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Single cell–resolution western blotting

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This protocol describes how to perform western blotting on individual cells to measure cell-to-cell variation in protein expression levels and protein state. Like conventional western blotting, single-cell western blotting (scWB) is particularly useful for protein targets that lack selective antibodies (e.g., isoforms) and in cases in which background signal from intact cells is confounding. scWB is performed on a microdevice that comprises an array of microwells molded in a thin layer of a polyacrylamide gel (PAG). The gel layer functions as both a molecular sieving matrix during PAGE and a blotting scaffold during immunoprobing. scWB involves five main stages: (i) gravity settling of cells into microwells; (ii) chemical lysis of cells in each microwell; (iii) PAGE of each single-cell lysate; (iv) exposure of the gel to UV light to blot (immobilize) proteins to the gel matrix; and (v) in-gel immunoprobing of immobilized proteins. Multiplexing can be achieved by probing with antibody cocktails and using antibody stripping/reprobing techniques, enabling detection of 10+ proteins in each cell. We also describe microdevice fabrication for both uniform and pore-gradient microgels. To extend in-gel immunoprobing to gels of small pore size, we describe an optional gel de-cross-linking protocol for more effective introduction of antibodies into the gel layer. Once the microdevice has been fabricated, the assay can be completed in 4–6 h by microfluidic novices and it generates high-selectivity, multiplexed data from single cells. The technique is relevant when direct measurement of proteins in single cells is needed, with applications spanning the fundamental biosciences to applied biomedicine.

INTRODUCTION

No two cells are the same. Cell-to-cell variation affects biological systems, from development[1] to stem cell biology[2] to cancer[3,4]. Unfortunately, conventional bulk measurements mask the biology occurring in each individual cell[5]. Single-cell measurement techniques capture cell-to-cell variation, and microfluidic tools have a central role in bringing biochemical assays to single-cell resolution. The small device length scales and precision fluid control can maintain high local concentrations of single-cell lysates[6]. In particular, RNA sequencing and genotyping on ten to thousands of single cells is possible, owing to microwell arrays and pneumatic microfluidic valves that control RT–PCR[1,7–11]. These tools are making contributions such as elucidating genome diversity and identifying mutations that occur during gametogenesis[1]. The study of events that occur at low frequency has also benefited[5]. Isolation of rare circulating tumor cells with micropillar arrays[12,13] and RNA analysis in those cells offer the prospect of an enhanced cancer taxonomy for clinical medicine. Nevertheless, even with large strides in single-cell genomics and transcriptomics, nucleic acid expression levels do not always correlate with protein expression levels[14,15], which drive cell fate. Consequently, advances in single-cell proteomic assays will complement advances in nucleic acid and imaging assays. For a more in-depth treatment of microfluidic single-cell analyses—including signaling dynamics[16] and lineage tracing using microscopy[17]—we direct the reader to a recent review of these topics[6].

Here we describe a single-cell resolution western blot assay with enhanced selectivity as compared with immunoassays[18,19], which rely on only antibody binding for detection[20–23]. Performing a protein sizing (electrophoretic mobility) stage with a subsequent immunoassay stage can identify protein isoforms and off-target signals that are not discernible with immunoassays alone. scWB performs thousands of concurrent single-cell western blots on one microdevice within a 4–6 h workflow. scWB has been applied to studies ranging from stem cell differentiation[18] to variations in cancer cell responses[19].

Development of the protocol

The scWB protocol presented here builds upon the efforts of our group to miniaturize western blotting assays for rapid, sensitive and selective quantitative analysis of protein expression[24]. The scWB assay leverages microfabrication techniques to pattern microwells that accommodate single cells in a thin PAG[18]. A key innovation is UV immobilization (blotting) of resolved proteins in the sieving gel[25], thus maintaining high local protein concentrations for in-gel immunoprobing. Furthermore, photopatterning of hundreds to thousands of 1-mm-long pore-gradient microgels[26] extends scWB to analyses of wide molecular mass ranges[27]. We have characterized cell lysis, PAGE and immunoprobing performance of scWB[19,28], and critical insight from our findings is included throughout this protocol. scWB is designed to be an economical assay compatible with bench-top equipment commonly found in well-equipped life science laboratories and is adaptable to the needs of users seeking to directly assay proteins in single mammalian cells.

Overview of the procedure

The procedure begins with a step-by-step guide to scWB microdevice fabrication, including surface silanization of standard glass microscope slides and epoxy-based negative photoresist (SU-8) mold casting of microwells in a thin PAG layered on the glass microscope slide. Options are provided to create either a PAGE gel of uniform pore size, using chemical polymerization with an SU-8 silicon (Si) mold, or a PAGE gel of gradient pore size, using a grayscale photopatterning technique with an SU-8 glass mold. To facilitate in-gel immunoprobing of immobilized proteins with large antibodies, we also describe an alternative gel formulation that incorporates an acid-labile cross-linker chemistry[27].

Once the microdevice has been fabricated, the scWB assay workflow comprises five stages (**Fig. 1**): sedimentation of cells, in-well chemical cell lysis and protein solubilization, PAGE of each cell lysate, photoactivated protein immobilization and in-gel immunoprobing.

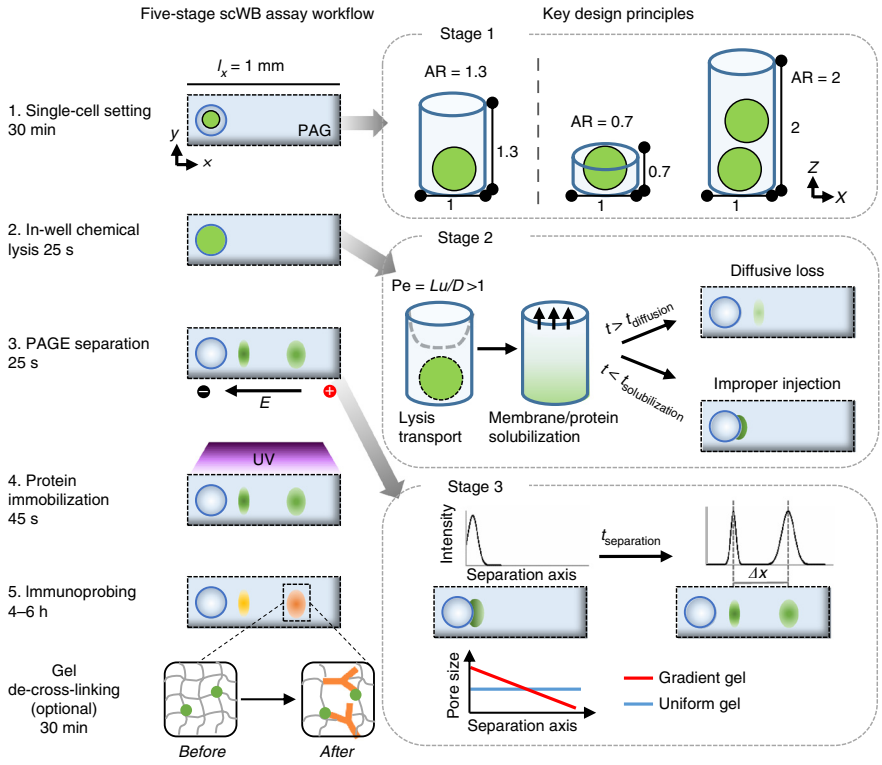

Figure 1 | Single-cell western blotting (scWB) workflow and principles. Left panel: the five-stage assay uses a microwell with surrounding PAG, which acts as both a molecular sieving matrix during PAGE and a blotting scaffold after gel photoactivation. In-gel immunoprobing is performed in the molecular sieving matrix, with or without acid-labile de-cross-linking of the hydrogel. Right panel: design considerations include (Stage 1) optimization of the microwell geometry for single-cell occupancies. An optimal microwell aspect ratio (AR) allows settling of single cells into each microwell (AR ~1.3), whereas shallow microwells (AR ≤ 0.7) allow cells to wash out during the PBS washing step and deep microwells (AR ≥ 2) allow more than one cell to settle into each microwell. (Stage 2) The lysis time (t) is selected to strike a balance between diffusive lysate losses ($t_{diffusion}$) and protein solubilization ($t_{solubilization}$). At the top 2/3 of the microwell (dashed gray line) where the Peclet number (Pe) or ratio of convective to diffusive transport (Lu/D) is >1, the proteins are transported out of the microwell. Here, u is the velocity of the buffer over the microwell, D is the diffusivity of the protein and L is the microwell diameter. With $t > t_{diffusion}$, protein diffusive loss may be observed. When $t > t_{solubilization}$, insoluble protein does not enter the PAG, with the protein retained at the edge of the PAG microwell. (Stage 3) Optimization of the PAGE performance depends on the molecular mass of targets, sample preparation, PAGE duration ($t_{separation}$), PAG pore size and applied electric field strength. Unlike uniform-pore-size gels, the pore size of a pore-gradient microgel decreases from large to small along the separation axis. As with all protein assays, exact conditions must be empirically determined for each application.

Sedimentation of cells. Cells are settled into an array of microwells molded in a thin sheet of PAG layered on a microscope slide. As passive, gravity-based settling seats cells in microwells, the cell size distribution among a population can result in zero, one or multiple cells in each microwell[18]. Matching the dimensions of the microwell to the suspended cells reduces the likelihood of two cells occupying a single microwell after the wash cycles (Experimental design section). Using neural stem cells, we have achieved up to 46% single-cell occupancy[18]. The cell occupancy may not be a Poisson distribution because of size exclusion from the microwells[18]. Microwell occupancy can be correlated to housekeeping protein expression level by scWB[18].

In-well chemical cell lysis and protein solubilization. Application of the lysis/electrophoresis buffer to the cell-laden microwells should be rapid to synchronize initiation of cell lysis across the entire array and so as to minimize diffusive losses of solubilized protein from the open microwells. Numerical modeling of the buffer pouring process (convective buffer addition) for a 30-µm-deep microwell reveals a recirculating flow in the top 2/3 of the microwell, which washes solubilized protein out of the microwells[18]. As demarcated by the dashed gray line in **Figure 1**, this top region supports a Peclet number >1 (Pe = Lu/D, ratio of convective to diffusive transport), which suggests that convective flow dominates diffusion in this region. Here u is the velocity of the buffer over the microwell, D is the diffusivity of the protein and L is the microwell diameter. As described in the Experimental design section, optimization of this step yields sufficiently solubilized proteins while minimizing diffusive losses that can bring the final protein concentration below the limit of detection (LOD).

PAGE of each single-cell lysate into the gel surrounding each microwell. By adding a protein separation (PAGE) step before an immunoassay, the western blot reports distinct physicochemical properties (i.e., molecular mass, charge-to-mass ratio), as well as target recognition by antibody probe. Reporting two characteristics gives the western blot higher selectivity than that afforded by an immunoassay alone. At the most basic level, the PAGE separation spatially separates the target from the confounding background signal.

Photoactivated protein immobilization onto the PAG. Rapid and efficient protein immobilization is required for suitable scWB performance. Photoactivated protein immobilization preserves the protein separation and reduces diffusional losses of protein from the gel and into the bulk buffer solution after electrophoresis. Protein immobilization relies on a UV-initiated hydrogen abstraction reaction to covalently bind proteins to a photoreactive *N*-(3-((4-benzoylphenyl) formamido)propyl) methacrylamide[29] (BPMAC) molecule in the PAG layer. Optimization of benzophenone incorporation and photoactivation is necessary, as described in the Experimental design section below.

In-gel immunoprobing. Immunoprobing of the PAG gel is performed by incubating the gel with solutions of primary antibodies and then with fluorescently labeled secondary antibodies. After incubation, the gel is incubated in a wash solution for >30 min to remove any antibody probes that are not bound to the covalently immobilized protein peaks[18]. At equilibrium, the concentration of probes in the gel layer is lower than that of probes in the solution phase, owing primarily to size-based exclusion of the probe from

TABLE 1 | Benchmarking of single cell–resolution protein assays.

Assay	Multiplexing (targets per cell)	Throughput (number of cells)	Selectivity	Integration with other assays
scWB	~11 targets (through chemical stripping and reprobing)	Thousands	Immunoaffinity and protein sizing	FACS sorting[18] and phenotypic imaging[19]
Immunofluorescence	Five (conventional filter sets[38]; 61 with quenching[39])	100 to thousands	Immunoaffinity alone	Nucleic acid measurement[45]
Mass cytometry	~37 (protein targets, as well as nucleic acid targets[67])	1,000 cells/s[34]	Immunoaffinity alone	Nucleic acid measurement[68]
Barcode of immobilized antibodies	11 cytoplasmic or membrane proteins[36]; 42 secreted proteins[40]	120 cells/device[36]	Immunoaffinity alone	Nucleic acid[48] and metabolomics measurement[49]
Proximity ligation assay	~22 protein targets[41]	Approximately hundreds[41]	Immunoaffinity alone	Nucleic acid measurement[41,44,69] and flow cytometry[70]
Flow cytometry	~17 targets[32]	10,000 minimum; ~20,000/s for FACS	Immunoaffinity alone	Cell sizing[71] and nucleic acid measurement[72]

the gel. We have measured an in-gel probe concentration of <20% of the solution concentration (i.e., $[Ab_{gel}] = K_{partition}[Ab_{solution}]$, where $[Ab_{gel}]$ is the in-gel concentration of the antibody probe, $[Ab_{solution}]$ is the solution concentration of the antibody probe and $K_{partition}$ is the partition coefficient). We have estimated $K_{partition}$ as 0.17 for antibody probing in an 8%T, 2.6%C PAG (where %T is the total amount of acrylamide and %C is the ratio of cross-linker mass to total monomer mass in the gel)[18]. As described in current limitations of scWB, partitioning of antibodies can have an impact on assay sensitivity, and this exclusion effect reduces the range of gel densities/pore sizes suitable for PAGE[28].

Comparison with existing single-cell protein assays

Workhorse single-cell protein assays include immunocytochemistry (ICC), flow cytometry, mass cytometry, proximity ligation assays and antibody barcode assays (**Table 1**)[30–37]. Such techniques report protein expression in single cells with target multiplexing (~10 protein targets) and high throughput (thousands of single cells) to enhance understanding of key cancer signaling pathways and other biological systems[36,37]. Selection of the optimal single-cell proteomic tool is dependent on the question and sample type of interest. Spectral constraints of first-generation immunofluorescence (IF), flow cytometry and immobilized antibody barcode assays limited multiplexing to ~4–5 distinct fluorescent labels[38]. In subsequent generations, multiplexing has been expanded by including chemical quenching in IF (61 targets[39]) and by performing barcoding using spatially separated, immobilized antibodies (11 cytoplasmic and membrane protein targets[36], and 42 secretomic targets[40]). Furthermore, multiplexing has benefited from optimized fluorescence detection in flow cytometry (17 targets[32]) and microfluidic fluid handling in proximity ligation assays (22 targets[41]). Although flow cytometry[33] and mass cytometry[34] have throughputs of thousands of cells per second, substantial cell losses make measuring small starting populations (<1,000 cells) and rare cells difficult[34].

Multiomics approaches for the study of interacting molecular components also benefit from single cell–resolution protein assays (**Table 1**)[42,43]. Both proximity ligation assays and ICC simultaneously detect protein and nucleic acid targets[41,44,45]. Mass cytometry simultaneously measures protein and cellular parameters such as live/dead[46] assays and cell cycle[47]. Finally, antibody barcode chips integrate genomics, metabolomics and targeted proteomic measurements for single cells[48,49].

Although powerful, existing single-cell assays for endogenous, unlabeled proteins rely solely on simple immunoassays (detection based on recognition of a target protein by antibody probes, **Table 1**). Immunoassays perform well only when a highly specific antibody probe for the protein target is available. When an antibody probe recognizes off-target proteins (nonspecific background binding), false signal and false localization can result[21]. In fact, recent studies suggest that nonspecific binding partially underpins irreproducible results (e.g., in 47 of 53 seminal findings in cancer biology[20]). Furthermore, up to 25% of epigenetic marker antibodies bind nonspecifically[22], thus hindering accurate quantification of target proteins. Consequently, advances in high-selectivity protein assays are important either for targets that lack a specific antibody (e.g., myriad protein isoforms[23]) or when background (or an unexpected signal) confounds interpretation.

Advantages and applications of scWB

Adaptability is a hallmark of scWB. By optimizing device and assay parameters, scWB can accommodate a wide range of protein targets, as well as a range of cell lines and dissociated single cells from primary cell samples (Experimental design section). To adapt the target selectivity of scWB, PAGs of uniform pore size are suitable for protein targets spanning 44 to 270 kDa[18]. In a study of differentiation of neural stem cells, scWB performed using a uniform PAG identified off-target antibody binding of a phosphorylated ERK antibody (off-target: 100 kDa; ERK: 44 kDa) and a putative isoform of nestin ($nestin_{\beta}$: 270 kDa; $nestin_{\alpha}$: 114 kDa). The two targets are indistinguishable using ICC[18].

PAGs of gradient pore size are more suitable for protein targets that span a wide molecular mass range and include neighboring targets of similar mass[27,50]. To investigate HER2-related signaling in breast cancer cells, we used a gradient PAG with scWB for targets spanning more than an order of magnitude in molecular mass (four targets from 25 to 289 kDa)[27]. To mitigate nonuniform in-gel immunoprobing observed in gradient gels, we developed

a gel de-cross-linking technique (**Fig. 1**)[27]. The de-cross-linked gel improves in-gel immunoprobing of small-molecular-mass proteins located in PAG regions with small pore sizes (ANTICIPATED RESULTS).

The scWB assay can be adapted to measure multiple protein targets as one assay or as a component of a larger analytical workflow (i.e., concatenated to whole-cell imaging or fluorescence-activated cell sorting (FACS)). Nine reprobing rounds allow one scWB assay to report tens of protein targets from each single cell[18]. After the PAGE separation stage, proteins are covalently bound to the benzophenone-containing PAG via UV-activated protein immobilization (Experimental design section). The robust covalent linkage facilitates serial chemical stripping and reprobing rounds for each gel, as well as long-term storage (~7 months) of the gel[19]. Taken together, these two attributes make iterative biological hypothesis testing on the same cell population possible, unlike one-shot single-cell protein measurements such as flow cytometry and mass cytometry.

The open design of the scWB device allows the assay to be appended to FACS and phenotypic imaging (**Table 1**). In one example, interfacing of scWB with a FACS nozzle allowed us to assay a subpopulation of 200 FACS-sorted cells[18]. In another example, intact human glioblastoma cells (U373 MG) were dosed with a chemotherapeutic (daunomycin) and monitored with whole-cell phenotypic imaging before scWB analysis of signaling proteins[19]. Apoptotic cells were identified via measurements of annexin V by fluorescence imaging and cleaved caspase-8 by scWB[19]. The two data sets—representing a phenotypic intact cell assay and an intracellular signaling assay—with P-glycoprotein quantification stratified cells into normal and drug-resistant subpopulations[19]. Correlation of intact cell phenotype with targeted proteomics signaling data could provide a more sophisticated taxonomy for describing rare cell responses in inherently heterogeneous populations, such as those found in tumors.

Current limitations of scWB

As with any protein assay—including conventional slab-gel western blots—the suitability of scWB is dictated by characteristics of the biological sample and protein targets. With that context in mind, here we restrict our discussion to limitations in throughput, analytical sensitivity and in-gel immunoprobing (the latter being specific to gradient microgels).

Although it is capable of handling small starting cell populations, the maximum throughput of scWB is ~10^3 cells per microdevice (**Table 1**). The throughput maximum is set by a trade-off between the total surface area of a standard microscope slide (75 × 25 mm) and the surface area needed for each single-cell assay (0.4 × 1 mm). Standard microscope slides are compatible with commercial microarray scanners. Although separation distances can be reduced below 1 mm, the peak capacity will concomitantly reduce.

Given a lower LOD of ~27,000 protein copies per cell[18], scWB detects the top 50% of proteins (most abundant) in the mammalian proteome[18,51]. The LOD of scWB is on par with that of conventional flow cytometry (**Table 1**). The estimated LOD is set by the detection modality (fluorescence microarray scanner, fluorophore-labeled secondary antibodies); nonspecific background signal; and diffusive protein loss during lysis and PAGE. To detect low-abundance proteins, inclusion of detection chemistries with high-performance sensitivity, reduction of nonspecific background signal and minimization of diffusive losses are a good starting point.

The use of in-gel immunoprobing presents unique constraints on the scWB assay. Analytical sensitivity and quantification are both affected by the local, in-gel antibody probe concentration. Of primary concern is size-exclusion-based thermodynamic partitioning[52,53], which lowers the local in-gel antibody concentration relative to the free solution concentration[18]. The degree of partitioning of macromolecules into porous hydrogels decreases exponentially with the polymer volume fraction[52]. Depending on the pore size of the gel and antibody binding affinity, partitioning can lower the immunoprobed protein signal below the LOD[27,28]. In scWB, the GFP immunoprobing signal decreased by >10× when gel pore size was decreased from an 8%T PAG to a 12%T PAG[27].

Although the dependence of immunoprobing efficacy on local gel properties is important when designing a uniform-pore-size scWB assay, the in-gel immunoprobing limitation becomes critical when using a gradient in PAG pore size along the PAGE separation axis[27]. To mitigate nonuniform in-gel immunoprobing behavior in this latter case, we developed an acid-labile ketal cross-linker to toggle the gel of nonuniform pore size to a gel of more uniform pore size to reduce the spatial bias of immunoprobing (**Fig. 1**)[27] (see PROCEDURE, Steps 51–55). As an important caveat, de-cross-linking is not recommended for immunoprobing in gels of uniform pore size. As has been reported by others[54] and also observed by us, de-cross-linking of surface-constrained hydrogels of uniform pore size leads to swelling and PAG surface nonuniformities, which, in turn, lead to a nonuniform antibody background that confounds scWB signal interpretation. We have not observed gel deformation after de-cross-linking when applied to gradient microgels.

Experimental design

Optimizing single-cell isolation and settling. Maximizing the number of microwells with single-cell occupancies depends on the physical characteristics of both the cells and the microwells. We recommend that the diameter and depth of the microwells be 1.2–1.5 times larger than the approximate diameter of the cells in suspension, giving an aspect ratio of ~4:3 (depth:diameter; **Fig. 1**). Deeper microwells can hold more than one cell, and shallow microwells result in cells being dislodged during the PBS washing steps, a prime mechanism of cell loss (see PROCEDURE, Step 37). The microwell diameter is controlled by the photomask features, whereas the depth of the microwells is controlled by the height of the SU-8 micropillar. We follow standard photolithography methods for SU-8 mold fabrication[18]. An example mask design file is provided to assist users who do not have computer-aided design experience (**Supplementary Fig. 1**; **Supplementary Data 1**). Alternatively, the SU-8 mold can be purchased from fabrication foundries. We have provided details for SU-8 mold fabrication on a glass wafer in the PROCEDURE, Steps 1–15.

Optimization of *in situ* chemical lysis of cells seated in microwells. To minimize fluid handling in the time-sensitive scWB workflow, we use a dual-function buffer that is suitable for both cell lysis and protein PAGE. The dual-function buffer satisfies the requirements for whole-cell lysis (i.e., ionic detergents, high temperature) and electrophoresis (i.e., low conductivity, alkaline pH). The lysis/electrophoresis buffer includes detergents to disrupt the cell membrane (i.e., SDS, sodium deoxycholate and Triton X-100 buffered with Tris-glycine at pH 8.3)[18]. Reducing agents are omitted, as most intracellular proteins do not contain

disulfide bonds[55]. For some protein targets (e.g., DNA-binding proteins), increasing the SDS concentration to 1% or adding urea can improve protein solubilization. A performance trade-off exists: high SDS concentrations (>2%) may solubilize proteins effectively, yet increase the buffer conductivity, which reduces separation performance, owing to Joule heating of the fluid and enhanced protein diffusivity.

We have experimentally measured diffusion of EGFP into the convective region and out of the microwell and found a loss of 40.2 ± 3.6% protein during the lysis step (at 4 °C lysis)[18,19]. To synchronize cell lysis across the microwell array, the lysis buffer should be poured rapidly (15–20 ml/s) over the array from a height of ~10 cm. Although protein loss increases approximately ninefold with higher temperature (50 °C lysis), separation performance is improved. For example, β-Tubulin (βTub; 50 kDa) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 35 kDa) are unresolvable in a 10%T gel with 4 °C lysis, but they are nearly baseline-resolved with 50 °C lysis (ANTICIPATED RESULTS)[19]. Diffusion of solubilized protein from the microwell during lysis is reduced by using a glass lid[19]. Real-time imaging of membrane solubilization aids in the selection of lysis conditions when using a new cell line or protein target[19] (**Fig. 2**). Thus, real-time imaging of cell lysis coupled with diffusion time-scale estimates yields a starting point for optimization. The goal of optimization is to time the lysis step to balance solubilizing and/or denaturing proteins (as is needed for high-performance separations) while retaining protein within each microwell (as is needed for high-performance detection sensitivity).

Optimization of protein separation by PAGE. To optimize PAGE separation performance, we tune the pore size of the molecular sieving PAG, the PAGE separation axis length and the applied electric potential. A uniform-pore-size gel is suitable when a small number of targets with sufficiently different molecular masses are of interest. A large-pore-size gel effectively sieves large proteins, whereas a small-pore-size gel separates smaller species (**Fig. 3**). When adjusting parameters during single-cell PAGE development,

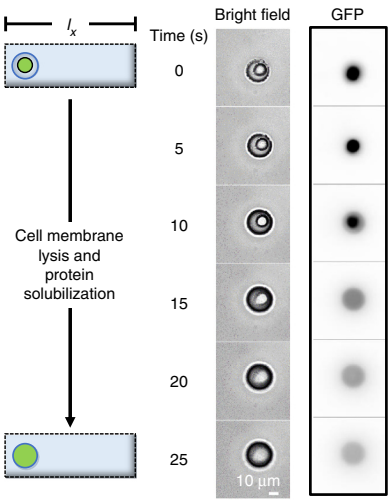

Figure 2 | Real-time imaging of in-well chemical lysis of GFP-expressing U373 glioblastoma cells (U373-GFP). Bright-field and fluorescence imaging of U373-GFP cells show that the GFP protein started to fill the microwell at 15 s, indicating the optimum lysis time (15–25 s) required to lyse the cell membrane and solubilize proteins while reducing diffusive loss of proteins from the protein-permeable microwell[28]. The l_x represents separation axis.

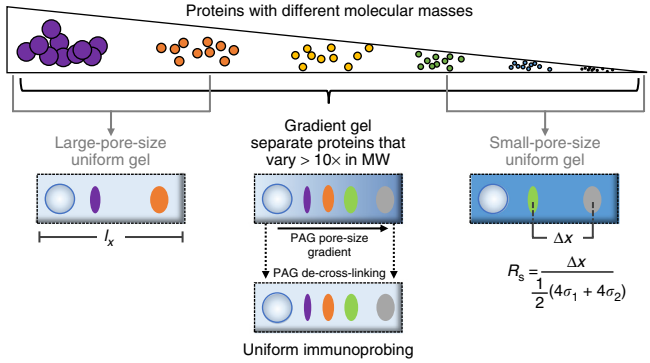

Figure 3 | Selection of suitable PAGE separation conditions. The large-pore-size, uniform PAG can resolve protein pairs having a large molecular mass difference (4%T for proteins >100 kDa; 8%T for proteins ranging from 50 to 300 kDa) within a 1 mm separation lane (l_x). The small-pore-size uniform gel can resolve small-molecular-mass protein pairs (10%T for proteins ranging from 50 to 100 kDa; 12%T for proteins ranging from 20 to 50 kDa). The separation resolution (R_s) is determined by the distances between protein peaks (Δx) and protein band width (4σ).

we quantify the degree to which two neighboring proteins are separated by the nondimensional 'separation resolution' (R_s):

$$R_s = \frac{\Delta x}{\frac{1}{2}(4\sigma_1 + 4\sigma_2)}$$

where Δx is the peak-to-peak displacement and the peak width is 4σ (σ, standard deviation of a Gaussian fit to a protein peak; **Figs. 1** and **3**). When $R_s > 1.5$, two targets are baseline-resolved and thus detectable. Increasing the separation axis length (l_x in **Fig. 3**) for a fixed gel and applied potential increases the peak-to-peak displacement between two targets, Δx, by extending the PAGE duration. If the separation axis length is increased, two corollary considerations arise. First, increasing the length of the PAGE separation axis decreases the pitch of the microwell array and, in turn, decreases the number of microwells (and cells assayed) for a fixed device

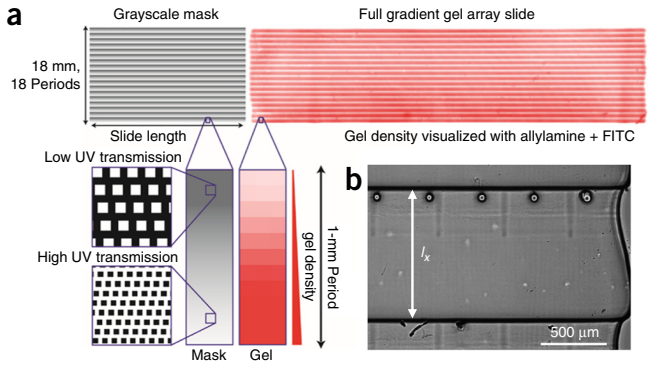

Figure 4 | One-step grayscale photopatterning of a scWB device creates 1-mm-long pore-gradient microgels, with each large-to-small-pore-size gel aligned to a microwell. (**a**) The pore-size gradient is modulated by attenuating the UV dose for gel photopolymerization through a grayscale chrome mask (shown left, in grayscale). Thousands of pore-gradient microgel regions cover the microscope slide (shown right, in red). (**b**) By aligning a micropillar array on a glass SU-8 mold to the grayscale chrome mask, one can fabricate microwells in line with the periodic pore-gradient microgel array. In scWB, the proteins separate along the separation axis (l_x) with a large-to-small-pore-size gradient.

TABLE 2 | Performance and fabrication limitations of various scWB gel formulations.

Sieving matrix	Separation performance: Proteins with small mass range	Separation performance: Proteins with large mass range	In-gel immunoprobing	Probing in dense PAGs > 12%T	Fabrication difficulty	Susceptibility to gel damage with handling
Uniform	Suited	Less suited	Uniform	No	Easier	Low
Pore-gradient	Less suited	Suited	Nonuniform	No	More difficult	Medium
Pore-gradient with de-cross-linking	Less suited	Suited	Uniform	Yes	More difficult	High

footprint (i.e., standard microscope slide). In a uniform-pore-size gel having a 1-mm separation length, we have resolved molecular mass differences of 33% (ref. 19). Second, long-duration PAGE assays can lead to buffer temperature increases (via Joule heating), and thus can exacerbate diffusive protein losses during PAGE—protein losses adversely affect the LOD of scWB.

A pore-gradient microgel is suitable for a complex mixture of targets, some of which differ only slightly in electrophoretic mobility (because of similar molecular masses)[27]. Importantly, a pore-gradient microgel presents a spectrum of sieving conditions and can locally increase Δx even for a heterogeneous sample with a large molecular mass range (**Fig. 3**). Conventional slab-gel pore-gradient gels are fabricated by spatially patterning different polymer precursor solutions[56,57]. In the scWB microdevice, we fabricate pore-gradient gels by spatially varying the polymerization kinetics of a single polymer precursor solution[27]. Grayscale photolithography is one approach to locally controlling polymerization kinetics, across a large area.

In a one-step process, we use an SU-8 glass mold and grayscale photopolymerization technique to create thousands of ~1-mm-long pore-gradient microgels on a microscope slide, with each separation lane precisely aligned with a microwell (**Fig. 4**). Grayscale photopatterning uses a component stack consisting of a UV light source, a UV filter, a glass plate, a chrome mask with a pattern of nonuniform opacity, an SU-8 glass mold for microwell casting, a layer of gel precursor solution and a silanized glass microscope slide (PROCEDURE). Fundamentally, the photopolymerization kinetics and final gel pore size are sensitive to the UV dose at the gel precursor solution; thus, to ensure device-to-device reproducibility, we recommend using a fixed-thickness component stack, and carefully measuring and documenting the UV dose (through the glass plate and SU-8 glass mold) for each fabrication condition. A wide range of pore-gradient microgel profiles are possible, with the note that gels of exceptionally large pore size (<4%T) at the head of the separation axis can compromise the structural integrity of the microwell. Thus, the local PAG pore size can be modulated through acrylamide monomer and cross-linker precursor concentrations; grayscale chrome mask opacity and patterning; and starting UV dose. Important fabrication considerations and limitations of uniform, pore-gradient and pore-gradient de-cross-linking gels are detailed in **Table 2**. The special cross-linker, *N*,*N*-((1-methylethylidene)bis(oxy-2,1-ethanediyl))diacrylamide (abbreviated as diacrylamide ketal, DK) used in the de-cross-linking gel was synthesized in a one-step reaction, and the procedure is described in the **Supplementary Note** (ref. 27).

An R_s value exceeding 1.0 makes objective identification of protein peaks feasible. One way to increase Δx between neighboring peaks—and thus to increase R_s—is by increasing the strength of the applied electric field, E. The electric field for a one-dimensional conductor with homogeneous electrical conductivity and permittivity is $E = \phi / L$, where ϕ is the voltage applied across the conductor and L is the length of the conductor. Thus, E can be increased by either increasing the applied voltage ϕ or reducing the distance L between the electrodes. The primary limitation in increasing E is Joule heating, which is resistive heating generated in the conductive buffer medium. Joule heating reduces separation performance through temperature-mediated increases in analyte diffusivity[58,59]. The heat flux (Q) generated during PAGE for an electrophoresis chamber with a uniform cross section is

$$\dot{Q} = \frac{\Phi^2}{L^2}\sigma_c H$$

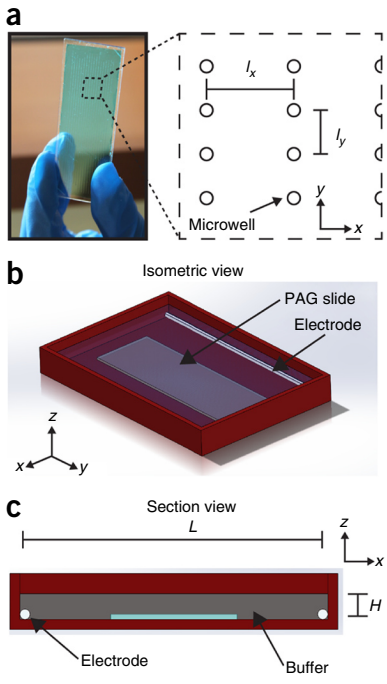

Figure 5 | Optimization of PAGE separation performance depends on the scWB device geometry and electrical interfacing. (**a**) Photograph and schematic of the PAG slide. Increasing the length of the separation axis (l_x) increases the PAGE separation resolution. The *y*-axis pitch (l_y) should be sufficient to minimize diffusive peak overlap from an adjacent lane. We recommend l_y = 400 µm as a good starting value. Rendering of the electrophoresis chamber with (**b**) isometric and (**c**) *x*–*z* plane section views. Decreasing the height (H) of the buffer and the spacing (L) between electrodes applies higher electric fields to the scWB.

Box 1 | scWB training using a purified protein solution ● TIMING 40 min

Several key steps of the scWB assay (lysis, PAGE and UV immobilization) are completed in rapid succession (within 2 min). We suggest that users familiarize themselves with handling the system using a solution of fluorophore-labeled purified proteins before using a cell suspension. Using epifluorescence microscopy, the process of PAGE can be visualized in real time to improve implementation. In addition, practicing with a solution of purified proteins with molecular masses corresponding to the final protein will allow the user to rapidly optimize protein PAGE conditions (time, electric field and PAG pore size). We have used Alexa Fluor-488-labeled purified trypsin inhibitor and Alexa Fluor-555-labeled purified BSA in 7–10%T PAG[19].

To perform scWB PAGE with purified proteins, follow Step 27 of the PROCEDURE for PAG slide fabrication and then proceed to Steps 34–50 with the following modifications:

- Disregard the cell settling (Steps 28–33) and directly incubate a solution of 1 µM purified protein (in PBS) on the PAG layer for 30 min, using the process described in Steps 56–61. The protein solution will preferentially partition into the microwells both before and during the PAGE steps.
- Perform PAGE ~2–5 s immediately after introducing the cold lysis/electrophoresis buffer.
- After protein immobilization, wash the PAG slide in TBST for 30 min (Steps 47–50). The PAG slide should be dried and imaged using a microarray scanner or other fluorescence imaging modality (see Step 70: scWB PAG slide imaging, for the selection of an imaging system).

where σ_c is the conductivity of the buffer and H is the height of the buffer in the electrophoresis chamber[60] (**Fig. 5**). To maximize E while minimizing Joule heating, the conductivity σ_c and height H of the lysis/electrophoresis buffer should be minimized. Ideally, use the minimum lysis/electrophoresis buffer volume required to fully cover the PAG slide and complete the electrical circuit. We typically use a buffer height of ~0.5 cm, an applied electric potential of 200 V and an electrode spacing of 5 cm (see **Supplementary Fig. 2** and **Supplementary Data 2** for the design of the electrophoresis chamber).

Optimization of photoactivated protein immobilization in the PAG layer. We suggest adding the BPMAC at a 3-mM final concentration to the PAG precursor solution and matching the illumination source to the BPMAC absorbance peak at 350–360 nm. Equilibration of the photocapture reaction requires ~45 s in the scWB PAG matrix using UV exposures of ~40 mW cm^{-2} (~1.8 J cm^{-2})[24]. A lower UV dose may require longer photocapture equilibration times, thus increasing protein loss from the gel. Notably, photocapture efficiency (fraction of analyte covalently bound to the PAG matrix) is affected by pH, protein state and molecular mass[24,25]. Higher photocapture is observed for proteins at basic pH[25], for proteins treated with SDS and for proteins of larger molecular mass (97.5 ± 0.7% for a 116-kDa protein versus 75.2 ± 0.8% for a 21-kDa protein)[24].

Recommended positive and negative controls for scWB. Before embarking on a full-scale experiment, we recommend that users familiarize themselves with the whole apparatus by performing electrophoretic separations of purified proteins, as described in **Box 1**. Furthermore, to validate cell lysis and protein PAGE (i.e., the power supply is functional, the electrophoresis buffer formulation is correct and the gel fabrication is successful), we recommend running a positive control using a GFP-expressing cell line that is closely related to the cell type of interest and imaging the lysis and PAGE process, as shown in **Figure 2**. When performing the full scWB assay, probing for housekeeping proteins (e.g., βTub and GAPDH) is a critical positive control that demonstrates cell lysis, protein PAGE, protein immobilization and subsequent immunoprobing functioned properly. In addition, to confirm putative protein peaks, ensure that R^2 is ≥ 0.7 for the Gaussian fit and signal-to-noise ratio (SNR) is ≥ 3, as described in the PROCEDURE. If available, cell lines that do and do not express a target of interest can be used as a positive and negative control, respectively.

MATERIALS

REAGENTS

- Applicable cell line: the human U373 glioblastoma cell line (U373 MG) and U373-GFP cell line are used in this protocol. The U373-GFP cell line is comprised of U373 MG cells stably transduced with GFP by lentiviral infection (multiplicity of infection = 10). Both U373 MG and U373-GFP cell lines were kindly provided by S. Kumar's laboratory[19]. In this protocol, we show both successful and failed scWB experiments performed using U373 MG cells (ANTICIPATED RESULTS). We have also demonstrated that scWB can be successfully applied in other mouse (i.e., mouse neural stem cell[18]) and human cell lines (i.e., breast cancer cell SKBR3 (ref. 27), as well as clinical human breast cancer samples[27] **! CAUTION** The 'U373' human glioblastoma cell line used in this study is not the original U373 but a misidentified U251 human glioblastoma cell line. The 'U373' is genetically nondistinct from the U251 based on the criteria of the American Type Culture Collection (ATCC), the International Cell Line Authentication Committee and other references[61,62]. We used the GFP protein in U373-GFP cells for system characterization only, without any biological interpretation (i.e., real-time in-well lysis (**Fig. 2**) and comparison of uniform and pore-gradient scWB (ANTICIPATED RESULTS))
  ! CAUTION Cell lines should be regularly checked to ensure that the cells are neither misidentified nor infected with mycoplasma.
- 3-(Trimethoxysilyl)propyl methacrylate (98%, Sigma-Aldrich, cat. no. 440159) **! CAUTION** 3-(Trimethoxysilyl)propyl methacrylate is combustible. Handle it with gloves in a chemical fume hood.
- Acetic acid (ACS grade, ≥99.7%, Sigma-Aldrich, cat. no. 695092)
  ! CAUTION Acetic acid is highly flammable, and it may cause severe skin burns and eye damage. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- Methanol (ACS grade, ≥99.7%; Sigma-Aldrich, cat. no. 179337)
  ! CAUTION Methanol is highly flammable and is toxic on inhalation, on contact with skin and if swallowed. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- Tetramethylethylenediamine (TEMED; Sigma-Aldrich, cat. no. T9281)
  ! CAUTION TEMED is highly flammable, is corrosive and is toxic on inhalation, on contact with skin and if swallowed. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.

- Ammonium persulfate (APS; Sigma-Aldrich, cat. no. A3678)
- β-Mercaptoethanol (Sigma-Aldrich, cat. no. M3148) **! CAUTION** β-Mercaptoethanol is toxic on inhalation, on contact with skin and if swallowed, and it is hazardous to the aquatic environment. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- 2,2-Azobis(2-methyl-*N*-(2-hydroxyethyl) propionamide) (VA-086; Wako Chemical)
- *N*,*N*-((1-methylethylidene)bis(oxy-2,1-ethanediyl))diacrylamide (DK) was synthesized in-house[27].
- Acrylamide/bis-acrylamide, 30% (wt/wt) solution (BioReagent, suitable for electrophoresis, 37.5:1; Sigma-Aldrich, cat. no. A3699) **! CAUTION** This material is highly toxic, carcinogenic and teratogenic. Avoid direct contact, and review and understand all Material Safety Data Sheet (MSDS) information.
- Acrylamide/bis-acrylamide, 40% (wt/wt) solution (BioReagent, suitable for electrophoresis, 29:1; Sigma-Aldrich, cat. no. A7802) **! CAUTION** This material is highly toxic, carcinogenic and teratogenic. Avoid direct contact, and review and understand all MSDS information.
- Acrylamide, 40% (wt/wt) solution (for electrophoresis; Sigma-Aldrich, cat. no. A4058) **! CAUTION** This material is highly toxic, carcinogenic and teratogenic. Avoid direct contact, and review and understand all MSDS information.
- Triton X-100 (Fisher Scientific, cat. no. BP-151)
- SDS (BioReagent, suitable for electrophoresis, for molecular biology, ≥98.5% (wt/vol); Sigma-Aldrich, cat. no. L3771) **! CAUTION** SDS is a flammable solid and is harmful if swallowed or inhaled. Avoid contact with skin and eyes, and handle it with gloves.
- Sodium deoxycholate (≥97% (wt/vol), Sigma-Aldrich, cat. no. D6750) **! CAUTION** Sodium deoxycholate is harmful if swallowed or if inhaled. Avoid contact with skin and eyes, and handle it with gloves.
- 0.5 M Tris-HCl, pH 6.8 (Teknova, cat. no. T1568)
- 1.5 M Tris-HCl, pH 8.8 (Teknova, cat. no. T1588)
- Premixed 25× Tris-glycine transfer buffer (Novex, cat. no. LC3675)
- 10× Tris-CAPS Anode Buffer (Boston BioProducts, cat. no. BP-192)
- Premixed 10× Tris-glycine electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine; Bio-Rad, cat. no. 161-0734)
- Deionized water (ddH_2O, 18.2 MΩ, obtained using an ultrapure water system from, e.g., Millipore)
- BPMAC, *N*-(3-((3-benzoylphenyl)formamido)propyl) methacrylamide can be custom-synthesized by PharmAgra Labs (cat. no. PAL0603)[19,27,28] or a positional isomer (para-form, *N*-(3-((4-benzoylphenyl)formamido)propyl) methacrylamide) of the BPMAC was synthesized in-house[18,24].
- Tris-buffered saline with Tween 20 (20× TBST, Santa Cruz Biotechnology, cat. no. sc-281695)
- PBS, pH 7.4 (Gibco, cat. no. 10010-023)
- BSA (heat-shock fraction, protease free, fatty acid free, essentially globulin free, pH 7, ≥98% (wt/vol); Sigma-Aldrich, cat. no. A7030)
- Petroleum jelly (Cumberland Swan Petroleum Jelly, cat. no. 18-999-1829)
- Gel Slick solution (Lonza, cat. no. 50640)
- Photoresist SU-8 2025 (MicroChem, cat. no. Y111069) **! CAUTION** SU-8 2025 is flammable, and it may cause severe skin and eye irritation. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- SU-8 developer (MicroChem, cat. no. Y020100) **! CAUTION** SU-8 developer is toxic, combustible and flammable, and it may cause severe skin and eye irritation. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- 75% (wt/vol) titanium diisopropoxide bis(acetylacetonate) in isopropanol (Sigma-Aldrich, cat. no. 325252) **! CAUTION** Titanium acetylacetonate is flammable and has acute toxicity (oral, dermal, inhalation). Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- Isopropyl alcohol (Sigma-Aldrich, cat. no. W292907) **! CAUTION** Isopropyl alcohol is flammable, and it may cause skin and eye irritation. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- Acetone (Sigma-Aldrich, cat. no. 320110) **! CAUTION** Acetone is flammable, and it may cause skin and eye irritation. Avoid contact with skin, eyes and clothing, and handle it with gloves in a chemical fume hood.
- 36.5–38% (wt/wt%) Hydrochloric acid (HCl; Fisher Scientific, cat. no. A144) **! CAUTION** Concentrated HCl is a strong acid, and it causes burns by all exposure routes. Use it only under a chemical fume hood with proper personal protective equipment.
- Appropriate antibodies.

EQUIPMENT

- Standard cell culture equipment, including laminar flow hood (e.g., SterilGARD III; The Baker Company, cat. no. SG-603), humidified tissue culture incubators at 37 °C and 5% CO_2 (e.g., Heracell 150i; Thermo Fisher Scientific), water bath at 37 °C (e.g., Isotemp; Thermo Fisher Scientific), refrigerator (e.g., Sanyo Labcool) and freezer (e.g., StableTemp; Cole-Parmer) to grow cells
- Standard cell culture disposables, including sterile tissue culture flasks (e.g., BioLite 75-cm^2 flask; Thermo Fisher Scientific) and sterile serological pipettes (e.g., 5-ml serological pipettes; Falcon, cat. no. 356543) to grow cells
- 10-cm or 15-cm Petri dishes (VWR, cat. no. 25384-342)
- Bath sonicator (Bransonic 220; Branson Ultrasonics)
- Vacuum line and nitrogen gas line
- Centrifuge (Thermo Scientific, Sorvall ST 8 Small Benchtop Centrifuge, cat. no. 75007200)
- Centrifuge tubes, 15 ml (Thermo Scientific Nunc, 15 ml conical sterile polypropylene centrifuge tubes, cat. no. 339651)
- SU-8 Si mold for uniform gel and glass mold for pore-gradient microgel (see **Supplementary Fig. 1** for 1:1 scaled mask design and **Supplementary Data 1** for original .dxf file). The SU-8 glass mold fabrication details have been described previously[27] and are detailed in the PROCEDURE. The mold is precoated with GelSlick before use to increase the hydrophobicity.
- Gel electrophoresis chamber (see **Supplementary Fig. 2** for 1:1 scaled electrophoresis chamber design and **Supplementary Data 2** for original .stl file). The scWB electrophoresis chamber can be fabricated out of acrylonitrile butadiene styrene with a fused deposition molding three-dimensional printer (e.g., MakerBot Replicator 2×).
- Rotator (Thermo Scientific, Compact Digital Waving Rotator, cat. no. 88880021)
- Razor blades (VWR, cat. no. 55411-050) **! CAUTION** Razor blades are sharp. Handle them with care.
- Plain glass slide (25 × 75 × 1 mm; VWR, cat. no. 16004-422)
- Diamond scribing pen (Ted Pella, cat. no. 54468)
- Slide breaker (e.g., Fletcher's Running and Nipping Pliers for Glass & Acrylic, Fletcher-Terry Company, cat. no. 06-111)
- Cell strainer (Falcon tube with cell strainer cap, 35-μm polyester (PET) filter, BD, cat. no. 352235)
- Light microscope (MFL-06 Duo-scope Microscope)
- UV illumination system (Hamamatsu Lightning Cure LC5) **! CAUTION** UV light is hazardous. Appropriate personal protective equipment should be worn while using a UV source.
- Imaging software (Metamorph)
- Power supply (Bio-Rad Powerpac Basic)
- Four-well rectangular slide plate (Thermo Scientific NUNC, cat. no. 267061)
- Five-place slide mailer (Heathrow Scientific, cat. no. HS159836)
- Fluorescence microarray scanner (i.e., Genepix 4300A)
- Epi-fluorescence microscope system, including microscope (Olympus IX71 inverted fluorescence microscope), CCD camera (Andor, iXon+ EMCCD camera) and mercury lamp light source (Lumen Dynamics, X-cite)
- Image analysis software (NIH ImageJ)
- Mini centrifuge (VWR, Galaxy mini centrifuge)
- Manual staining assembly—staining dish and rack handle (Thermo Scientific, cat. no. 110) or 20 slide glass staining dish with removable slide rack (Wheaton 900200)
- Microscope slide storage box (VWR, cat. no. 82003)
- Chrome mask (aBeam Technologies). The design and purchase of the chrome mask have been described for fabricating pore-gradient microgel[27].
- Near-UV light source (OAI). An upward-facing UV source is required to follow the alignment protocol described here.
- Long-pass filter plastic sheet (390 nm, no. 39–426, Edmund Optics)
- Spin coater (Brewer Science)
- Mask aligner (OAI Series 200 aligner, San Jose, California)
- 3-Inch-diameter silicon wafer (University Wafer, cat. no. 1196)
- 3-Inch-diameter glass wafer (University Wafer, cat. no. 1610)
- Ultrapure water system, e.g., Millipore
- Nitrogen gun
- Diamond-tipped pen

REAGENT SETUP

10% (wt/vol) APS Dissolve 10 mg of APS in 100 μl of ddH_2O. Store the solution at 4 °C for short-term (<7 d) storage. ▲ **CRITICAL** Freshly prepare the solution before use.

10% (vol/vol) TEMED Dissolve 10 µl of TEMED in 90 µl of ddH_2O. Store it at 4 °C for short-term (<7 d) storage. ▲ CRITICAL Freshly prepare the solution before use.
5% (wt/vol) SDS Dissolve 0.5 g of SDS in 10 ml of ddH_2O. Store it at room temperature (20–25 °C). The maximum recommended storage time is 6 months.
5% (vol/vol) Triton X-100 Dissolve 0.5 ml of Triton X-100 in 10 ml of ddH_2O. Store it at room temperature. The maximum recommended storage time is 6 months.
100 mM BPMAC Dissolve 70.048 mg of BPMAC in 2 ml of DMSO. Prepare one 100-µl aliquot per 0.65-ml Eppendorf tube. Store aliquots at −20 °C. Shield the solution from light. Avoid freeze and thaw cycles. The maximum recommended storage time is 3 months.
1× TBST 1× TBST is used as a washing buffer for in-gel immunoprobing steps. Add 50 ml of 20× TBST to 950 ml of ddH_2O to make a 1× TBST solution. Store the buffer solution at 4 °C. The final concentration of Tween 20 in 1× TBST is 0.05%. The maximum recommended storage time is 3 months.
2% (wt/vol) BSA/TBST Antibody dilution buffer for in-gel immunoprobing steps. Dissolve 2 g of BSA in 100 ml of 1× TBST. Store the solution at 4 °C. The maximum recommended storage time is 3 months.
3% (wt/vol) VA-086 photo-initiator solution Dissolve 30 mg of VA-086 in 1 ml of ddH_2O. ▲ CRITICAL Freshly prepare the solution before use.
1× Tris-CAPS solution Add 50 ml of 10× Tris-CAPS to 450 ml of ddH_2O to make a 1× Tris-CAPS solution. Store the buffer solution at room temperature. The maximum recommended storage time is 3 months.
1% (vol/vol) HCl solution Add 5 ml of concentrated HCl (36.5–38% (wt/wt%)) to 495 ml of ddH_2O. Store the 1% (vol/vol) HCl solution in a secondary container in the corrosive cabinet of the fume hood. The maximum recommended storage time is 6 months.
400 mM DK solution Dissolve 108.1 mg of lyophilized DK (270.32 Da) in 1 ml of 1× Tris-CAPS solution. Shake the solution until the contents have dissolved. Prepare aliquots of the solution in microcentrifuge tubes. Store the tubes in a dark environment at −20 °C for long-term storage. The maximum recommended storage time is 3 months.
Silane solution For a 400-ml silane solution, add 80 ml of 3-(trimethoxysilyl)propyl methacrylate and 120 ml of acetic acid to 200 ml of ddH_2O in a glass Erlenmeyer flask. Mix the solution well and degas it in the sonicator. ▲ CRITICAL Freshly prepare the solution before use.
Dual-function lysis/electrophoresis buffer For a 500-ml lysis/electrophoresis buffer solution, add 25 ml of 10× Tris-glycine electrophoresis buffer (final concentration, 0.5×), 2.5 g of SDS (final concentration, 0.5% (wt/vol)), 1.25 g of sodium deoxycholate (final concentration, 0.25% (wt/vol)) and 500 µl of Triton X-100 (final concentration, 0.1% (vol/vol)) to 474.5 ml of ddH_2O in a 500-ml glass container. Dissolve the contents and mix the buffer well before use. Store it at 4 °C. The maximum recommended storage time is 3 months.
Harsh stripping buffer For a 100-ml harsh stripping buffer solution, add 12.5 ml of 0.5 M Tris-HCl pH 6.8 buffer (final concentration, 62.5 mM Tris-HCl), 2 g of SDS (final concentration, 2% (wt/vol)) and 800 µl of β-mercaptoethanol (final concentration, 0.8% (vol/vol)) to 87 ml of ddH_2O in a 100-ml glass container. Dissolve the contents and mix the buffer well before use. Store the buffer at room temperature in a chemical fume hood. The maximum recommended storage time is 3 months.
Gel precursor solution For a 10%T, 2.7 %C gel precursor solution, add 25 µl of 1.5M Tris-HCl pH 8.8 buffer (final concentration, 75 mM Tris-HCl), 166.7 µl of 30%T acrylamide/bis-acrylamide solution and 15 µl of 100 mM BPMAC (final concentration, 3 mM) to 265.3 µl of ddH_2O in a 1.5-ml Eppendorf tube. Vortex to mix the solution. Spin down at 2,000*g* and room temperature for 3 s to remove solution from the cap of the Eppendorf tube by using a mini centrifuge, then carefully puncture the cap of the Eppendorf tube with a syringe needle and attach a vacuum line to degas the solution in a sonicator for 6 min. Add 10 µl of 5% (wt/vol) SDS (final concentration, 0.1% (vol/vol)), 10 µl of 5% (vol/vol) Triton X-100 (final concentration, 0.1% (vol/vol)), 4 µl of 10% (wt/vol) APS (final concentration, 0.08 % (vol/vol)) and 4 µl of 10% (vol/vol) TEMED (final concentration, 0.08% (vol/vol)) to the degassed gel precursor solution. Mix the solution well without introducing bubbles and load it immediately in between the glass slide and the wafer. ▲ CRITICAL Freshly prepare the solution before use.
Pore-gradient microgel precursor solution For an 11%T, 3.3%C pore-gradient precursor solution, add 183.3 µl of 30%T acrylamide/bis-acrylamide solution, 166.7 µl of 3% (wt/vol) VA-086 (final concentration, 1% (wt/vol)) and 15 µl of 100 mM BPMAC (final concentration, 3 mM) to 135 µl of ddH_2O in a 1.5-ml Eppendorf tube. Vortex to mix the solution. Spin down at 2,000*g* and room temperature for 3 s to remove solution from the cap of the Eppendorf tube by using a mini centrifuge, then carefully puncture the cap of the Eppendorf tube with a syringe needle and attach a vacuum line to degas the solution in a sonicator for 6 min. ▲ CRITICAL Freshly prepare the solution before use.
99:1 De-cross-linking gel precursor solution For a 12%T pore-gradient precursor solution with a molar cross-linker ratio of 99:1 DK to methylene bisacrylamide, add 20 µl of 25× Tris-glycine transfer buffer (1× final concentration), 138.4 µl of 40%T acrylamide solution, 2.7 µl of 40%T acrylamide/bis-acrylamide solution (233 nmol of bisacrylamide), 166.7 µl of 3% (wt/vol) VA-086 (final concentration, 1% (wt/vol)), 57.6 µl of 400 mM diacrylamide ketal (23.1 µmol DK) and 15 µl of 100 mM BPMAC (final concentration, 3 mM) to 99.6 µl of ddH_2O in a 1.5-ml Eppendorf tube. Vortex to mix the solution. Spin down at 2,000*g* and room temperature for 3 s to remove the solution in the Eppendorf cap by using a mini centrifuge, then carefully puncture the cap of the Eppendorf tube with a syringe needle and attach a vacuum line to degas the solution in a sonicator for 6 min. ▲ CRITICAL A 99:1 de-cross-linking gel precursor solution is critical to the success of the experiments. Freshly prepare the solution before use.
Adhesion primer The adhesion primer used here is 2% (wt/vol) titanium acetylacetonate. Dilute the 75% (wt/vol) titanium diisopropoxide bis(acetylacetonate) in anhydrous isopropanol. ▲ CRITICAL Freshly prepare the primer before use.

PROCEDURE

Glass–SU-8 fabrication (optional) ● TIMING ~1 h per wafer

! CAUTION The SU-8 photoresist, SU-8 developer, acetone and isopropyl alcohol are all toxic and flammable. Perform all the steps inside a chemical fume hood with the sash lowered and wear the proper personal protection equipment, including a fire-resistant lab coat and nitrile gloves. It is recommended that the fabrication be performed in a cleanroom to prevent contamination by dust particles.

▲ CRITICAL Here we detail the steps for fabricating ~30-µm-height SU-8 micropillars on top of a glass wafer using SU-8 2025, as used in previous publications[27,63]. We recommend using SU-8 2025 for fabricating a 30- to 60-µm height. Additional details about SU-8 are available from the MicroChem website (http://www.microchem.com/pdf/SU-2000DataSheet2025thru2075Ver4.pdf), including a standard procedure for Si-SU-8 fabrication, which should be used instead of Steps 1–15 if scWB is to be performed using uniform microgels.

1| Dehydrate a 3-inch glass wafer by placing it on a 110 °C hot plate for a minimum of 10 min. Once it is dehydrated, transfer the 3-inch glass wafer to the chuck center of the spin coater. Apply a vacuum and verify that the wafer is strongly adhered to the chuck.
▲ CRITICAL STEP A clean glass wafer is extremely important for subsequent uniform SU-8 coating. The wafer can be cleaned by rinsing it with acetone, followed by isopropyl alcohol. The wafer should be fully dehydrated before coating.

2| Use the dropper to uniformly distribute the adhesion primer on the wafer.

3| After 30 s in contact with the glass wafer, remove the adhesion primer by spinning, as shown in the table below:

Recommended coating condition	Spinning speed (r.p.m.)	Acceleration (r.p.m./s)	Time (s)
Distribute adhesion primer on the wafer	500	100	5
	3,000	500	30

4| Immediately after the spin is complete, pour ~5 ml of SU-8 2025 onto the wafer and spin as shown in the table below to create a uniform 30-µm base SU-8 layer on the wafer:

Recommended coating condition	Spinning speed (r.p.m.)	Acceleration (r.p.m./s)	Time (s)
Distribute SU-8 evenly on the wafer	500	100	30
Achieve the desired SU-8 height	2,500	500	30

5| Soft-bake the wafer on a 110 °C hot plate for 3 min. Allow the wafer to cool to room temperature before proceeding to Step 6.

6| Place the wafer on the mask aligner and expose it at 360 nm for 250 mJ cm^{-2}. No photolithography mask is applied in this step, as the purpose is to create a uniform SU-8 base layer.
▲ **CRITICAL STEP** The exact UV exposure power and time is instrument-dependent. The UV exposure dose recommended here follows the MicroChem guidelines.

7| Bake the wafer on a 110 °C hot plate for 5 min. Allow the wafer to cool to room temperature before proceeding to Step 8.

8| Place the wafer on the chuck center of the spin coater and make sure that the wafer adheres by applying a vacuum.

9| Pour ~5 ml of SU-8 2025 onto the wafer and spin as shown in the table below to create a second uniform 30-µm-height SU-8 layer on the wafer:

Recommended coating condition	Spinning speed (r.p.m.)	Acceleration (r.p.m./s)	Time (s)
Distribute SU-8 evenly on the wafer	500	100	30
Achieve the desired SU-8 height	2,500	500	30

10| Soft-bake the wafer on a 65 °C hot plate for 6 min. Allow the wafer to cool to room temperature before proceeding to Step 11.

11| Place the wafer on the mask aligner and expose it at 365 nm for 250 mJ cm^{-2} through the desired mask.
▲ **CRITICAL STEP** For the U373 glioblastoma cell line used to generate the data in the ANTICIPATED RESULTS, we designed the photolithography mask to have microwells with a 30-µm diameter. An example mask design file is provided to assist users who do not have computer-aided design experience (**Fig. 5a**; **Supplementary Fig. 1**; **Supplementary Data 1**).

12| Place the wafer on a 65 °C hot plate for 3 min. Ramp the hot plate up to 90 °C and hold that temperature for 7 min. Afterward, allow the wafer to cool to room temperature before proceeding to Step 13.

13| Prepare a SU-8 developer bath for development and place it on a rotator. Submerge the wafer with the SU-8 side facing up. Gently swirl the SU-8 developer bath for 2–2.5 min.
▲ **CRITICAL STEP** Over- and underdeveloping the SU-8 will lead to deformed micropillars. To check whether the development is complete, remove the wafer from the SU-8 developer bath and spray it with isopropyl alcohol. If a white film appears during the isopropyl alcohol rinse, additional development time is required. Perform development in 20-s increments. For fine SU-8 features, we recommend using the spray bottle to spray the SU-8 developer on the wafer for an extra 30 s.

14| After development, rinse the wafer with water and dry it with a nitrogen gun.

15| Place the wafer on a 110 °C hot plate to hard-bake for 10–15 min. Allow the wafer to slowly cool to room temperature.
▲ **CRITICAL STEP** Performing the hard-bake step will increase wafer durability. Ensure that the wafer is cooled down slowly; otherwise, the wafer may crack due to the thermal stress.

Batch silanization of glass slides ● TIMING 40–50 min per batch
! CAUTION The 3-(trimethoxysilyl)propyl methacrylate, methanol and acetic acid are all flammable, and methanol is toxic. Perform Steps 19–24 inside a chemical fume hood with a lowered sash and while wearing proper personal protection equipment, including a fire-resistant lab coat and nitrile gloves.
16| Score the corner of the glass microscope slides (75 × 25 mm) with a diamond-tipped pen. The mark will reference the methacrylate-functionalized glass side (silanized slide), which is facing down when the score is positioned in the upper right corner of the slide.

17| Arrange the marked microscope slides in a removable slide rack (i.e., 30-slot rack) and place the slide rack in a glass slide staining dish. Two glass slides can be placed back to back in a single slot. In this configuration, ensure that the side to be silanized is in contact with the silane solution.

18| Prepare the silane solution and degas it for 30 min.

19| Pour the degassed silane solution into the glass slide staining dish. Ensure that the silane solution fully covers all the slides.

20| Tap the glass slide staining dish to remove all the bubbles trapped in between the slides. Let the silanization reaction proceed for 30 min.

21| Remove the slide rack and place it in a glass slide staining dish containing methanol. Hold the handle of the slide rack and gently shake it (with the glass slides submerged in methanol) to remove any residual silane solution.

22| Repeat the washing step in Step 21 with fresh methanol.

23| Remove the slide rack from the methanol solution and place it in a container containing fresh ddH_2O. Hold the handle of the slide rack and gently shake it (with the glass slides submerged in ddH_2O) to remove methanol.

24| Repeat Steps 22 and 23 two more times in the same methanol and ddH_2O containers.

25| Place the slides in a container with fresh ddH_2O. One at a time, remove the slides from the ddH_2O and quickly dry them with a nitrogen gun.

26| Store the silanized slides in a standard slide storage box. The silane solution can be reused to coat a total of three batches of glass slides.
■ **PAUSE POINT** The silanized slides can be stored at room temperature for up to 2 months.

scWB PAG slide fabrication ● TIMING 20–30 min per slide
27| Based on the required PAGE separation conditions (**Fig. 3**), the user should prepare either uniform (option A) or pore-gradient (option B) PAG slides.
(A) Uniform scWB PAG slide fabrication ● TIMING 20–30 min per slide
(i) Tape an SU-8 mold onto the lab bench to hold it in place during processing.
(ii) Make the gel precursor solution and degas it with a bath sonicator and vacuum line to eliminate bubbles (**Fig. 6a**).
(iii) Add detergents (SDS and Triton X-100) and initiators (APS and TEMED) to the gel precursor solution and mix it well without forming bubbles. Pipette a 250-µl droplet near one of the short edges of the SU-8 mold (**Fig. 6b**).
(iv) Hold a silanized slide with the silanized side facing down. Lower one of the short edges until the silanized slide is in contact with the SU-8 mold and the precursor droplet. The slide should be at an ~30° angle. Slowly lower the slide until the silanized slide is flat against the SU-8 mold (**Fig. 6c**).
(v) After loading, press gently on the slide to squeeze excess precursor from the gap and to ensure that the micropillars on the SU-8 mold are in contact with the slide (**Fig. 6d**). Depending on the amount of the initiator added, the chemical polymerization process can be completed in ~15 min.
? TROUBLESHOOTING

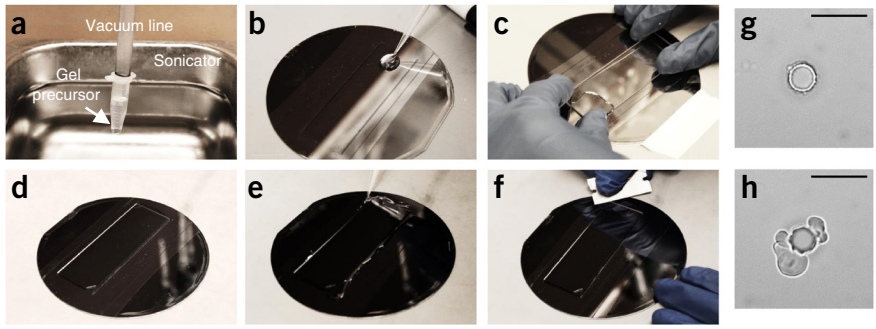

Figure 6 | scWB PAG slide fabrication. (**a**) Step 27A(ii): degas the gel precursor solution. (**b**) Step 27A(iii): pipette the gel precursor solution onto the SU-8 mold. (**c**) Step 27A(iv): place a silanized slide against the SU-8 mold. (**d**) Step 27A(v): gently press the silanized slide to remove excess solution. (**e**) Step 27A(vi): rehydrate the edge of the slide with PBS. (**f**) Step 27A(vii): detach the PAG slide from the SU-8 mold with a razor blade. Fabrication of the PAG slide is complete. Bright-field micrographs show a well-formed (**g**) and deformed (**h**) microwell. Scale bars, 100 µm. The PAG slide in the image is a standard microscope slide (25 × 75 mm).

(vi) After chemical polymerization of the PAG, add 1–2 ml of PBS to rehydrate the edge of the slide and facilitate release of the slide from the SU-8 mold (**Fig. 6e**).

(vii) Gently slide a razor blade underneath the slide, and lift the blade straight up to detach the PAG slide from the SU-8 mold (**Fig. 6f**).

! CAUTION The gel precursor solution, containing acrylamide, APS and TEMED, is acutely toxic following oral or inhalation exposure, and is a skin irritant. The acrylamide is also neurotoxic. The SU-8 mold needs to be rinsed in running ddH_2O after each use to remove any residual precursor solution.

? TROUBLESHOOTING

(viii) Check the PAG slide under a bright-field microscope to ensure the integrity of the microwells (**Fig. 6g**,**h**).

? TROUBLESHOOTING

(ix) Place the PAG slide in a rectangular four-well slide plate filled with PBS before use. The side of the slide with the PAG layer should face up.

■ PAUSE POINT The PAG slides can be stored in PBS at 4 °C for up to a week before use.

(B) Pore-gradient scWB PAG slide fabrication ● TIMING 15–20 min per slide

(i) Tape a chrome mask onto the borosilicate glass plate (chrome side facing up) to hold it in place during the alignment and exposure steps (**Fig. 7a**).

(ii) Place the hydrophobic SU-8 glass mold on top of the gradient chrome mask with the SU-8 micropillars facing up.

(iii) Pipette a droplet of the pore-gradient gel precursor solution or 99:1 de-cross-linking gel precursor solution atop the SU-8 glass mold near one of its short edges (similar placement to that depicted in **Fig. 6b**). The volume of the droplet should be sufficient to fill the mold when the glass slide is added, with little to no excess. A 150-µl droplet volume is typical, but it will vary with the height of the micropillars on the SU-8 mold and the size of the silanized slide used.

(iv) Hold a silanized slide with the silanized side facing down. Lower one of the short edges until the silanized slide is in contact with the SU-8 mold and the precursor droplet. The slide should be at an ~30° angle. Slowly lower the slide until the silanized slide is flat against the SU-8 glass mold.

(v) After placing the silanized slide atop the mold, press gently on the slide to squeeze excess precursor from the gap and to ensure that the micropillars on the SU-8 mold are in contact with the slide. Remove excess precursor solution with a task wipe.

▲ CRITICAL STEP Excess solution can lead to the silanized slide slipping on the mold when the setup is moved—resulting in a misaligned array.

(vi) Holding the base borosilicate glass plate, slowly transfer the plate to a bright-field microscope stage.

(vii) First by eye, align the micropillars on the SU-8 glass mold with the grayscale gradient on the chrome mask (**Fig. 7b**). After rough alignment, use the microscope to verify the alignment and adjust as necessary. Confirm on both the left and right sides of the array that the micropillars are similarly aligned to the grayscale mask (as demonstrated in **Fig. 7c**).

▲ CRITICAL STEP Misalignment of the SU-8 micropillars and the grayscale mask opacity gradient will result in fabrication of PAG separation lanes that each have a different pore-gradient microgel relative to each respective microwell, thus resulting in unwanted protein mobility variation among the different PAGE separation lanes (**Fig. 7d**).

(viii) Carefully move the entire assembly onto the UV system. If the mold slips with respect to the mask, repeat the previous alignment step (Step 27B(vii)).

(ix) Apply UV light to photopolymerize the pore-gradient microgel. From the UV light source, the component stack should be arranged in the following order: UV filter, glass plate, chrome mask, SU-8 glass mold, gel precursor solution and silanized slide.

▲ CRITICAL STEP Optimization of the UV exposure conditions will be required for each new condition. UV exposures that are of insufficient duration or intensity will result in under-photopolymerization of the pore-gradient microgel. Underpolymerized gels will lead to deformation of the microwell and poor cell settling in the deformed microwells (**Fig. 7e**). Over-photopolymerization of the pore-gradient microgel will create a small pore size at the head of the microgel, thus resulting in incomplete electrophoretic injection of protein into the PAG.

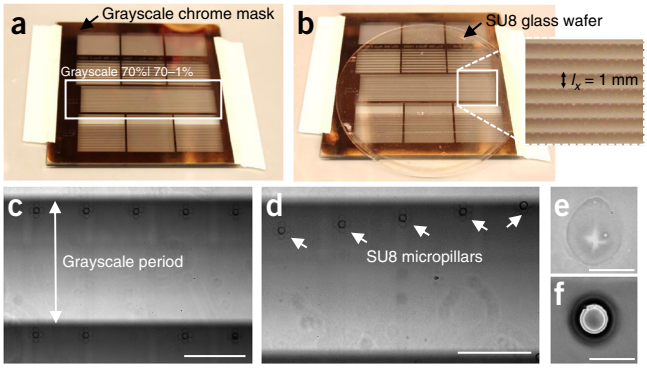

Figure 7 | Pore-gradient PAG slide fabrication. (**a**) Step 27B(i): tape the chrome mask in place. (**b**) Step 27B(vii): align the SU-8 glass mold to the chrome mask. Determine whether the SU-8 micropillars are aligned (**c**) or misaligned (**d**) to the grayscale gradient; scale bars, 500 µm. The arrows point to SU-8 micropillars under the microscope. After photopolymerization, a bright-field micrograph shows a shallow and deformed microwell (**e**) that suggests under-photopolymerization of the PAG near the microwell; scale bar, 100 µm. (**f**) Bright-field micrograph shows a circular, well-formed microwell, indicating suitable photopolymerization conditions; scale bar, 100 µm.

(x) Carefully move the whole assembly to the benchtop.

(ix) Gently slide a razor blade underneath the slide and lift straight up to detach the PAG slide from the SU-8 mold.

(xii) Check the pore-gradient PAG slide under a bright-field microscope to ensure the integrity of the microwells (**Fig. 7e**,**f**). **? TROUBLESHOOTING**

(xiii) Place the pore-gradient PAG slide in a rectangular four-well slide plate with the gel side facing up. If the pore-gradient microgel contains DK cross-linker (DK PAG slide), fill the plates with the 1× Tris-CAPS buffer solution. Exchange the Tris-CAPS buffer with PBS before proceeding to cell settling (Steps 28–33).
■ **PAUSE POINT** The gradient PAG slides can be stored in buffer at 4 °C for up to a week before use. scWB of pore-gradient PAG slides is performed using the same procedures (Steps 28–50) as for uniform PAG slides.

Settling of cells into microwells ● TIMING 30–60 min

▲ **CRITICAL** When working with mammalian cells, Steps 28–33 should be performed in a biohazard cabinet in an enclosed laboratory facility. All work with cell lines should comply with institutional and governmental biosafety regulations.

28| Use an ~80% confluent monolayer of cells in a T75 flask. With respect to U373 glioblastoma cells, an 80% confluent T75 flask corresponds to ~2 × 10^6 cells. As a total of 2 × 10^5 cells gives adequate settling per PAG slide, one T75 flask is sufficient to perform ten different scWB experiments. A full microscope slide (75 mm x 25 mm) is used as a standard here; however, a half slide (37.5 × 25 mm) can also be used for rapid prototyping. A half slide can be made by using a diamond scribe to score a silanized slide and a slide breaker to break the slide in half either before or after PAG polymerization.

29| Harvest the cells from the flask using a general tissue culture procedure. Pellet the cells by centrifugation and resuspend the cells in ice-cold PBS with a concentration of ~1 × 10^6 cells per ml.

30| If applicable, stain the cells for cell imaging using standard methods[19]—e.g., nucleus staining (Hoechst 33342) or apoptotic cell staining (Alexa Fluor-647-conjugated annexin V).

31| Filter the cell suspension through a cell strainer with a 35-µm PET filter cap to create a single-cell suspension.
▲ **CRITICAL STEP** Obtaining a single-cell suspension is important for settling cells at one cell per microwell occupancies, as aggregated cells are excluded from the microwells during settling (**Fig. 8a**). If cell clumping or aggregation is observed, filter the cell suspension through the cell strainer. The selection of the filter cap is cell-size dependent.

32| Remove the PAG slides (from Step 27A or B) from PBS. With a task wipe, dry the glass side of the PAG slide. Place the slide in a Petri dish with the PAG side up.

33| Pipette 200 µl of filtered cell suspension (containing ~2 × 10^5 cells) and gently disperse the cell suspension on top of a PAG slide. Periodically, check the cell settling efficiency using bright-field microscopy (**Fig. 8b**). The cell settling efficiency (microwell occupancy) is defined in **Box 2**. Gently tap the Petri dish to agitate the cell suspension. The 10 min of settling is sufficient for most cell lines tested. Place the Petri dish with cells on ice to maintain cell viability.
▲ **CRITICAL STEP** Continue directly to the 'Single-cell PAGE and photoblotting' procedure (Steps 34–50). Some protein expression and/or post-translational modifications may be altered over time[64,65].
? TROUBLESHOOTING

Single-cell PAGE and photoblotting ● TIMING 5–10 min per slide

! CAUTION Appropriate precautions should be taken to protect users from UV exposure.

34| Turn on the UV illumination system in advance to stabilize the UV light bulb. Set the power to a UV dose sufficient for subsequent protein photocapture to the PAG (Experimental design section).

35| Transfer 10–15 ml of lysis/electrophoresis buffer to a 15-ml conical tube and preheat it in a 50–55 °C water bath.

36| After confirming sufficient cell settling under a bright-field microscope (Step 33), remove excess cell suspension by tilting the PAG slide at a 45° angle.

37| Gently apply a steady stream of 1 ml of PBS by pipetting to the higher edge of the tilted PAG slide to gently wash excess cells off the surface of the PAG slide (**Fig. 8c**).

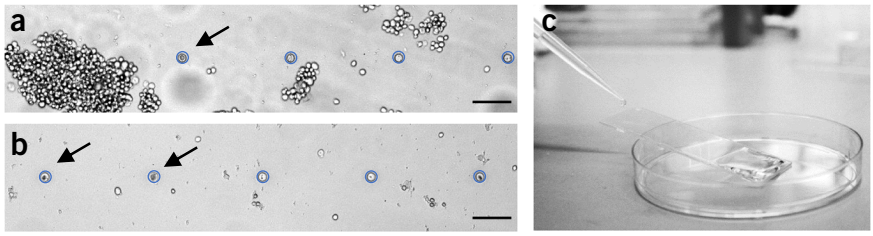

Figure 8 | Examples of poor and ideal single-cell settling into microwells. (**a**) Bright-field micrograph of microwell array and cells shows that U373 cells may aggregate during settling and lead to unfavorable cell settling into the microwells. (**b**) Bright-field micrograph of microwell array and cells shows good cell settling into microwells with limited cell clumping. Scale bars, 100 µm; blue circles indicate microwells, and arrows point to microwells with a single cell per microwell occupancy. (**c**) Step 37: PBS wash of excess cells from the PAG slide.

38| Verify by visual inspection with a light microscope that the majority of cells on the surface have been removed (**Fig. 8b**). Repeat the rinsing process (Step 37) if excess cells are observed on the gel surface.
▲ **CRITICAL STEP** Do not tilt the scWB slide to more than 90°, flush the PBS solution rigorously or flush the PBS solution down the middle of the PAG slide, as these handling processes may remove settled cells from the microwells.
? TROUBLESHOOTING

39| Apply a 1-cm-diameter spot of petroleum jelly to the bottom of the scWB electrophoresis chamber to temporarily adhere the PAG slide to the chamber.

40| Place the PAG slide in the scWB electrophoresis chamber with the PAG layer facing up.

41| If applicable, perform whole-cell imaging (**Box 2**).

42| Connect the electrophoresis chamber to the high-voltage power supply.

43| Set up the electrophoresis power supply to provide a constant voltage. The suggested E is 40 V cm^{-1}.

44| Pour 50 °C preheated lysis/electrophoresis buffer rapidly over the PAG slide to fill the electrophoresis chamber. Immediately upon pouring, start timing the lysis duration.
▲ **CRITICAL STEP** The lysis/electrophoresis buffer should be applied quickly so that cells lyse simultaneously. Abrupt pouring of the lysis/electrophoresis buffer from >15 cm above the electrophoresis chamber in a fully vertical orientation may dislodge cells and wash out the protein lysate. We suggest pouring the lysis/electrophoresis buffer from the short side of the electrophoresis chamber at a ~30° tilt to minimize the number of dislodged cells[19].

45| At 25 s of lysis duration, apply the electric field to initiate PAGE. For separating GAPDH and βTub in a 10%T, 2.7%C PAG, a 30-s electrophoresis duration is a good starting point.
! CAUTION The high-voltage power supply can be the source of a lethal electrical current. Please consult the instruction manual before use and use proper safety precautions. Confirm that the power supply is properly and safely connected to the electrophoresis chamber.

46| Immediately at PAGE completion, apply UV light (350–360 nm, ~1.8 J cm^{-2})[18] to immobilize the separated proteins in the PAG.
! CAUTION Use proper UV protection, such as UV-blocking goggles and UV-blocking face shields to protect the user and surrounding personnel from UV illumination.
! CAUTION Confirm that the electric field is turned off immediately after electrophoresis and before handling the electrophoresis chamber.
▲ **CRITICAL STEP** Apply UV to immobilize the proteins immediately after PAGE; otherwise, the proteins will rapidly diffuse out of the PAG and adversely affect LOD.

47| After protein immobilization, carefully remove the PAG slide from the electrophoresis chamber using tweezers.

48| Using a task wipe, wipe off the petroleum jelly from the glass side of the PAG slide.

Box 2 | Whole-cell imaging in microwells ● TIMING variable

After cell settling, we recommend imaging the intact cells to count the number of cells in each microwell (occupancy). To perform whole-cell imaging in the microwells, place the PAG slide onto a microscope stage adaptor with the gel layer facing up. Pipette ~1 ml of PBS onto the PAG layer to keep the cells hydrated during the image acquisition. An automated, motorized stage with commercial software to stitch individual images into a composite image of an entire microscope slide (e.g., MetaMorph SlideScan or Zeiss ZEN Tiles features) should be used. A 4× objective is sufficient to determine cell microwell occupancy (i.e., one, two or more cells in a single microwell). The total time required for imaging is dependent on the magnification, imaging area and speed of the motorized stage. The cell settling efficiency (microwell occupancy) is defined as the number of cell-occupied microwells normalized to total microwells in a scWB PAG slide. Besides cell counting, whole-cell imaging with a specific cell stain (i.e., live or apoptotic cell staining) can provide the correlation of phenotypic traits with proteomic readouts from subsequent scWB of cell lysate[19]. After imaging, remove excess PBS by tilting the PAG slide, and then place the PAG slide into the electrophoretic chamber and continue to 'Single-cell PAGE and photoblotting' (Steps 41–50).

49| Place the PAG slide into a rectangular four-well slide plate filled with TBST buffer.

50| Place the plate on the rotator for at least 30 min to remove residual lysis/electrophoresis buffer, as residual SDS will affect antibody and antigen binding during the subsequent in-gel immunoprobing process (Steps 56–69).
■ **PAUSE POINT** The PAG slide can be stored in 1× TBST at 4 °C for short-term storage (~1 week). For long-term storage (>1 week), rinse the PAG slide three times with ddH_2O to remove salts retained on the PAG slide, gently blow-dry by nitrogen gun and store the slide in a slide mailer at room temperature protected from light.

De-cross-linking of DK PAG slides ● TIMING 30 min
! **CAUTION** A 1% (vol/vol) HCl, pH 1.1, solution is used here to de-cross-link the DK PAG slide. Always handle the solution in a secondary container inside the fume hood while using personal protective equipment.
▲ **CRITICAL** Perform Steps 51–55 only if you are using DK PAG slides. For uniform or pore-gradient PAG slides fabricated using methylene bisacrylamide and no DK, proceed directly to in-gel immunoprobing (Steps 56–69).
51| Place the DK PAG slides into a glass container with 1% (vol/vol) HCl.

52| Incubate the DK PAG slides for 30 min to complete the de-cross-linking reaction[27].
▲ **CRITICAL STEP** Performing the de-cross-linking reaction for <10 min may result in partially de-cross-linked DK gels, resulting in spatially nonuniform immunoprobing.

53| Carefully remove and place the DK PAG slides into a Petri dish filled with ddH_2O. Gently rinse three to four times.

54| Carefully remove and place the DK PAG slides into a rectangular four-well slide plate filled with TBST buffer.

55| Place the plate on the rotator for at least 30 min for buffer equilibration before performing in-gel immunoprobing (Steps 56–69).
■ **PAUSE POINT** The DK PAG slides can be stored in 1× TBST at 4 °C for short-term storage (~1 week). For long-term storage (>1 week), rinse the DK PAG slides three times with ddH_2O to remove salts, gently blow-dry with a nitrogen gun and store each DK PAG slide in a slide mailer at room temperature protected from light.

In-gel immunoprobing ● TIMING 4–6 h
56| Prepare 100 µl of primary antibody immunoprobing solution by diluting stock solution of primary antibodies in 2% (wt/vol) BSA/TBST solution. 1–5 µg of primary antibody per slide is a recommended starting mass, although each assay must be optimized. Place the prepared primary antibody solution on ice before use. The identity of primary and secondary antibodies used in our reported scWB studies are listed in **Supplementary Table 1**.

57| To perform immunoprobing on a single PAG slide, place two pieces of tape slightly shorter than the width of the PAG slide onto a clean surface, such as a plain 50 × 75 mm glass slide. The tape pieces will act as spacers to hold the scWB slide slightly off the glass surface, creating a gap in which the Ab solution will wick in via capillary action. It is noted that two PAG slides can be immunoprobed simultaneously with the gel sides facing each other.

58| Remove the PAG slide from the TBST and remove excess TBST by tilting the slide and wiping the glass side of the PAG slide with a task wipe. Do not touch the gel side of the PAG slide, as damage to the PAG may result.

59| Place the PAG slide onto the tape with the gel side facing down (**Fig. 9a**).

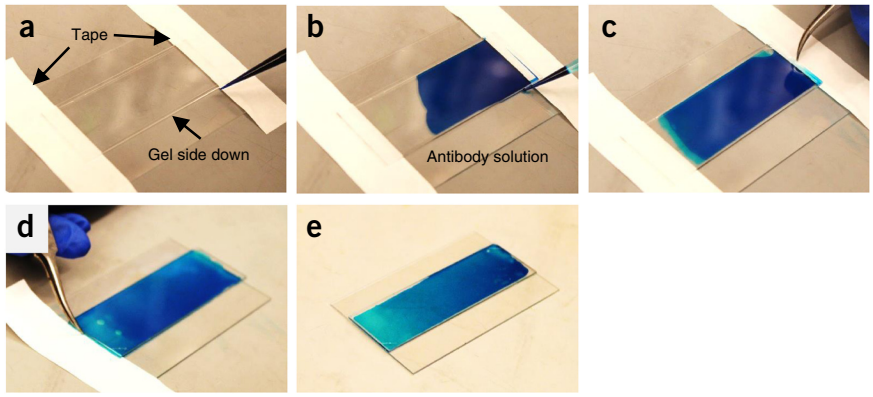

Figure 9 | Handling of the scWB device during immunoprobing. Bright-field images of blue dye visualize the scWB immunoprobing handling procedure. (**a**) Step 59: place lab tape on a clean glass surface and place the short sides of the slide on the tape to lift the PAG slide up with the gel side facing down. (**b**) Step 60: use capillary action to load the antibody solution between the two layers. (**c**–**e**) Step 61: remove tape strips to release the scWB device for the next assay steps. The PAG slide in the image is a standard microscope slide (25 × 75 mm).

60| Inject prepared primary antibody solution by pipetting into the air-filled gap between the PAG slide and the glass (**Fig. 9b**).

61| Gently move both sides of the tape off the glass to deliver a uniform antibody solution (**Fig. 9c**–**e**).
▲ **CRITICAL STEP** Do not create bubbles in the gap, which will result in local antibody depletion and increased immunoprobing variance.

62| Incubate the primary antibody at room temperature for 1–2 h. This time interval is a general recommendation, with the exact duration determined by optimization.

63| Gently remove the PAG slides and place them in a rectangular four-well slide plate filled with TBST buffer.

64| Place the rectangular four-well slide plate on a rotator for a 10- to 30-min wash cycle. This time interval is a general recommendation, with the exact duration determined by optimization.

65| Repeat the washing steps (Steps 63–64) twice more using fresh TBST.

66| Prepare 100 µl of secondary antibody immunoprobing solution per pair of slides by diluting the stock solution of the secondary antibody in 2% (wt/vol) BSA/TBST solution. 1–5 µg of secondary antibody is the recommended starting mass, although each assay must be optimized. Place the prepared secondary antibody solution on ice before use.

67| Repeat Steps 57–62 to prepare the slides for secondary antibody probing. Incubate the slides at room temperature for 1 h.
▲ **CRITICAL STEP** Fluorophore-conjugated secondary antibodies are used, so cover the slides with aluminum foil to shield them from light exposure.

68| Perform TBST washing, as described in Steps 63–65.

69| Remove TBST and rinse the PAG slides with ddH_2O three times to remove salts contained on the PAG slides. Gently blow-dry the slides with a nitrogen gun. The PAG slides are ready for imaging.
? TROUBLESHOOTING
■ **PAUSE POINT** The PAG slides can be stored dehydrated in a slide mailer at room temperature, protected from light, for more than 4 months with negligible probing signal reduction[19].

scWB PAG slide imaging ● TIMING varies with imaging apparatus
70| After incubation of the scWB PAG slide with primary and fluorescently labeled secondary antibodies, quantify proteins by fluorescence imaging. The scWB PAG slide can be imaged with a fluorescence imaging system such as a fluorescence microarray scanner or inverted epifluorescence microscope. The imaging system should have an ~5 µm/pixel spatial resolution; compatibility with a standard microscope slide and an adjustable *z* axis (depth) focus; a robust image stitching algorithm, as stitching artifacts will affect quantification; and a LOD down to ~0.5 nM for Alexa-Fluor-labeled secondary antibodies (27,000 molecules in a 50-µm-diameter spot in a 40-µm-thick gel), thus suitable for detection of ~50% of the mammalian proteome[18]. Representative imaging data are shown in the ANTICIPATED RESULTS.

scWB PAG slide stripping ● TIMING 30 min
71| Place the PAG slide into the slide mailer.

72| Fill the slide mailer with harsh stripping buffer. Tightly close the slide mailer cap.
! CAUTION β-Mercaptoethanol is acutely toxic following oral, inhalation or dermal exposure. Always handle the solution in a fume hood while using personal protective equipment. Seal the slide mailer to prevent solution leakage during the stripping process.

73| Place the slide mailer in a 55 °C water bath for at least 30 min. Ensure that the level of the water bath is higher than that of the harsh stripping buffer inside the slide mailer in order to achieve uniform stripping.
? TROUBLESHOOTING

74| Remove the slide mailer from the water bath. Pour the harsh stripping buffer into a labeled waste container.
! CAUTION β-Mercaptoethanol is toxic with chronic exposure and is also acutely toxic to aquatic life. Please consult your institutional safety guidelines for the appropriate disposal method.

75| Rinse the PAG slides at least three times with ddH_2O.
▲ CRITICAL STEP Image the PAG slides after stripping to ensure thorough removal of the antibodies (i.e., undetectable protein signal or SNR <3).

76| If intending to perform another round of immunoprobing, place the PAG slides in a rectangular four-well slide plate with TBST on the shaker for at least 30 min to reconstitute the proteins before repeating Steps 56–69. Otherwise, gently blow-dry the slides with a nitrogen gun and store them in a slide mailer at room temperature, shielded from light exposure. Slides can be probed up to nine times[18].
■ PAUSE POINT The PAG slides can be stored dehydrated for more than 7 months for future reprobing rounds[19].

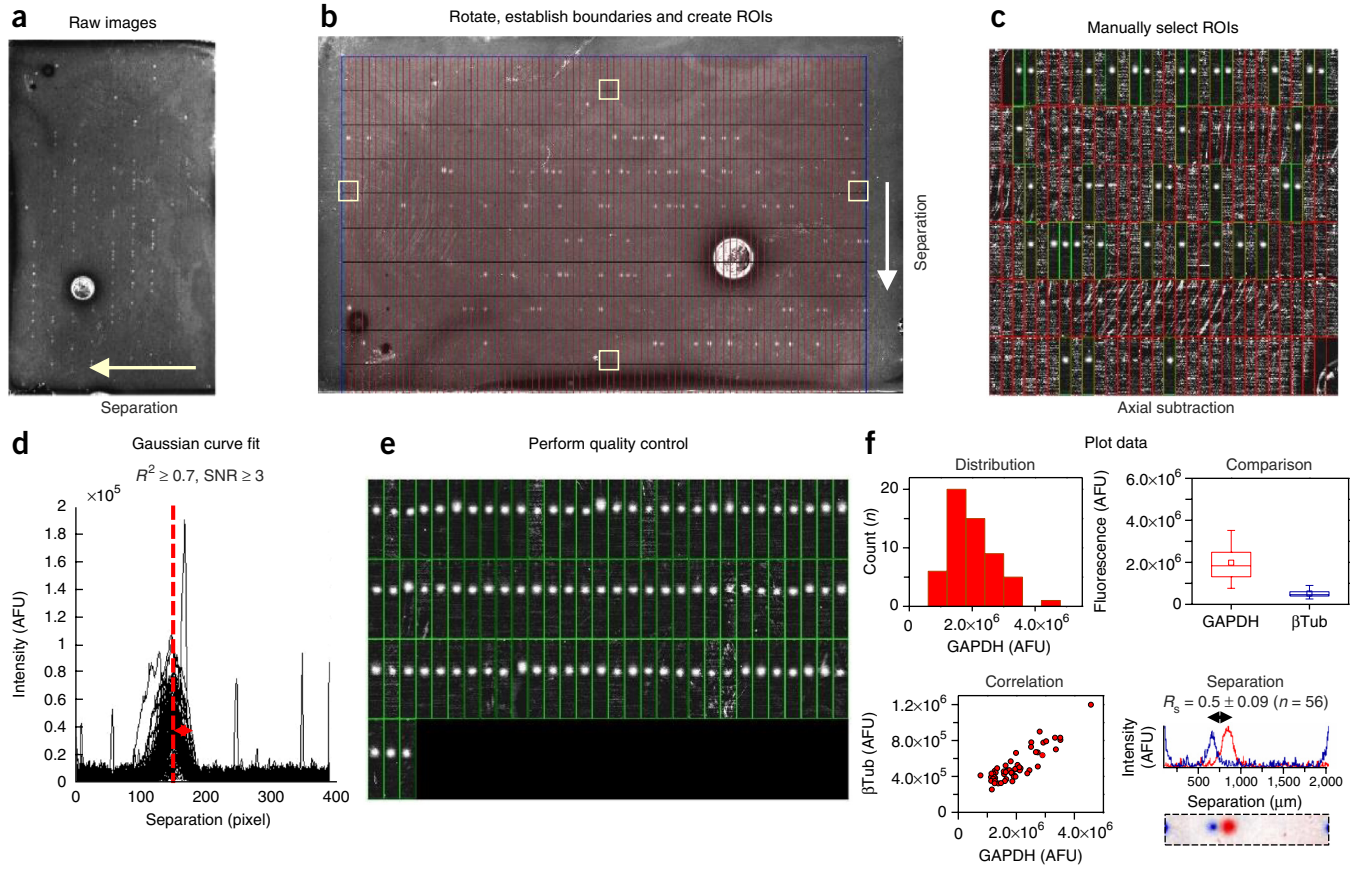

Figure 10 | The scWB image analysis workflow. (**a**) Step 77: a raw image of the GAPDH scWB signal is acquired from a fluorescence microarray scanner. (**b**) Steps 78–80: using in-house MATLAB code, the image is rotated and segmented into ROIs. (**c**,**d**) Steps 82 and 83: after manual selection of ROIs, Gaussian curve fitting extracts assay parameters (including peak location, peak width and area under the curve from individual separations). (**e**) Step 84: ROI images are selected based on acceptable curve fitting ($R^2 \geq 0.7$ and SNR ≥ 3). (**f**) Step 85: finally, the individual protein data collected from each single cell is plotted for analysis. AFU: arbitrary fluorescence unit. In scWB, 1 pixel corresponds to 5 μm.

scWB image analysis (optional) ● **TIMING ~1 h per slide with multiple protein targets**

▲ **CRITICAL** Here we describe an algorithm for analyzing the scWB data with an automated analysis script. The scWB data can also be analyzed one separation at a time using ImageJ or custom code using MATLAB, R scripts or other analysis platforms.

77| *Open raw scWB image files*. Start with the raw images (**Fig. 10a**). Images should be full resolution and bit depth to ensure accurate quantification. The image produced from the microarray scanner used in this protocol is an ~ 80 Mb TIFF file with an ~25 × 75 mm image (5,000 × 15,000 pixels, with 5 μm per pixel).

78| *Align the image*. Using the microwells as fiducial markers, align all images of a single slide (i.e., all slide scans). Use an 'affine transform' to map all images to a reference. Image alignment is commonly performed with MATLAB (Image Processing Toolbox) or ImageJ (Landmark Correspondences).

79| *Define array boundaries*. scWB is an array format, so the boundaries are the topmost and bottommost, as well as the furthest left and furthest right microwells (**Fig. 10b**).

80| *Create regions of interest (ROIs)*. Inter-microwell spacing is uniform across the scWB array. Therefore, the array can be segmented into ROIs of identical size, where each ROI corresponds to one single-cell protein separation.

81| *Perform background subtraction*. To quantify proteins in the scWB array, we recommend local background subtraction based on the protein intensity profile within each ROI. The background subtraction can be performed by either subtracting the average background intensity for the entire ROI (averaged subtraction) or by subtracting the intensity in the background signal at each pixel along the separation axis (axial subtraction). For scWB separations with pore-gradient microgels, we recommend axial background subtraction, owing to the different background signal along the separation axis[27].

82| *Manually select ROIs with housekeeping proteins (optional)* (**Fig. 10c**). While optional, we recommend creating an ROI mask to identify microwells containing cells. Eliminating from further analysis all microwells that do not contain cells saves subsequent analysis time and aids analysis of weak or absent protein target signals (e.g., positive GAPDH, but negative HER2 signal in MCF-7 cells).

83| *Perform Gaussian fitting* (**Fig. 10d**). During protein PAGE, the protein diffuses as it migrates through the sieving matrix. Protein peaks are typically Gaussian distributions along the separation axis[66]. For each ROI, fit a Gaussian distribution to the intensity profile along the separation axis. The protein peak center, peak height, peak width (4σ) and area under curve (AUC) can now be extracted from the Gaussian fitting. The protein expression is measured from the AUC within 4σ. The R_s between two target proteins is calculated as described earlier (Experimental design section).

84| *Perform quality control* (**Fig. 10e**). After fitting, verify the results by viewing images selected from the best fits (e.g., $R^2 \geq 0.7$, SNR ≥ 3).

85| *Plot data* (**Fig. 10f**). Plot single-cell-level protein data in histograms to show protein distributions; in box plots to compare the expression of two targets or different experimental conditions; and in scatter plots to view correlation between two targets. Further statistical analyses can be used based on a user's needs and specific questions.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
27A(v)	There are bubbles trapped in the PAG	The glass slide or the wafer is not clean	Confirm that both the glass slide and the wafer are free of dust. The glass slide can be cleaned with methanol and then rinsed with distilled water and blown dry
		The gel precursor solution is not fully degassed	Confirm that there are no bubbles in the gel precursor solution before use
27A(vii)	The PAG peels off of the glass slide	The glass slide is not fully silanized	Confirm that the glass slides are clean and methacrylate-functionalized before use

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
	The PAG adheres to the SU-8 mold	The SU-8 mold does not have a complete layer of dichlorodimethyl silane	Confirm that the SU-8 mold is silanized before use
	The PAG does not polymerize	Wrong concentration or old APS or TEMED is used for polymerization	Prepare fresh APS or TEMED
		O_2 inhibits polymerization	Confirm that the gel precursor solution is degassed or perform polymerization under N_2 purge
27A(viii)	Microwells are deformed (**Fig. 6h**)	The glass slide moves during polymerization	Ensure that no component moves during polymerization
		The PAG slide moves during detachment from the SU-8 mold	Hold the SU-8 mold in place while peeling up the gel
27B(xii)	Shallow microwells (**Fig. 7e**)	Low UV dose	Increase the UV exposure time, UV intensity or the concentration of the gel precursor solution
	Deformed microwells	The SU-8 micropillars are misaligned to the grayscale gradient period (**Fig. 7d**)	Confirm that the SU-8 micropillars are aligned to the grayscale gradient using bright-field microscopy
		The SU-8 micropillars are aligned to the edge of the grayscale gradient	Confirm that the SU-8 micropillars are aligned 20–50 μm from the edge of the grayscale gradient (**Fig. 7c**)
33	Poor cell settling	Not enough cells are used for settling on the PAG slide	Prepare the cell suspension at a higher cell density
		Cell clumping	Filter the cells through a cell strainer multiple times and settle the cells immediately after filtering
		The microwell size and depth are not optimized	Fabricate a range of microwell diameters to select the microwell size for the cell size under study
38	Poor cell settling	Too many cells are rinsed off during washing	Gently rinse off excess cells during the washing step and check frequently under the microscope
69	No probing signal	Poor cell settling	Optimize the cell density and microwell geometry
		The lysis time is too long	Increased lysis time will increase protein diffusional loss during lysis; lysis time-dependent experiments may be needed to optimize this parameter
		No UV photo-immobilization	Confirm that the BPMAC is fresh and added into the gel precursor solution during polymerization
			Confirm that the UV light is on and the UV dose for protein immobilization is optimized. A housekeeping protein with high abundance can be used as a positive control
		Primary antibodies are not target-specific	Verify the specificity and species reactivity of the primary antibody
		Secondary antibodies do not recognize primary antibodies	Confirm that species-specific secondary antibodies are used
		Low-affinity antibody	Increase the primary antibody concentration

(continued)

TABLE 3 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
		Low-abundance protein	Identify cell lines with higher expression levels of the target protein as positive controls
		The PAG slides are overstripped	Confirm the harsh stripping buffer composition and temperature. Reduce the stripping time, if needed
		The detection system does not match the fluorophore used	Verify that the excitation wavelength and emission filter cube are suitable for the secondary antibodies used; also verify that the focal plane is correct
	Unexpected punctate signal	Aggregates from the secondary antibodies	Spin down the secondary antibodies (e.g., 10,000*g*) before use
		Dust on the PAG slide	Confirm that the PAG slides are probed and washed in a clean environment
	High background	Primary or secondary antibody concentration is too high	Use a lower concentration of the primary or secondary antibodies
		Insufficient washing	Wash the PAG slides with fresh TBST 2–3 times after each incubation step
	Poor protein injection	The PAG pore size is too small for large proteins	Reduce and optimize the PAG pore size
	Dispersed protein signal	Insufficient cell lysis or protein solubilization	Slightly increase the cell lysis time or increase the SDS concentration
	Protein electro-migrates to an unexpected location	Insufficient solubilization or dissociation of protein complexes	Slightly increase the cell lysis time or increase the SDS concentration
	Off-target probed signal	The primary or secondary antibodies are not target-specific	Verify the specificity and species reactivity of the antibodies
	Protein overrun to the next row of microwells	The electrophoresis time is too long	Slightly reduce the electrophoresis time
	Target protein bands are not resolved	Insufficient cell lysis or protein solubilization	Slightly increase the cell lysis time or increase the SDS concentration
		The PAG pore size or electrophoresis time is not optimized	Optimize the PAG pore size and electrophoresis time to fit the target proteins
73	Detectable protein signal after stripping	The antibodies are not fully removed during the stripping process	Increase the stripping time
			Prepare fresh harsh stripping buffer and reapply
			Confirm that the water level of the water bath is higher than the level of harsh stripping buffer solution in the slide mailer
			Confirm that the temperature is 55 °C

● TIMING

Steps 1–15, glass-SU-8 fabrication (optional): ~1 h per wafer
Steps 16–26, batch silanization of glass slides: 40–50 min per batch
Step 27, scWB PAG slide fabrication: 20–30 min per slide
Steps 28–33, settling of cells into microwells: 30–60 min

Steps 34–50, single-cell PAGE and photoblotting: 5–10 min per slide
Steps 51–55, de-cross-linking of DK PAG slides: 30 min
Steps 56–69, in-gel immunoprobing: 4–6 h
Step 70, scWB PAG slide imaging: time varies with imaging apparatus
Steps 71–76, scWB PAG slide stripping: 30 min
Steps 77–85, scWB image analysis (optional): ~1 h per slide with multiple protein targets
Box 1, scWB training using a purified protein solution: 40 min
Box 2, whole-cell imaging in microwells: variable

ANTICIPATED RESULTS

Single-cell western blotting

The overall utility of scWB derives from a capacity for direct protein measurement in single cells, with a selectivity that exceeds that of immunoassays. As demonstrated here (**Fig. 11**), single-cell PAGE and UV-activated protein immobilization are completed within 10 min and resolve two housekeeping proteins, GAPDH and βTub, from single U373 glioblastoma cells in a 10%T PAG within a 1-mm separation distance. Two assay stages—cell lysis and protein PAGE—are critical to the success of the scWB assay. First, optimized cell lysis conditions are essential for efficient protein solubilization and minimal protein loss. Hot lysis buffer (50 °C) helps dissociate the native tetramer forms of GAPDH (mobility = 0.27 ± 0.14 × 10^{-5} cm^2 V^{-1} s^{-1}) into monomer GAPDH (mobility = 6.11 ± 0.27 × 10^{-5} cm^2 V^{-1} s^{-1}; **Fig. 11a**). Second, the PAGE condition must be optimized for the protein targets of interest. Using GAPDH and βTub as a model system, we perform PAGE on a 10%T uniform PAG and achieve an R_s = 0.5 ± 0.09 at 30-s electrophoresis time (E = 40 V cm^{-1}) or R_s = 0.95 ± 0.06 at 34-s electrophoresis time (**Fig. 11b**). Each application may require different gel pore sizes, electrophoresis times and gel architecture to achieve optimal separation performance. Selection of an appropriate starting point for the PAGE conditions and optimization should follow instructions in the Experimental design and TROUBLESHOOTING sections of this protocol. By following the recommended positive and negative controls for scWB and verifying proper microwell fabrication (**Figs. 6** and **7**) and experimental conditions (**Figs. 11** and **12**), the user can optimize this protocol to the device and assay conditions that are most suitable for their own experimental inquiry.

Pore-gradient microgel and immunoprobing

The scWB is especially useful in target multiplexing, as a pore-gradient microgel can simultaneously resolve protein pairs with similar electrophoretic mobility even when protein targets span a wide molecular mass range. **Figure 12** illustrates a pore-gradient PAGE analysis of three targets (mTOR, GAPDH and GFP) that completes in a 1-mm separation length; however, in the uniform gel, the GFP electro-migrates beyond the 1-mm separation lane and locates at the second microwell, resulting in protein loss from diffusion out of the microwell and nonquantifiable data.

During immunoprobing of proteins photo-immobilized in the pore-gradient microgel, we observe low immunoprobing efficiency at the small-pore-size region of the PAGE separation axis. Thus, only mTOR, which locates at the large-pore-size region of the PAG, yields detectable immunoprobed signal, whereas GAPDH and GFP are undetectable. Spatially nonuniform introduction of antibody probe into the pore-gradient microgel is mitigated by de-cross-linking of the pore-gradient microgel before immunoprobing (**Fig. 12**, right panel; see PROCEDURE Steps 51–55).

On balance, scWB is an adaptable protein assay that is suitable for assessing cell-to-cell variation in protein-mediated cell functions for a range of protein targets. Distinct from other single-cell protein assays, the single-cell protein PAGE step

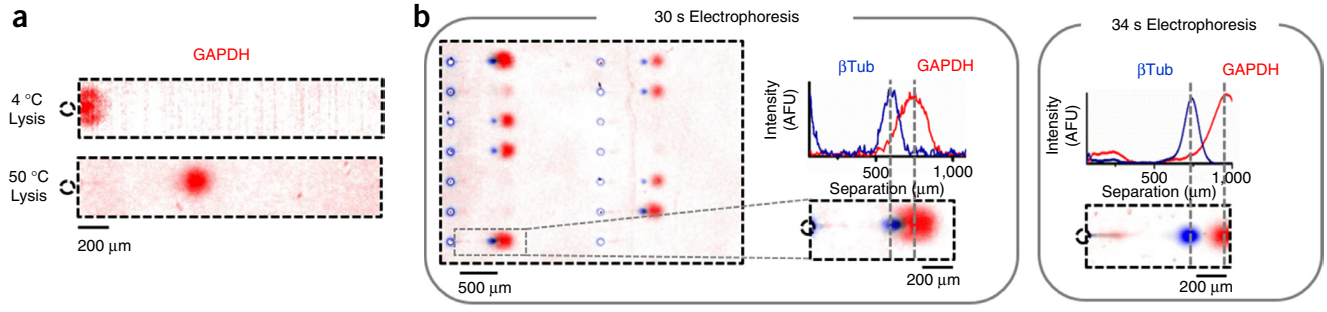

Figure 11 | scWB reports GAPDH and βTub expression in single U373 glioblastoma cells. (**a**) False-color fluorescence micrographs show that low-temperature lysis conditions (4 °C) cannot fully dissociate GAPDH protein (red) to monomer, as suggested by the peak location compared to that of the 50 °C lysis condition. (**b**) False-color fluorescence micrographs show that, with elevated temperature (50 °C), 25-s lysis duration and 30-s electrophoresis times, the GAPDH and βTub (blue) are partially resolved in 10%T, 2.7%C molecular sieving gel (R_s = 0.5 ± 0.09). With longer electrophoresis times (34 s), the GAPDH and βTub are nearly fully resolved (R_s = 0.95 ± 0.06).

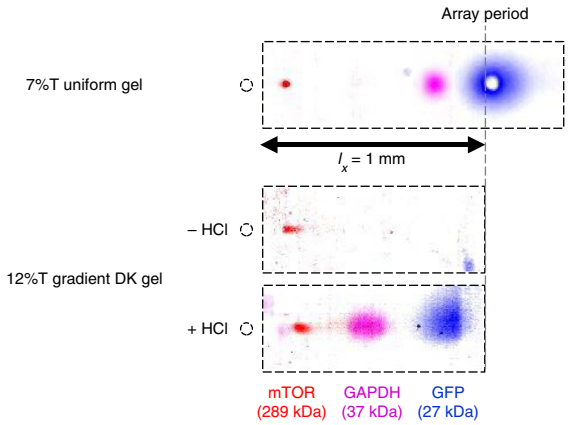

Figure 12 | Comparison of uniform versus pore-gradient scWB readouts. The uniform PAG resolves mTOR (red, 289 kDa), GAPDH (magenta, 37 kDa) and GFP (blue, 27 kDa) (top). However, the GFP protein overruns the 1-mm separation lane. A comparable pore-gradient microgel resolves all three proteins in the 1-mm separation lane (bottom). The GAPDH and GFP proteins are undetectabe by immunoprobing in the pore-gradient microgel without prior gel de-cross-linking using an HCl solution. Lysis duration for U373-GFP cells = 25 s; PAGE at E = 40 V cm^{-1} is 15 s for both uniform PAG (7%T, 2.7%C) and pore-gradient PAG (12%T precursor with 99% DK cross-linker). The grayscale chrome mask contained a 200-µm uniform 70% grayscale region contiguous to an 800-µm region housing a 70–1% grayscale gradient. The pore-gradient DK PAG slides are de-cross-linked with pH 1.1 HCl and rinsed with 1× TBST before immunoprobing.

confers a selectivity that is enhanced over immunoassays alone, thus opening new means to identify important proteins and protein isoforms. Importantly, our assay and device is designed for adoption and adaptation by a broad range of users seeking new tools to address both fundamental and applied questions in protein signaling at the single-cell level.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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