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Measuring expression heterogeneity of single-cell cytoskeletal protein complexes

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Multimeric cytoskeletal protein complexes orchestrate normal cellular function. However, protein-complex distributions in stressed, heterogeneous cell populations remain unknown. Cell staining and proximity-based methods have limited selectivity and/or sensitivity for endogenous multimeric protein-complex quantification from single cells. We introduce micro-arrayed, differential detergent fractionation to simultaneously detect protein complexes in hundreds of individual cells. Fractionation occurs by 60 s size-exclusion electrophoresis with protein complex-stabilizing buffer that minimizes depolymerization. Proteins are measured with a ~5-hour immunoassay. Co-detection of cytoskeletal protein complexes in U2OS cells treated with filamentous actin (F-actin) destabilizing Latrunculin A detects a unique subpopulation (~2%) exhibiting downregulated F-actin, but upregulated microtubules. Thus, some cells may upregulate other cytoskeletal complexes to counteract the stress of Latrunculin A treatment. We also sought to understand the effect of non-chemical stress on cellular heterogeneity of F-actin. We find heat shock may dysregulate filamentous and globular actin correlation. In this work, our assay overcomes selectivity limitations to biochemically quantify single-cell protein complexes perturbed with diverse stimuli.
Over 80,000 protein complexes comprised of interacting proteins regulate processes from proteostasis to transcription. A critical set of multimeric protein complexes form the cell cytoskeleton: actin filaments, microtubules, and intermediate filaments. For example, actin dynamically polymerizes and depolymerizes between monomeric G-actin (−42 kDa) and filamentous F-actin to determine cell morphology, motility, and proliferation. F-actin is considered the functional actin species in the cytoskeleton. Thus, the F-actin ratio (or F-actin abundance divided by total actin) is a metric for cytoskeletal integrity. F-actin levels can be increased in metastatic cancer cells, thus underpinning the design of oncology drugs that disrupt F-actin filaments. In addition, microtubule-stabilizing chemotherapeutics (e.g., taxanes) are widely used in the treatment of numerous cancers (e.g., breast, lung, and prostate). However, the development of taxane-resistant cell subpopulations requires further advances to screen drugs that target the cytoskeleton. Quantifying the distribution of cytoskeletal protein complexes in single cells would inform drug development and help elucidate stress-induced cancer transformations. To understand cytoskeletal protein-complex expression heterogeneity, no existing method combines the needed detection sensitivity, throughput, and selectivity for multimeric protein complexes in single cells. Single-cell, bottom-up mass spectrometry has been demonstrated with identification of up to 1000 protein groups from individual cells with the nanoPOTS system. Bottom-up mass spectrometry digests proteins and cannot determine protein-complex stoichiometry like top-down mass spectrometry of intact proteins. However, lossy sample fractionation in top-down mass spectrometry limits the identification of protein complexes from low-cell number samples. Indeed, with nanoPOTS integrated with top-down mass spectrometry, only ~170 of over a million possible proteoforms were detectable from ~70 pooled HeLa cells. Thus, while highly multiplexed, top-down mass spectrometry currently lacks single-cell resolution for protein complexes. Targeted approaches such as proximity ligation assay and FRET achieve single-cell sensitivity and can assess cellular heterogeneity with flow cytometry readout. Indeed, with SIFTER gel (0.5 mm thick) minimized resistive heating that could prematurely depolymerize or dissociate protein complexes. Owing to the arrayed format and open microfluidic design, hundreds of fractionation separations are performed simultaneously. After fractionation and bi-directional electrophoresis, both the depolymerized protein complex (e.g., F-actin, microtubule, and/or intermediate filament) and monomer (e.g., G-actin, β-tubulin, and/or vimentin) states are blotted (immobilized) in distinct gel regions abutting each microwell. Protein complex and monomer states are quantified by in-gel immunoblotting, allowing target multiplexing. We applied SIFTER to four basic questions. First, do two well-studied actin-targeting drugs (Latrunculin A and Jasplakinolide) induce variation in F-actin complex-levels in single cells compared to controls? Second, as a corollary, does Latrunculin A yield cellular phenotypes distinct from controls with different organizations of other cytoskeletal protein complexes, such as microtubules and intermediate filaments? Third, what is the distribution of the F-actin ratio across a population of single cells? Fourth, how does heat shock, another cellular stress, shift the F-actin ratio distribution and coordination between F- and G-actin at the single-cell level? We show SIFTER is a versatile method for understanding cellular heterogeneity—at single-cell resolution—in protein-complex levels in response to perturbation.

Results

SIFTER design principles and characterization. To selectively detect cytoskeletal protein complexes from single cells, we integrate differential detergent fractionation, electrophoretic separation, and immunoassay steps into a single microdevice. An important set of dynamic protein complexes comprise the cytoskeleton, including F-actin filaments, microtubules (MT), and intermediate filaments (IF; Fig. 1a). Two design considerations are central to our measurement of dynamic protein complexes: (1) discerning the protein complexes from monomers, and (2) maintaining protein complexes during fractionation. For the first design consideration, we focus on the F-actin filament, which is the smallest and most dynamic of the three cytoskeletal protein complexes. Each filament can be composed of up to hundreds of globular G-actin monomers (koff ~ 0.2−10 s−1 in vivo), F-actin averages ~2.7 MDa (versus MT at ~178 MDa with 1 μm average MT length29 and 1625 tubulin heterodimers per μm of MT30, and IF at ~30 MDa for typical μm-scale IF31 at >30 kDa per nm of filament32). F-actin polymerization proceeds rapidly once four G-actin monomers are incorporated in a filament. Steady-state polymerization (koff ~0.1−5 μM−1 s−1)38 yields a distribution of filament masses. While the F-actin mass distribution below
~2700 kDa is unknown in vivo, F-actin is highly enmeshed. Thus, discerning F- (>160 kDa) vs. G-actin (42 kDa) requires coarse size cutoff (~hundreds of kDa), which should also fractionate MT and IF. On the second design consideration, rapid F-actin depolymerization occurs below the critical concentration of total actin (~0.2–2.0 μM in vivo). To maintain local concentrations of actin above the critical concentration demands <~10× dilution during the assay, as cellular total actin is ~10–100 μM. Thus, the SIFTER fractionation gel contains microwells with ~108× smaller reaction volume versus bulk ultracentrifugation to minimize dilution. The microwells accommodate gravity-sedimented single cells27 within the fractionation gel (Fig. 1a). The open SIFTER device is suited to the rapid serial introduction of buffers via interchangeable hydrogel lids to first lyse cells and stabilize protein complexes during fractionation, and then depolymerize or dissociate protein complexes to spatially separate monomers from protein complexes (Fig. 1b, c).

To report both the state (protein complex vs. monomer) and the amount of specific protein complexes per cell, SIFTER comprises five assay steps (Fig. 1c). First, single trypsinized cells are gravity-settled in the microwell array (from a cell suspension27) and lysed in an F-actin stabilization buffer.
delivered by the hydrogel lid, creating a lysate containing the monomers and complexes. Second, protein complexes are fractionated from the smaller monomers by polyacrylamide gel electrophoresis (PAGE, 60 s), during which large protein complexes are size-excluded from the gel and retained in each microwell. Monomers electrophoresed into the gel and are immobilized (blotted) using a UV-induced covalent reaction to the gel polymer network. Covalent immobilization to the gel prevents monomer diffusion that would broaden the protein peak and result in protein loss out of the gel. Third, to depolymerize the complexes retained in each microwell, a protein-complex depolymerization buffer is introduced by another hydrogel lid. Fourth, we electrophoresed the now depolymerized complexes into a region of the gel separate from the immobilized monomers, where depolymerized complexes are in turn immobilized. Fifth, in-gel immunoprobing (~5 h) detects the immobilized populations of monomer and monomer depolymerized from the complexes. We use a fluorescently labeled antibody probe against the protein (i.e., anti-actin antibody probe to detect F- and G-actin, and an anti-vimentin antibody probe to detect vimentin monomers and intermediate filaments).

To maintain intact protein complexes in each microwell during PAGE fractionation, the F-actin stabilization buffer slows the natural depolymerization kinetics. The non-ionic detergent Triton X-100 at ~1% v/v lyses the cell and minimally alters in vitro polymerization rates of actin. Addition of 2 mM MgCl₂ stabilizes F-actin complexes, as Mg²⁺ binds G-actin to lower depolymerization rates. Consequently, only ~2% of total F-actin depolymerizes per minute in mammalian cells lysed in stabilization buffer, compatible with our goal to fractionate in ~1 min. Cell lysis depends on the diffusion of Triton X-100 micelles, which require ~10 s to reach the bottom of the microwells. Imaging release of monomeric G-actin fused to fluorescent GFP from GFP-actin expressing breast cancer cells (MDA-MB-231 GFP-actin) within a microwell confirms a 45 s lysis yields only ~2.5–4× dilution of total actin to remain above the actin critical concentration (Fig. 1d). Important to minimizing F-actin-complex depolymerization during the assay, SIFTER completes cell lysis and fractionation in <5 min, or ~40× faster than bulk ultracentrifugation.

Validation and benchmarking SIFTER. We first validated SIFTER by fractionating and quantifying the G-actin monomer vs. F-actin complexes in single MDA-MB-231 GFP-actin cells. We selected GFP-actin expressing cells to utilize fluorescence imaging to optimize cell lysis (Fig. 1d) and PAGE conditions. Immunoprobing for GFP yields distinct Gaussian protein peaks corresponding to GFP G-actin (G) on the left and GFP F-actin (F) to the right of each microwell (Fig. 1e). The area-under-the-curve of F-actin and G-actin peaks corresponds to the F-actin (F) and G-actin (G) protein fraction abundances, respectively. By design, the target peak is identified using a combination of reactivity with immunoprobe and migration distance (size). For immunoblots where dispersed signal between the target actin peak and microwell is both detectable and resolvable, the off-target signal is excluded from quantification. Immunoblots with non-Gaussian target signal are omitted from data analysis. We attribute the dispersed signal to either (or both): (i) cross-reactivity of the fluorescent antibody probes with smaller proteins and cellular material or (ii) injection dispersion arising from likely incomplete protein solubilization (including dissociation of the filamentous actin we study here). Full solubilization of the F-actin filament may not be complete for all cells in the short 45 s lysis and solubilization period, a duration that is dictated by diffusive losses of protein out of the microwell prior to electrophoresis.

for each cell. The MDA-MB-231 GFP-actin fusion cell average

\[
F_{\text{ratio}} = F/(F + G)
\]

(1)

for each cell. The MDA-MB-231 GFP-actin fusion cell average

\[
F_{\text{ratio}} = 0.45 \pm 0.10 \text{ (standard deviation; } n = 578 \text{ cells, from } N = 3 \text{ SIFTER devices, in reasonable agreement with } F_{\text{ratio}} \sim 0.5 \text{ for MDA-MB-231 from bulk ultracentrifugation. With SIFTER, the } F_{\text{ratio}} \text{ coefficient of variation is 22%, revealing single-cell variation obscured in the bulk assay. } F_{\text{ratio}} \text{ variation measured by SIFTER includes cellular variation, such as the inverse correlation between the } F_{\text{ratio}} \text{ and cell volume. For example, cells grown in microniches that controllably decrease cell volume by half undergo a similar magnitude increase in } F_{\text{ratio}} \text{ and decrease in } G-\text{actin (which should correspond to } \sim 2\times \text{ increase in } F_{\text{ratio}}\text{). Further, the F-actin stabilization buffer also maintains IF complexes (Fig. 1f, Supplementary Note 1). As such, we define and quantify an IF ratio:}

\[
IF_{\text{ratio}} = IF/(IF + VIM_{\text{monomer}})
\]

(2)

from the area-under-the-curve of the peaks, where VIM_{\text{monomer}} is the amount of native vimentin monomer and IF is the amount of depolymerized intermediate filament in arbitrary fluorescence.
directly observed PAGE of we determined the gel composition needed to fractionate F-actin MB-231 GFP-actin cells have an average IF structure to the cell, the primary function of IF. We distinct resolution is to detect small sub-populations of cells with errors are standard deviations from one SIFTER device). We confirm the actin state of the species in the microwell by imaging PAGE of U2OS cells expressing RFP-Lifeact, a common marker for F-actin. The microwell retains the F-actin complexes (Fig. 2c), with signal decrease attributable to diffusive losses of RFP-Lifeact-bound G-actin out of the microwell and photobleaching. We hypothesize two factors lead to no observed F-actin electromigration into the gel, including RFP-Lifeact bound dimers. First, small oligomers are a minor fraction of F-actin due to substantial dissociation rates. Second, highly crosslinked filaments remain enmeshed within the cytoskeleton even in lysed cells. Further, we expect that free RFP-Lifeact would diffuse out of the microwell during cell lysis if present. Thus, we confirm that SIFTER fractionates F-actin complexes from single cells. Importantly, size exclusion may fractionate other protein complexes by adjusting the %T, as >99% of F-actin retention of F-actin complexes in the microwell (repeated for a total of 3 cells). A schematic of heating in the microwell is outlined with a dashed line in the micrograph and intensity profile. C Intensity profiles (top) and false-color fluorescence micrographs of single RFP-Lifeact U2OS cells in microwells (dashed outline; only F-actin is fluorescent) upon lysis in F-actin stabilization buffer. PAGE results in retention of F-actin complexes in the microwell (repeated for a total of n = 3 cells). D Left: schematic of heating in the fractionation gel (gray) and gel lid (yellow) upon applying a current, i. Right: plot of temperature as a function of elapsed PAGE time under the F-actin stabilization lysis buffer gel at 30 V cm⁻¹ (n = 3; black diamonds). Source Data are available as a source data file.

Fig. 2 Size-based fractionation and efficient heat dissipation at the microscale provides molecular specificity to fractionate F-actin complexes from single cells. A Left: schematic of fractionation using polyacrylamide gel electrophoresis (PAGE) to separate F-actin complexes from G-actin monomers. Right: estimated molecular mass cutoff as a function of gel density (%T). The shaded region is the molecular mass range of 99.9% of non-interacting protein species comprising the mammalian proteome, with notations indicating G-actin (42 kDa, solid green line) and average F-actin (~2700 kDa, dashed green line) molecular masses. B False-color fluorescence micrographs and corresponding intensity profiles during electrophoresis (30 V cm⁻¹) of MDA-MB-231 GFP-actin single-cell lysates in F-actin stabilization buffer; 76 ± 3% of the fluorescence remains in the microwell (n = 4, error is the standard deviation). Microwell is outlined with a dashed line in the micrograph and intensity profile. C Intensity profiles (top) and false-color fluorescence micrographs of single RFP-Lifeact U2OS cells in microwells (dashed outline; only F-actin is fluorescent) upon lysis in F-actin stabilization buffer. PAGE results in retention of F-actin complexes in the microwell (repeated for a total of n = 3 cells). D Left: schematic of heating in the fractionation gel (gray) and gel lid (yellow) upon applying a current, i. Right: plot of temperature as a function of elapsed PAGE time under the F-actin stabilization lysis buffer gel at 30 V cm⁻¹ (n = 3; black diamonds). Source Data are available as a source data file.

units. The IF_ratio indicates the fraction of vimentin actively giving structure to the cell, the primary function of IF. We find MDA-MB-231 GFP-actin cells have an average $IF_{ratio} = 0.63 \pm 0.11$ (error is the standard deviation; n = 168 cells, from N = 4 SIFTER devices measured on the same day). The significance of determining metrics such as $F_{ratio}$ and $IF_{ratio}$ with the single-cell resolution is to detect small sub-populations of cells with distinctive filament and monomer distributions, especially the phenotypes that arise in response to stresses. Observed cell-to-cell variation in $F_{ratio}$ and $IF_{ratio}$ raises the intriguing question of whether cells compensate levels of one cytoskeletal protein complex for another. We investigate differential stress responses and compensation of cytoskeletal protein complexes in this work.

To validate monomer vs. protein-complex detection specificity, we determined the gel composition needed to fractionate F-actin (the smallest of the three cytoskeletal protein complexes) and directly observed PAGE of fluorescently labeled actin from single-cell lysates. The molecular mass cutoff for the gel depends on the total acrylamide concentration (%T). Based on native PAGE, the SIFTER cutoff for an 8%T gel is ~740 kDa (Supplementary Fig. S1 and Fig. 2a), or larger than 42 kDa G-actin, but smaller than an average ~2700 kDa F-actin. During PAGE of MDA-MB-231 GFP-actin cells (in which GFP is fused to both G- and F-actin), actin species indeed fractionate at the microwell edge (Fig. 2b). Within 45 s of PAGE, the G-actin Gaussian protein band completely injects a mean distance of 350 ± 16 μm into the polyacrylamide gel (with mean peak width of 66 ± 8 μm, n = 275; errors are standard deviations from one SIFTER device). We confirm the actin state of the species in the microwell by imaging PAGE of U2OS cells expressing RFP-Lifeact, a common marker for F-actin. The microwell retains the F-actin complexes (Fig. 2c), with signal decrease attributable to diffusive losses of RFP-Lifeact-bound G-actin out of the microwell and photobleaching. We hypothesize two factors lead to no observed F-actin electromigration into the gel, including RFP-Lifeact bound dimers. First, small oligomers are a minor fraction of F-actin due to substantial dissociation rates. Second, highly crosslinked filaments remain enmeshed within the cytoskeleton even in lysed cells. Further, we expect that free RFP-Lifeact would diffuse out of the microwell during cell lysis if present. Thus, we confirm that SIFTER fractionates F-actin complexes from single cells. Importantly, size exclusion may fractionate other protein complexes by adjusting the %T, as >99% of individual proteins of the mammalian proteome would not be size-excluded by a smaller pore-size 10%T gel.

We further validate SIFTER maintains protein complexes during fractionation without PAGE-induced temperature rise that may depolymerize or dissociate protein complexes (e.g., as shown in vitro or in certain cell types above 45 °C[16–18]). An electrical current passing through conductive buffer produces heat ( Joule heating) during PAGE, which can increase the temperature if not efficiently dissipated. The temperature difference, $\Delta T$, between the surrounding medium and the conductor varies along the height axis, z, of the conductor:

$$\Delta T = E^2 \sigma \left( \frac{a^2 - z^2}{2k} \right)$$ (3)
where $E$ is the electric field strength ($V \text{ m}^{-1}$), $\sigma_c$ is the buffer conductivity ($S \text{ m}^{-1}$), $2a$ is the height and $k$ is the thermal conductivity of the conductor ($W \text{ m}^{-1} \text{C}^{-1}$)\(^4\). Due to large temperature rises during electrophoresis in F-actin stabilization buffers containing MgCl\(_2\) ($\sigma_c \sim 0.13 \text{ S m}^{-1}$), $E$ is limited to $-2$ to $10$ V cm\(^{-1}\) for $120$ to $480$ min in native slab gels\(^5\), or $18$ V cm\(^{-1}\) in capillaries\(^6\). In SIFTER, the anticipated $\Delta T$ at 30 V cm\(^{-1}\) is $-0.02 ^\circ \text{C}$ ($2a \sim 0.54 \text{ mm}$) vs. $-6.18 ^\circ \text{C}$ increase in a slab gel ($2a \sim 5$ mm; Supplementary Fig. S2). Indeed, we measure constant room temperature using liquid crystal temperature sensors under the hydrogel lid during PAGE at 30 V cm\(^{-1}\) with SIFTER (Fig. 2d).

Thus, we confirm SIFTER maintains endogenous protein complexes without Joule heating with $\sim 100 \times$ faster fractionation than in Latrunculin A (LatA)\(^7\). In conventional imaging of F-actin with three actin subunits\(^8\) to lower the number of actin subunits needed throughput analysis\(^9\). The latter may be overcome in the case of cells\(^10\), the concentration is high enough to induce decreased fluorescence (Fig. 3a) in agreement with previous findings\(^11\).

As with Jpk, we utilized a high enough LatA drug concentration to induce visible changes in phalloidin fluorescence. To assess variation in cell response to LatA, we benchmarked the distribution of F-actin levels from LatA treatment in SIFTER versus flow cytometry of trypsinized, fixed, and phalloidin-stained U2OS cells. By flow cytometry, we find the median F-actin complex level of DMSO control cells is significantly higher than the LatA treatment median by $1.9x$ (Mann–Whitney $P$-value $< 0.0001$, Fig. 3d, $n = 9203$ control cells, and $n = 5114$ LatA-treated cells). With SIFTER, we observe the median F-actin complex level in DMSO control cells is significantly higher than the LatA treatment median by $1.5x$ (Mann–Whitney $P$-value $< 0.0001$, Supplementary Fig. S3 and Supplementary Note 2). Thus, SIFTER does not measure as large a decrease in F-actin levels upon LatA treatment as flow cytometry of fixed and phalloidin-stained cells. One reason SIFTER may report smaller decreases in F-actin levels upon LatA treatment (while still maintaining statistical significance) is due to run-to-run variation observed across assay replicates (each replicate shown in Supplementary Fig. S4).

Unlike Jpk, LatA treatment corresponds with an increase in F-actin CQV as CQV\(_{\text{LatA, U2OS}} = 0.49 \text{ vs. CQV}_{\text{DMSO control, U2OS}} = 0.32 \text{ by SIFTER (a 1.5x increase)}$ and CQV\(_{\text{LatA, U2OS}} = 0.30 \text{ vs. CQV}_{\text{DMSO control, U2OS}} = 0.23 \text{ by flow cytometry (a 1.3x increase)}$. Previously, phalloidin staining revealed a single F-actin complex phenotype from $\sim 200$ sparsely seeded cells treated with 250 nM LatA\(^6\). Here, the CQV increase upon LatA exposure suggests differential cell tolerance to LatA potentially due to the almost $10x$ higher LatA concentrations utilized here. Thus, SIFTER circumvents competitive binding or cell segmentation challenges to quantify variation in drug effects on F-actin complexes at the single-cell level.

The high CQV\(_{\text{LatA, U2OS}}$ from SIFTER prompted us to further investigate cellular variation in response to LatA treatment. It is not currently possible to quantify the variation in the other cytoskeletal protein complexes, IF and MT with flow cytometry, as vimentin and tubulin antibodies would bind both the monomer and protein complexes in the cell. However, with SIFTER, co-detection of multiple protein complexes, IF and MT with flow cytometry, as vimentin and tubulin antibodies would bind both the monomer and protein complexes in the cell. However, with SIFTER, co-detection of multiple protein complexes within the same cell is possible, using antibodies raised against different species, or with a chemical stripping and re-probing approach developed previously\(^7\).
intermediate with another Latrunculin, LatB. The counteracting increase in α (MT, of orientations. The cytoskeletal protein complexes F-actin, microtubules and representative intensity profiles from SIFTER on single BJ fibroblast cells treated with the indicated concentration of Jpk. The scale bar is 100 μm. Microwell is outlined with a dashed line in the intensity profile and in the micrograph. e Violin plot of F-actin levels quantified from three different SIFTER devices with the indicated total number of single cells. Median F-actin AFUs are 11053 for control, 6876 for 100 nM Jpk, and 5343 for 200 nM Jpk. Boxplot box edges are at 25th and 75th percentile, the middle point is the median, and whiskers extend to the minimum and maximum values of the data set. Mann−Whitney U Test p-value < 0.0001 with Dunn−Sidak correction for multiple comparisons. f Violin plot of F-actin levels quantified from four different SIFTER devices with the indicated total number of single cells. Boxplot box edges are at 25th and 75th percentile, the middle point is the median, and whiskers extend to the minimum and maximum values of the data set. Medians are 42105 for control (n = 1584), 3454 for control (n = 1051), and 200 nM Jpk (n = 444), blue) for LatA. Mann−Whitney U Test p-value < 0.0001. Source Data are available as a source data file.

differential expression of other cytoskeletal protein complexes. Second, we asked whether LatA induced unique cell subpopulations. The cytoskeletal protein complexes F-actin, microtubules (MT, of α- and β-tubulin subunits), and intermediate filaments (IF, of vimentin or keratin subunits) have both redundant and distinct functions in maintaining cytoskeletal integrity (Fig. 4a). Such redundancy⁶⁸ yields increased IF to counteract F-actin destabilization of mesenchymal cells⁶⁹ with only 1 h treatment with another Latrunculin, LatB. The counteracting increase in intermediate filament levels occurs because keratin intermediate filament-regulating genes become differentially expressed⁶⁹. Yet, quantification of cytoskeletal changes remains a challenge in single cells by microscopy due to segmentation artifacts and low signal-to-noise ratio (SNR) from immunohistochemistry and phalloidin staining⁷⁰,⁷¹.

To understand the concerted effects of 1 h LatA drug treatment on F-actin, MT, and IF, we performed same-cell, target-multiplexed SIFTER (Fig. 4b and Supplementary Fig. S5). We assess the expression relationships between the three protein complexes in the DMSO vehicle control cells (n = 201 single cells) by Spearman rank correlation, and obtain ρ = 0.70 for MT vs. F-actin, ρ = 0.72 for F-actin vs. IF, and ρ = 0.59 for MT vs. IF (Supplementary Fig. S6; p < 0.0001 for each correlation). The correlation values suggest the coordination of cytoskeletal protein-complex levels across a large proportion of cells. A follow-up agglomerative hierarchical clustering analysis reveals
Fig. 4 Multiplexed SIFTER detects subpopulations of cells with altered cytoskeletal protein complexes in response to F-actin destabilization. 

**a** Schematic of the cell cytoskeleton composed of F-actin, intermediate filaments (IF) and microtubules (MT), and the unknown effects of Latrunculin A (LatA) on IF and MT. 

**b** Representative false-color fluorescence micrographs and intensity profiles from SIFTER. F-actin (green), MT (blue), and IF (orange) are electrophoresed to the right of the microwell. Protein quantification is performed by peak area integration. The scale bar is 100 μm. Microwell is outlined by a dashed line in the micrographs and intensity profiles. 

**c** Heat maps with dendrograms from agglomerative hierarchical clustering with Euclidean distance metric and Ward linkage for U2OS cells incubated in DMSO (n = 201 cells, three SIFTER devices) or 2 μM LatA (n = 507 cells, four SIFTER devices). Distinct sub-lineages used as bait groups A-D for CellFishing are shown with colored bars (blue, purple, teal, and lavender, respectively). Heatmap is standardized by row (mean at 0, and color gradations at units of standard deviation). 

**d** Spectral clustering projections and heatmaps depicting LatA treatment bait group cells (blue), DMSO control cells (gray), and fished out DMSO control cells (yellow). Source Data are available as a source data file.
sets of cells with distinct patterns of protein-complex expression (e.g., groups A−D, Fig. 4c).

Next, to elucidate whether any of the potential subpopulations shown in Fig. 4c (e.g., groups A−D) were unique to the LatA-treatment, we adapted the GeneFishing method72 for CellFishing. Using a group of co-expressed cells as bait, we attempt to fish out other cells from a candidate pool that present a similar protein-complex expression pattern to that of the bait cells. We do this through a semi-supervised clustering approach, coupled with subsampling to ensure robust discoveries. Here, groups of LatA-treated cells from hierarchical clustering that appear as unique phenotypes each define a set of bait cells, and the DMSO control cells define the candidate pool. If a group of bait cells does not identify any cells with similar phenotypes in the DMSO control cells, we assume the phenotype is unique to the LatA-treated cell population. We found that bait group A does not fish out DMSO control cells, while groups B-D are examples of bait groups that do (Fig. 4d). Groups B (−3% of LatA cells), C (−4% of LatA cells), and D (−3% of LatA cells) all fish out DMSO control cells (−0.5, 2, and 20% of the DMSO control cells, respectively) and thus represent phenotypes not exclusive to LatA treatment. Group B is marked by elevated MT and to a lesser extent, elevated IF compared to the average protein complex expression levels of the LatA-treated cells. Group C is characterized by increased IF. Group D expresses low F-actin, MT, and IF, which was a phenotype observed in a substantial number of both control and LatA cells as displayed in the heat map. Group A (−2% of LatA-treated cells) is characterized by elevated MT in response to F-actin destabilization and is only found in the LatA treatment cells. If MT compensates for F-actin perturbation in subpopulations of cells, such cells may be better equipped to maintain cytoskeletal integrity in response to stress. LatA causes an increase in the percentage of serum-starved fibroblasts expressing mature microtubules (from 40 to 70% of cells after one hour at 0.1 μM)73, a shift between two cell populations. Here, hierarchical clustering of multiplexed SIFTER reveals distinct subpopulations with unique cytoskeletal composition stratified by the expression of all three complexes. For example, IF levels distinguish Groups A and B with similar MT and F-actin levels (Fig. 4c, d). Our results open up questions such as whether increases in MT in LatA-treated cells correspond with changes in transcriptional or translational rates, and subunit stability or MT organization, which warrant further investigation.

Quantifying distributions of total actin and FRatio across cells.
To assess actin cellular heterogeneity, we asked: what are the statistical distributions of total actin and FRatio across cells? In order to assess statistical distributions across SIFTER replicates, we needed to prepare the cells at a fixed time after preparing the single-cell suspension, as detachment lowers the level of cytoskeletal integrity in response to stress. LatA causes an increase in the percentage of serum-starved fibroblasts expressing mature microtubules (from 40 to 70% of cells after one hour at 0.1 μM)73, a shift between two cell populations. Here, hierarchical clustering of multiplexed SIFTER reveals distinct subpopulations with unique cytoskeletal composition stratified by the expression of all three complexes. For example, IF levels distinguish Groups A and B with similar MT and F-actin levels (Fig. 4c, d). Our results open up questions such as whether increases in MT in LatA-treated cells correspond with changes in transcriptional or translational rates, and subunit stability or MT organization, which warrant further investigation.

Heat shock induces cellular heterogeneity in actin distribution.
To assess how a non-chemical stress perturbs (1) the FRatio distribution and (2) F- and G-actin coordination, we apply SIFTER to the study of heat shock. Cytoskeletal reorganization is a hallmark of disease states5, and protein-complex dysfunction is prominent in aging79 and during cellular stress80,81. Cell stress such as heat shock yields re-organization of F-actin in many, but not all cell types82. Indeed, with phalloidin staining, we observed a qualitative decrease in F-actin fluorescence of RFP-Lentiviral transformed MDA-MB-231 GFP-actin cells upon heat shock (Fig. 5a).

For more nuanced characterization of the FRatio distribution not possible with phalloidin staining, SIFTER reports the median FRatio in the heat-shocked cells was similar to control cells (0.53 vs. 0.59, respectively; Mann−Whitney p-value < 0.0001, Fig. 5b, c). However, the interquartile range of the FRatio in heat-shocked cells is ~1.5× that of control cells (0.17 vs. 0.11). We quantified the skew of the distribution with the Pearson’s moment coefficient of skew:

$$\mu_3 = \epsilon \left[ \frac{X - \mu}{\sigma} \right]^3$$

(5)

where \(\epsilon\) is the expectation operator, \(X\) is the random variable (here, FRatio), \(\mu\) is the distribution mean and \(\sigma\) is the standard deviation. We find \(\mu_3 = -0.40\) for the control data set, and −0.38 for the heat-shocked cells.

To understand if F- and G-actin levels remain coordinated upon heat shock, we quantified Spearman \(\rho\) (for F- and G-actin level correlation, Fig. 5d). The Spearman \(\rho\) decreased from 0.82 for the 49 control cells from one SIFTER device to 0.47 for 131 heat-shocked cells from two SIFTER devices. Across the two heat shock SIFTER devices, \(\rho = 0.73\) for device 1 (\(n = 22, \rho = 0.0002\)) and \(\rho = 0.41\) for device 2 (\(n = 109, \rho < 0.0001\)). We hypothesize heat shock SIFTER device 1 had too few cells that passed analysis quality control to capture the reduced correlation in F- and G-actin upon heat shock observed in device 2. We conclude that F-actin levels alone may not reveal actin cytoskeletal integrity: the Spearman \(\rho\) correlation of F- and G-actin also helps uncover differential stress response across the cell population.

Discussion
SIFTER maintains multimeric cytoskeletal protein complexes during fractionation to reveal monomer versus protein-complex states in single cells. From perturbation of actin with well-characterized drugs, we find LatA, but not Jpk (at the concentrations tested), results in increased F-actin expression heterogeneity as characterized by increasing CQV. To investigate the heterogeneity of LatA-treated cells, we extended SIFTER to a multiplexed readout of the three major cytoskeletal protein complexes (F-actin, microtubules, and intermediate filaments) simultaneously in each cell. We identified previously unknown cell subpopulations, such as the cluster with decreased F-actin.
sets of genes across subsets of cells, including some genes that are unknown. However, recent single-cell investigations have revealed that the origins of differential maintenance of the cytoskeleton are unknown. In the clonal population of U2OS osteosarcoma cells investigated, while some cells increase the expression of microtubules, intermediate filaments, and other cells in the population undergo a complete cytoskeletal collapse. Boxplot box edges are at 25th and 75th percentile, the middle point is the median, and whiskers extend to the minimum and maximum values of the data set.

We hypothesize that cell subpopulations with compensating overexpression of microtubules could be a metric for assessing whether a population of cells is at a non-chemical stress, we evaluated the impact of heat shock on the F-actin ratio of cells. Though missed by phalloidin staining, SIFTER uncovers a potentially marked decrease in the correlation of F- and G-actin upon heat shock. Our results present the possibility that SIFTER presents a more nuanced assessment of actin cytoskeletal integrity than phalloidin staining.

Cellular stresses, be they chemical, heat shock, hypoxia, or oxidative stress, are critical features of cancer biology. Understanding which protein complexes are differentially expressed in drug-susceptible versus drug-resistant cells, or in subsets of cells that metastasize will be critical to advancing cancer therapies. Thus, SIFTER unlocks the capability to assess single-cell heterogeneity in the expression of multimeric protein complexes, with broad applications across biology, potentially including protein complexes unrelated to the cytoskeleton.

The SIFTER assay presently is conducted with a well-characterized F-actin stabilization buffer for cell lysis and maintenance of cytoskeletal protein complexes during electrophoresis. However, no single buffer is ideal for the stabilization of all protein complexes, prompting careful optimization of detergent, salt (ionic species and concentration), buffer, and pH for immunoprecipitation of specific sets of protein complexes. We have not yet investigated alternative lysing buffers for SIFTER, such as certain immunoprecipitation buffers (e.g., containing 10–100 mM NaCl or KCl). Higher buffer salt concentrations than the F-actin stabilization buffer will increase buffer conductivity and we hypothesize could yield more extensive Joule heating that can dissociate protein complexes. Fabrication of thinner (<500 μm) hydrogel lids for efficient heat dissipation may be needed for PAGE in high salt buffers. Thus, further device or buffer optimization may be required to apply SIFTER to protein complexes beyond the cytoskeletal complexes investigated here.

The range of detectable and separable protein-complex sizes is set by a tradeoff between fractionation and immunoprobing. Denser gels compromise assay detection sensitivity because size-exclusion-based partitioning lowers the in-gel antibody probe concentration during the immunoaassay. Fractionation in decrosslinkable gel should allow isolation of up to hundreds of the known mammalian protein complexes with masses of ~295 kDa or greater in a 12%T gel (~7 or more protein subunits, assuming each subunit has the average mammalian protein size of 375 amino acids, or mass of ~40 kDa).

Another factor that determines which protein complexes are detectable with SIFTER is assay detection sensitivity. The cytoskeletal protein complexes investigated here are among the most abundant proteins in mammalian cells, often expressed at millions of copies. Utilizing an in-gel immunoaas for readout, we have previously detected down to 27,000 copies of protein in a protein band. As the SIFTER device is an open device design (vs. enclosed microchannels), protein is diffusively lost out of the microwell during cell lysis and out of the fractionation gel during electrophoresis. Such losses typically require proteins to be expressed at median copy number levels for mammalian proteins to be detectable in single-cell western blotting. While diffusive losses during SIFTER electrophoresis will be lower than in single-cell western blotting owing to efficient heat dissipation, protein fractionation inherently splits the amount of protein to be detected into the monomer and protein complex fractions. Thus, SIFTER likely requires proteins to be expressed above-median copy numbers for detection.

One major advantage of SIFTER over existing assays for protein complexes, such as FRET or proximity labeling, is that SIFTER measures endogenous proteins without requiring cell modifications. Thus, we anticipate SIFTER will be valuable in the measurement of protein complexes from clinical specimens. For example, our group previously introduced isolated circulating...
tumor cells into a microwell array single-cell western blot device for protein profiling. Circulating tumor cells are known to metastasize. With SIFTER, it would be informative to identify differentially expressed cytoskeletal protein complexes from circulating tumor cells to understand which protein complexes could be targets for small molecular inhibitors towards the prevention of metastasis.

For time-sensitive cytoskeletal re-organization or mechanosensitive protein complexes within the cytoskeleton (e.g., stress fibers and focal adhesions), the fractionation gel functionality can be extended to also serve as a cell culture extracellular matrix. On-chip culture can assay adherent cells without the perturbation of trypsinization. We anticipate that SIFTER can aid in evaluating snapshots of dynamic processes while cells are still adherent, such as cytoskeletal recovery from acute stress (e.g., heat shock, and hypoxia). In the present study, we trypsinized and gravity-settled heat-shocked cells for 10 min after the heat shock stress. The amount of time for cytoskeletal recovery from heat shock depends on the duration of the heat shock and cell type, as more fibroblasts or partially rounded cells (DMSO, D2438) were washed off at h and h at 43 °C. For shorter heat shock, or other stresses with faster recovery, growing and then stressing the cells on the SIFTER device will allow us to probe cytoskeletal protein-complex changes immediately after the stress, or at set times during the recovery. For mechano-sensitive cytoskeletal proteins, SIFTER may evaluate single-cell regulation of F-actin, MT, and IF in metastatic cancer cell subpopulations by quantifying dozens of cytoskeletal binding proteins with increased multiplexing by stripping and re-probing. Looking ahead, SIFTER could assist drug screens targeting diverse protein interactions, and fundamental study of cellular stress responses underpinning invasive and heterogenous cancer cells.

Methods

Chemicals. Tetramethylthielenediame (TEMED, T9281), 40% T, 3.4% acrylamide/bis-acrylamide (29:1) (A7802), N,N,N’,N’, ammonium persulfate (APS, A3678), sodium deoxycholate (NaDOC, D6750), sodium dodecyl sulfate (SDS, L3771), bovine serum albumin (BSA, A7030), diethothreitol (DTE, D8255), triton X-100 (X100), urea (U5578), β-mercaptoethanol (M3148), anhydrous magnesium chloride (MgCl2, 814733) and dimethylsulfoxide (DMSO, D2438) were provided by the Drubin lab at UC Berkeley. BJ fibroblasts only partially restore F-actin within 24 h after 1 h of trypsinization. Circulating tumor cells are known to metastasize; such as cytoskeletal recovery from acute stress (e.g., heat shock, and hypoxia). In the present study, we trypsinized and gravity-settled heat-shocked cells for 10 min after the heat shock stress. The amount of time for cytoskeletal recovery from heat shock depends on the duration of the heat shock and cell type, as more fibroblasts or partially rounded cells were washed off at h and h at 43 °C. For shorter heat shock, or other stresses with faster recovery, growing and then stressing the cells on the SIFTER device will allow us to probe cytoskeletal protein-complex changes immediately after the stress, or at set times during the recovery. For mechano-sensitive cytoskeletal proteins, SIFTER may evaluate single-cell regulation of F-actin, MT, and IF in metastatic cancer cell subpopulations by quantifying dozens of cytoskeletal binding proteins with increased multiplexing by stripping and re-probing. Looking ahead, SIFTER could assist drug screens targeting diverse protein interactions, and fundamental study of cellular stress responses underpinning invasive and heterogenous cancer cells.

Cell culture. All cell lines were authenticated by short tandem repeat profiling by the UC Berkeley Cell Culture facility and tested negative for mycoplasma. Naive U2OS cells were purchased from the UC Berkeley Cell Culture Facility. BJ fibroblasts expressing hTERT and Cas9 were provided by the Dillin lab. U2OS RFP-Lenti MDA-MB-231 GFP-Actin cells were purchased from the UC Berkeley Cell Culture facility and tested negative for mycoplasma. BJ fibroblasts only partially restore F-actin within 24 h after 1 h of trypsinization. Circulating tumor cells are known to metastasize; such as cytoskeletal recovery from acute stress (e.g., heat shock, and hypoxia). In the present study, we trypsinized and gravity-settled heat-shocked cells for 10 min after the heat shock stress. The amount of time for cytoskeletal recovery from heat shock depends on the duration of the heat shock and cell type, as more fibroblasts or partially rounded cells were washed off at h and h at 43 °C. For shorter heat shock, or other stresses with faster recovery, growing and then stressing the cells on the SIFTER device will allow us to probe cytoskeletal protein-complex changes immediately after the stress, or at set times during the recovery. For mechano-sensitive cytoskeletal proteins, SIFTER may evaluate single-cell regulation of F-actin, MT, and IF in metastatic cancer cell subpopulations by quantifying dozens of cytoskeletal binding proteins with increased multiplexing by stripping and re-probing. Looking ahead, SIFTER could assist drug screens targeting diverse protein interactions, and fundamental study of cellular stress responses underpinning invasive and heterogenous cancer cells.

SIFTER assay (step-by-step protocol provided on Protocol Exchange). Buffers and gel lid incubation: F-actin stabilization lysin buffer used was 10 mM Tris-HCl, 1% Triton X-100, 2 mM MgCl2, and 0.5 mM DTE (titrated to pH = 7.4) [92]. The experiment and allowed to dissolve at 75 °C. Hydrogel lids (15%, 3.3% C) were photopolymerized as previously described between Gel Slick-coated glass plates offset with a 500 μm spacer [1829]. Hydrogel lids were incubated overnight at 4 °C in either the F-actin stabilization or the depolymerization buffer (before urea or DTE addition). Upon complete preparation of the urea-containing depolymerization buffer, the buffer was introduced to the gel lids in a water bath set to 75 °C and incubated for ~30 min before beginning the experiments. F-actin stabilization buffers and gel lids were kept at room temperature. Gel lids and buffers were only stored for up to 2 weeks, and buffer solution was never re-used. Gel Slick acrylamide fractions (8% T and 3.3% C with 0% TBMAC incorporated) were polymerized on SU-8 micro-post molds as described elsewhere [27]. Trypsinization was performed for 3 min at 37 °C, and cells in PBS (10010049, Thermo Fisher Scientific, pH = 7.4, magnesium and calcium-free) settled in the microwell array for 10 min. Trypsinized cells were introduced to the microwell array in 1× PBS solution for passive gravity settling settling [72]. Every few minutes, the fractionation gel is gently slid back and forth to distribute cells across the gel. After 10 min, the gel is placed at a slight incline and excess cells are lightly rinsed off the gel surface with PBS. Each replicate experiment was run with a different 1 cm petri dish of freshly trypsinized cells in suspension.

For the fractionation separation, the fractionation gel device was pre-incubated in 10 mM Tris-HCl (pH = 7.5) briefly before the glass slide was adhered to the surface of a custom 3D-printed PAGE chamber with petroleum jelly. A custom heater with a 12 V PTC ceramic heating element (ELE4147, Bolen Tech) and PID temperature controller (ITC-106VH, Inkbird) was interfaced to the bottom surface of the PAGE chamber at 37 °C. For the F-actin stabilization hydrogel lid was then applied to the array and cell lysate proceeded for 45 s before the electric field was applied (30 V cm−1, 45 s for 42 kDa actin in U2OS or BJ fibroblasts, or 60 s for 69 kDa GFP-actin from the GFP-actin cells; Bio-Rad Powerpac basic power supply). Proteins were blotted, or bound to the fractionation gel, by UV-irradiated covalent immobilization, or to the BPA/DMAC gel lid. Proteins were immobilized in the fractionation gel (LCs, Hamamat, 100% power, 45 s). The electrode terminals were reversed, and the hydrogel lid was exchanged with depolymerization buffer gel hydrogel lid for 45 s. PAGE was performed for the same duration in the opposite direction before a final UV photo-immobilization step. PAGE was performed for the same duration in the opposite direction before a final UV photo-immobilization step (same UV power and duration). The gel was incubated with 50 μl of 1:10 dilution of the stock primary antibody in TBST for 2 h and then washed 2× for 30 min in 1× TBST. Donkey Anti-Rabbit IgG (H+L) Conjugated Alexa Fluor 647-labeled (A31573, Thermo Fisher Scientific), Donkey Anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555-labeled (A31570, Thermo Fisher Scientific), and Donkey Anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488-labeled (A31571, Thermo Fisher Scientific) were used at 1:200 dilution in TBST for a one-hour incubation after 5 min of centrifugation at 10,000 × g. Two more 30 min TBST washes were performed prior to drying the gels in a nitrogen stream and imaging with a laser microarray scanner (Genepix 4300A, Genepix Pro 7 software, Molecular Devices). When immunoprobing with rhodamine-labeled anti-actin Fab (see Supplementary Table 1) and Ab 200838, 1:5 dilutions were used. After 10 min of immunoprobing, the gels were peeled from the PAGE and washed 1× for 30 min in TBST. For multiplexed analysis of actin, vimentin, and β-tubulin protein complexes, actin and vimentin were immunoprobed together, the gels were chemically stripped and then re-probed for β-tubulin. Chemical stripping was performed for at least one hour at 55 °C. Gels were briefly rinsed in fresh 1× TBST then washed 1× in 1× TBST for at least one hour before re-probing.

Images were analyzed as described elsewhere [27]. Briefly, the images were median filtered utilizing the Remove Outliers macro in Fiji (pixel radius = 2 and threshold

Generation of RFP-Lenti MDA-MB-231 GFP-Actin Cells. MDM-MB-231 GFP-actin cells were a kind gift from the laboratory of Dr. David Drubin. Genome editing was performed at the genomic locus by integrating TagGFP (see Source Data for sequence) at the genomic locus for ACTB. Verification of genome editing was performed via PCR via and standard PCR and sequencing. Briefly, DNA was collected from cells using the Qiagen DNAeasy Blood and Tissue Kit (69506) as per the manufacturer’s guidelines. 100 ng of genomic DNA was used for PCR and sequencing was performed using standard sanger sequencing (primers provided in Supplementary Table S2). A schematic for genome editing is provided in Supplementary Fig. S5. In brief, MB-231 GFP-actin cells were infected with lentivirus containing CD510B-1_pCDH-CMV-ED1-Puro (SystemBio) modified to carry TagRFP (see Source Data for sequence) under the CMV promoter.
Heat shock treatment of cells incubated at 45 °C (VWR mini incubator, 10055-006) for heat shock, or at 37 °C in
were included in the
were washed twice with PBS and analyzed with
AlexaFluor 594 phalloidin in PBS supplemented with 2% BSA) at 4 °C for 30 min.
Flow cytometry analysis of phalloidin-stained cells
microscope controlled with Metamorph (Molecular Dynamics). Images were
semi-quantitative assay. Image analysis was performed in MATLAB R2019b.

Temperature measurement in SIFTER. Temperature sensors (liquid crystal
Type C, 30–60 °C with 5 °C intervals from ThermometerSite) were placed directly under the hydrogel lid (immersed in F-actin stabilization lysis buf-
ner). The temperature was monitored while applying 30 V cm⁻¹ across the elec-
trodes of the electrophoresis chamber without interfacing with the custom heater. Fluorescence imaging of cells in microwells, lysis, and PAGE: Imaging was performed via time-lapse epi-fluorescence microscopy on an Olympus IX50 and
inverted epifluorescence microscope (and thus the custom heater was not used as it would block the illumination path through the PAGE chamber). The microscope was controlled using Metamorph software (Molecular Devices) and images were recorded with a CCD camera (Photometrics Coolsnap HQ2). The imaging setup included a motorized stage (ASI), a mercury arc lamp (X-cite, Lumen Dynamics), and a Chroma XFL-10X2 filter (Omega Optical). Imaging was performed with a 10x magnification objective (Olympus UPlanFLN, NA 0.45 or UPLFLN10X2, NA 0.3) and 900 ms exposures with 1 s intervals with U2OS RFP-Lifeact, and 2 s exposure with 2 s intervals with MDA-MB-231 GFP-actin (1x pixel binning).

F-actin cell staining and drug treatment. Latrunculin A (Cayman Chemicals 10010630) was dissolved in DMSO as a 2 mM stock solution and stored at −20 °C until use. Jasplakinolide (Millipore-Sigma, 420107) was reconstituted in DMSO
being used as a 0.1% Triton X-100 (in 10 min at room temperature), and permeabilized with 0.1% DMSO in cell culture media for the same time as the drug-treated cells. Cells were fixed with 3.7% paraformaldehyde in 1x PBS (10 min at room temperature), and stained with Alexa Fluor 647-labeled phallolid (20 min at room temperature, Thermofisher Scientific, A2287).

Cells were imaged by epi-fluorescence with an Olympus IX70 inverted microscope controlled with Metamorph (Molecular Dynamics). Images were captured with an Andor iXon EM-CCD camera (emission wavelength set to one of the peaks of the Alexa Fluor 594 (0.6 NA) objective, a mercury arc lamp (X-cite exacut, Lumen Dynamics), and a Chroma 490/09 ET filter. Exposure time was 800 ms and pixel binning was 1x.

Flow cytometry analysis of phalloinid-stained cells. Fixed cells were incubated in permeabilization buffer (0.1% Triton X-100 in PBS) at room temperature for 10 min. Cells were then spun down and incubated in staining solution (66 mM AlexaFluor 594 phallolid in PBS supplemented with 2% BSA) at 4 °C for 30 min. Finally, cells were washed twice with PBS and analyzed with flow cytometry using BD LSRFortessa (and FlowJo v10.6 software). To analyze stained cells, single cells were gated by forward and side scatter (Supplementary Fig. S11). Only single cells were included in the fluorescence analysis.

Heat shock treatment of cells. MDA-MB-231 GFP-actin RFP-lenti cells were incubated at 45 °C (VWR mini incubator, 10055-006) for heat shock, or at 37 °C in a humidified 5% CO₂ incubator for 1 h prior to trypsinization and gravity settling in the fractionation gel.

Statistical analysis. Mann-Whitney test (with U test statistic) and Spearman rank correlation. QQ-plot generation with normal and gamma distribution was performed using pre-existing functions in MATLAB 2019b. All tests were two-

Cell fishing clustering analysis. Standardization is by row for both the LatA treated and DMSO control data sets (expression level, or Gaussian protein peak AUC, for each protein complex) with the mean at 0 and standard deviation of 1. Initial agglomerative hierarchical clustering was performed separately for the LatA treated and DMSO control data sets utilizing Euclidean distances, and the Ward linkage criterion (R version 3.6.1, NMF package). Distinct sub-clusters in the LatA treated data were further inspected as bait groups of cells inspired by the GeneFishing method described elsewhere2. We conducted an analogous analysis to GeneFishing, which we call Cell Fishing. Candidate cells from the DMSO control data sets were randomly split into subsets of 100 cells, and each subsample was pooled together with the bait cells to form a sub-dataset. Semi-supervised clustering is applied to each sub-
dataset using spectral analysis and a clustering algorithm based on the EM-fitted mixture Gaussian of two components model9 (R version 3.6.1, mclust package). The subsampling protocol was repeated 3000 times for a given bait set, and cells were considered fished out if they had a capture frequency rate of 0.999 or higher.

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Author contributions

J.V., L.L.H, R.H.-S., C.K.T., and A.E.H. designed the experiments. J.V. and L.L.H. performed SIFTER assay and analysis, and phalloidin staining of adherent cells. C.K.T. provided cell culture and cell line modification support and conducted cell staining of suspension cells and flow cytometry. Y.Z. and H.H. designed and performed Cell Fishing clustering analysis and normalization and subsampling to compare flow cytometry and SIFTER F-actin distributions. All authors wrote the paper.

Competing interests

The Authors declare the following competing interests: L.L.H., R.H.-S., Y.Z., C.K.T., H.H., and A.D. declare no competing interests. J.V. and A.E.H. are inventors on single-cell protein separation intellectual property, which may result in licensing royalties. A patent application (16/977,175, dispatched from preexam, not yet docketed) including separations in complex stabilizing or dissociating buffer was filed by The Regents Of The University Of California including A. E.H. and J.V. as inventors.

Additional information

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