiles. Thin (10-pixel for confocal and 5-pixel for light-sheet) borders...
between the ROI and background regions were used to assess the background-subtracted noise of the measurement to compute signal-to-noise ratios for each protein peak. Protein peaks corresponding to separation lanes were quantified (passing quality control if: (1) there was a segmented fluorescence spot, (2) the Gaussian fit to the z-intensity profile of the peak region had an R² of the Gaussian intensity profile fit of >0.7, and (3) the Gaussian fit had a signal-to-noise ratio (Gaussian peak fit amplitude divided by twice the standard deviation of the background-subtracted noise calculation region over the z region of the protein peak) of >3. A schematic representing the analysis workflow for tiled light-sheet images (similar to the workflow used for confocal images but with the addition of functionality to find and analyze separate lane ROI locations across multiple image tiles/series) is presented in Supplementary Figure 9.

**Reporting summary** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability** The datasets generated and analyzed as a part of this work are available in the Dryad repository in the main data publication (https://doi.org/10.6078/D1B13V) and linked data publications (https://doi.org/10.6078/D1N989, https://doi.org/10.6078/D1811G, https://doi.org/10.6078/D1HH16C). Source data for Fig. 1(g), 2(c–e), 3(d–b), 4(c–g), 5(d, e, j–o) and Supplementary Figs 1, 4, and 6 are also provided as a Source Data file. Source data are provided with this paper.

**Code availability** All custom analysis code generated as a part of this work is available from the corresponding author upon reasonable request. Custom MATLAB scripts central to this work are deposited in a GitHub repository: https://github.com/samanthagrast/projection_ep_analysis.

Received: 28 February 2020; Accepted: 20 October 2020; Published online: 04 December 2020

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**Acknowledgements**

This work was funded in part by the National Cancer Institute of the National Institutes of Health, Cancer Moonshot award, Grant Number: 1R33CA225296-01 to A.E.H. and by the Chan Zuckerberg Biohub. S.M.G. gratefully acknowledges the support of a post-doctoral fellowship from the Natural Sciences and Engineering Research Council of Canada (NSERC). A.P.M.E. gratefully acknowledges support from the National Science Foundation Graduate Research Fellowship Program (NSF GRFP). We sincerely thank Dr. Burcu Gümusçu Sefinc for her assistance with early proof-of-concept tests. Confocal and light-sheet imaging experiments were conducted at the CRL Molecular Imaging Center at UC Berkeley, supported by the Helen Wills Neuroscience Institute. We acknowledge all members of the Herr Lab at UC Berkeley, as well as Dr. Ben Smith at UC Berkeley and the group of Prof. Mark Pegram at Stanford University, for useful discussions and feedback. We are also grateful to the CRL Molecular Imaging Center staff as well as Kamran Ahmed and Eva Nichols for assistance with light-sheet imaging setup.

**Author contributions**

S.M.G. and A.E.H. designed the projection electrophoresis assay. S.M.G., A.P.M.E., and A.E.H. designed experiments. S.M.G. performed projection electrophoretic separations of purified proteins and single cells. S.M.G. and A.P.M. performed electrophoretic probing experiments. S.M.G. designed software and performed data analysis. All authors wrote the manuscript.

**Competing interests**

APM declares no competing interests. SMG and AEH declare the existence of a potential financial competing interest: SMG and AEH are co-inventors of University of California intellectual property regarding z-directional electrophoresis.

**Additional information**

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-020-19738-1.

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**Peer review information**

*Nature Communications* thanks Robert Kennedy and the other anonymous, reviewer(s) for their contribution to the peer review of this work.

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