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Materials and Methods

Reagents: Ammonium persulfate (APS; A3678), *N,N,N',N'*-tetramethylethylenediamine (TEMED; T9281), acrylamide/bisacrylamide solution (30% 37.5:1; A3699), Tris-HCl (pH 6.8; BBT-403; Boston Bioproducts), Tris-HCl (pH 8.8; T1588; Teknova), microscope glass slide (25 mm x 75 mm x 1mm; 48300-048; VMR), fibronectin (bovine plasma powder; F4759), rhodamine fibronectin (FNR01; cytoskeleton), *N*-[3-[(3-benzoylphenyl)formamido]propyl]methacrylamide (BPMAC, PharmAgra), Deionized water (ddH_2O; 18.2 mΩ; Millipore), dichlorodimethylsilane (440272), 3-(trimethoxysilyl)propylmethacrylate (440159), sodium dodecyl sulfate (SDS; L3771), sodium deoxycholate (D6750), Triton X-100 (X100), 10x Tris-glycine (25 mM, pH 8.3; 161-0734; Bio-Rad), phosphatase inhibitor cocktail (PI78442; FisherScientific), sucrose (S0389), bovine serum Albumin (A7030), Tris-buffered saline with Tween 20 (TBST, 10x; 9997S; Cell Signaling Technology), primary antibodies for β-tubulin (rabbit; ab6046; Abcam), for p-ERK (rabbit; 4370S; Cell Signaling Technology), and for p-p38 (rabbit; 4511S; Cell Signaling Technology), anti-rabbit secondary antibody (Donkey, AlexaFluor 555; A31572; Life Technologies), 2-mercaptoethanol (M3148), DMEM (DMEM(1x) + GlutaMAX-1 supplement; 10566-016; Gibco), fetal bovine serum (FBS; sterile; 100106; Gemini), penicillin/streptomycin (Pen Strep; 15140-122; Gibco), MEM non-essential amino acids solution (MEM NEAA; 100x; 11140050; Life Technologies), phosphate buffered saline (PBS; sterile, pH 7.4; 10010023; Gibco), Trypsin-EDTA (0.05%; 25300-054; Gibco), LIVE/DEAD® Viability/Cytotoxicity Assay Kit (L3224; Life Technologies), glass cover slip (No.1; 12545F; FisherScientific), cell strainer (sterile; 352235; Corning), AlexaFluor-555 conjugated ovalbumin (2 mg ml^{-1}; O34782; ThermoFisher), Silicon wafer (100-mm diameter; C04009; WaferPro), SU-8 photoresist (3050; Microchem), SU-8 developer (Y020100; Microchem), mylar mask with microwell features (CAD/Art Services). All the chemicals and reagents that are not specified with the manufacturer information were purchased from Sigma.

Fabrication of SU8 mold: To cast the polyacrylamide (PA) gel for *in situ* scWB, we fabricated the SU8 mold through the standard photolithography protocol.[1] The SU8 mold was designed to comprise arrays of micro-posts of ~50 μm in diameter (100 μm for characterization experiments) to allow for HeLa cell spreading in the microwell during the overnight culture. The spacing (~500 μm) between micro-post arrays was designed to prevent signal crosstalks between neighboring cells, while maintaining a high throughput.

Fabrication of fibronectin-functionalized PA gel: Fibronectin (FN) was used as the extracellular matrix (ECM) protein to functionalize microwells on the PA gel for on-chip cell culture. Other ECM proteins (e.g., collagen, gelatin, laminin) can also be used based on the same principle. FN-functionalization of PA gels was completed at the gel polymerization step. Specifically, FN solution of a particular concentration (in the range of 1-100 μg ml^{-1}, determined based on previous work[2]) was added to the precursor solution (mainly composed of acrylamdie, bis-acrylamide, benzophenol-methacrylamide, APS, and TEMED) of the PA gel. The mixture solution of FN and PA gel precursor was uniformly applied onto the SU8 mold. A silane-treated (for enhanced hydrophilicity) microscope glass slide was then added on top of the SU8 mold as the substrate of the *in situ* scWB device. The sandwich of SU8-precursor-glass slide was kept at room temperature for ~1 h to allow for the completion of the polymerization. Once the polymerization was completed, the gel was peeled off from the SU8 mold for *in situ* scWB experiments. Optionally, UV photocrosslinking can be implemented before or after the peeling to form covalent bonds between FN and the gel. To facilitate gel detachment from the SU8 mold, the sandwich of SU8-gel-glass slide can be immersed in DI water for ~5 min prior to peeling.

Cell culture on the gel: The HeLa cancer cell line was purchased from Cell Culture Facility at University of California Berkeley, and maintained in DMEM supplemented with fetal bovine serum (10%) and penicillin/streptomycin (1%) in a humidified incubator at 37 ºC under 5% CO_2. The HeLa cell line was tested mycoplasma negative and authenticated with short tandem repeat analysis. The 4-well plates (10766933; FisherScientific) and the gels used for cell culture were sterilized with 70% ethanol for at least 20 min in the tissue culture hood prior to in use. Cells were detached from the tissue culture plate through trypsin treatment at ~80% confluence, and resuspended into the fresh media to form a suspension of ~1 million cells ml^{-1}. The cell suspension was then filtered through a 35-μm membrane filter (08-771-23; FisherScientific) to eliminate multiple-cell clogs. After diluted down to ~12% of its original concentration, the cell suspension was loaded onto the *in situ* scWB devices that were housed in the chamber of a 4-well plate. After ~10 min settling, the gels were washed gently with warm PBS to remove the off-site cells (cells that were located outside the microwells). The fresh media (~6 ml) was added onto the device before we put the cell-loaded devices in the CO_2 incubator. The cells were cultured on the device overnight to restore from the trypsin-induced stress for a formation of the stable adhesive bonds to the microwells. The duration of the short-term culture was determined by the cell doubling time and the recovery rate of the adhesive bonds of the cells. For HeLa cells, the short-term culture was between 4 h (bond recovery time) and 24 h (doubling time). For the control experiments, cells were cultured from a suspension of the same density, and the standard cell culture protocol was followed.

Cell viability validation: A calcein AM/Ethidium homodimer-1 kit was used for the cell-viability assessment. The assay was performed with cells on different substrates before and after the overnight culture to measure the change of the viability arising from the different culture substrates. Substrates with the cells were washed with warm PBS prior to the viability staining. For staining, the cell-laden substrates were incubated in a mixture of calcein AM (10 μM in PBS) and Ethidium homodimer-1 (4 μM in PBS) at room temperature for ~20 min in the dark. At the end of the staining, the substrates were washed off the staining solution with warm PBS for fluorescence imaging. The live and dead cells were examined through distinct fluorescence channels (green and red) using the fluorescence microscope.

Cell spreading assessment: The evaluation of cell spreading was performed by examining the fluorescence micrographs of the calcein AM-stained cells. The staining protocol was similar as described in the viability validation assay, but the staining solution only included calcein AM. To ensure a precise calculation of the contour of the cells, the fluorescence micrographs were taken at a high

magnification (40x). Each micrograph of the cell was quantitated in the projected area and circularity (circularity = 4π x (area / perimeter^2)).

In situ scWB for protein measurements: After overnight culture (typically 8-12 h), the cells formed the new bonds with the microwells, and were ready for subsequent scWB experiments. Extracellular stimulation or imaging can be implemented to cells at this stage. For single-cell western blot, the *in situ* scWB device was quickly assembled in an electrophoresis chamber. A harsh lysis/electrophoresis buffer (1%w/v SDS, 0.5%w/v sodium deoxycholate, 0.1%v/v Triton X-100, 0.5x Tris-Glycine) that was heated at 75 ºC was immediately poured onto the device to lyse the cell and solubilize the proteins. After ~15 s of chemical lysis and solubilization, a DC electronic field of 40 V/cm was applied to the device to initiate the electrophoretic separation of the proteins. When the target proteins migrated to the desired location along the migration lane (typically ~12 s), the electronic field was turned off and the long-wavelength UV (360 nm, 1.8 kJ/cm^2) was applied to the device to immobilize the proteins onto the gel. After immunoprobing, the abundance of the target proteins was measured from the fluorescence of the fluorescently-labeled antibodies.

Osmotic stress experiments: Isotonic (300 mOsm), hypertonic (400, 500, 600 mOsm), and hypotonic (200 mOsm) solutions were prepared by mixing 300 mOsm, 600 mOsm, 900 mOsm, 1200 mOsm, and 0 mOsm (MilliQ water) sucrose solution, with cell culture media or PBS buffer. For *in situ* scWB, HeLa cells were cultured overnight on the *in situ* scWB device in the incubator before the implementation of the osmotic stress. Then the old medium was quickly removed from the device and replaced with an equal volume of osmotic solution. The cell-laden devices immersed in the osmotic solution were incubated in the CO_2 incubator for 60 min to induce the osmotic responses. Upon the induction of the osmotic stress, the cells were analyzed for the abundance of the phosphorylated proteins through *in situ* scWB as described above. To mitigate the potential phosphatase activities on the phosphorylated protein, a cocktail solution of phosphatase inhibitor was added to the lysis/electrophoresis buffer. For scWB of trypsinized cells from conventional 2D cell culture with stimulation (normal scWB), HeLa cells were cultured in T25 tissue culture flasks to ~80% confluence. Following the addition of the stress solution, the cells were cultured in the CO_2 incubator for 60 min to induce the osmotic responses. Upon the completion of the stress induction, the cells were trypsin dissociated (3 min) into a single-cell suspension. After 3-min centrifugation, the cells were resuspended in the osmotic solution (PBS adjusted with the sucrose solution of the corresponding osmolarity), filtered into individual cells, and settled in individual microwells of the unfunctionalized scWB device. The operation was performed on ice. For slab gel western blot, cells were cultured in 24-well plates (500 µl medium in each well) for 1~2 days before osmotic stress induction. Then 250-µl osmotic solution (300, 600, 900, 1200, or 0 mOsm) was added to the cell culture in each well. Following an incubation of a defined time, the medium in each well was aspirated and the ice-cold lysis buffer (100 µl, RIPA buffer (89900, ThermoFisher) + Protease/Phosphatase inhibitor cocktail (78442, ThermoFisher)) was immediately added to the well. After a 20-min 4 ºC incubation on the shaker, the cell lysates were transferred to the Eppendorf tubes for centrifugation (10000 rpm, 5 min, 4 ºC). Upon centrifugation, 32 µl of supernatant in each tube was transferred to a new tube containing 48 µl SDS sample buffer (2x SDS sample buffer (LC2676, ThermoFisher) + Reducing Agent (NP0009, ThermoFisher)). The samples were denatured at 90 ºC for 3 min and subjected to western blot analysis.

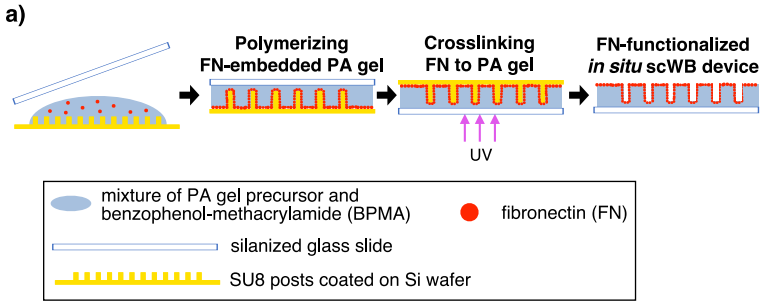
Fluorescence imaging: Stained cells in the viability assay and spreading evaluation were visualized via wide-field imaging. An Olympus IX71 inverted fluorescence microscope (equipped with ASI motorized stage, X-cite mercury lamp light source (Lumen Dynamics), and standard FITC and Cy5 filter cube (10x and 40x objectives)) was used for imaging. The bright-field and fluorescence micrographs were obtained using an iXon+ EMCCD camera (Andor Technology Ltd.) controlled by a MetaMorph software (Molecular Devices) with 10 ms exposure time. Confocal fluorescence micrographs were obtained using a confocal microscope (LSM 880-NLO-Airyscan, Zeiss) operated on ZEN 2.3 SP1. A water-immersion 20x objective (NA1.0, Zeiss W Plan APO 20x/1 DICIII) was used, and the scanning step in the z-stack imaging was 1 μm. The fluorescence images of the proteins in scWB experiments were collected by scanning the scWB devices or *in situ* scWB devices with a fluorescence microarray scanner (Genepix 4300A, Molecular Devices).

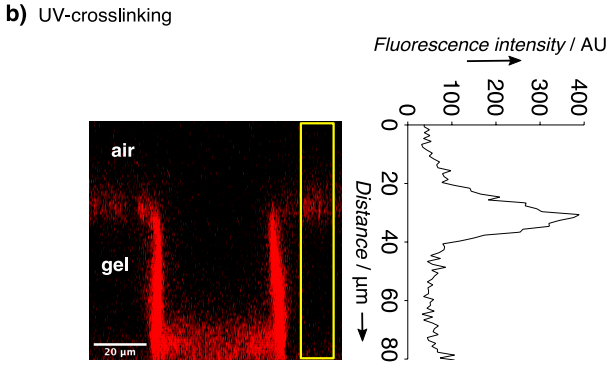
Data analysis: Data analysis for osmotic stress experiments was performed with Matlab (MathWorks) using an in-house analysis script. Briefly, background-subtracted signals of the proteins were integrated within the defined boundaries for AUC calculation. As a quality control, the protein peaks with SNR ≥ 3 were analyzed. All the other data analysis was performed with Fiji (NIH).

Statistical analysis: Mann-Whitney *U* test was used to evaluate the difference between the groups that do not follow the normal distribution, including the comparisons of the normalized phosphorylated protein abundance between the populations of iso- and hyper-osmotic conditions, the comparisons of the spreading levels between the cells cultured on different FN concentrations, and the comparisons of the viability between different culture conditions. Two-tailed students' *t* test was used to compare the variation of the FN* concentrations on the gels with different microwell diameters and FN* concentrations. Each experimental group contains >100 cells for statistical validity.

In situ scWB devices fabricated with and without covalent bonds

Fibronectin (FN) proteins are presumably retained on the surface of the polyacrylamide (PA) gel through the combined effects of hydrophobic adsorption[3] and hydrogen bonding.[4] The viscous nature of FN and its interaction with the PA gel are sufficient to hold FN to the gel without forming covalent bonds. To form stronger covalent attachment to the PA gel, FN can be crosslinked with the benzophenone of benzophenol methacrylamide (BPMA) -incorporated PA gel using UV irradiation[5] before peeling off the gel from the SU8 mold (Figure S1a). The characterization of the *in situ* scWB devices fabricated with (Figure S1b) and without (Figure S1c) covalent crosslinking indicates the successful coating of rhodamine-labeled FN (FN*) layer on the surface of both devices. Comparing the ratio of the FN* signal at the surface to the signal in the gel indicates a higher coating efficiency of the device without covalent crosslinking. The lower FN* intensity on the covalently-linked device likely arises from the lower FN*-transfer efficiency from the silicon mold to the gel when UV unintentionally activates the crosslinking between BPMA, FN* and silane-treated silicon wafer (via the methyl group).[6] For non-covalent ECM protein substrates, attached cells may displace or internalize the ECM proteins under induced starvation.[7] Thus, stronger covalent attachment of FN to the gel should be considered in long-term cell culture on chip. Since we don't intend for long-term culture on the device in this research, non-covalent FN is presumably not a major concern. To obtain devices with higher coating efficiency, we skipped the UV photo-capture in the fabrication in the osmotic stress measurements.





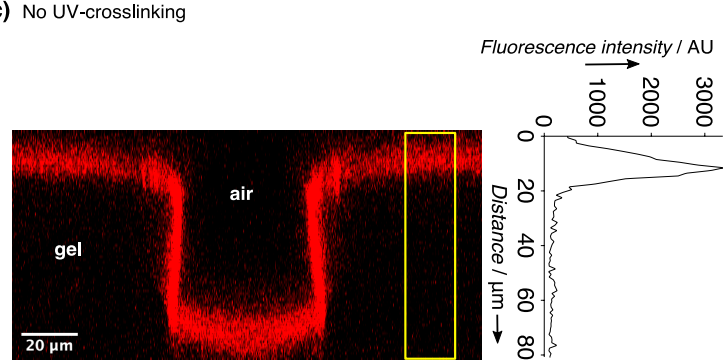


Figure S1. Comparison of the fibronectin (FN) coating on the *in situ* single-cell western blot (*in situ* scWB) devices with and without UV-initiated covalent bonding. **a)** The schematic of the device fabrication with Rhodamine-labeled FN (FN*) covalently attached to the gel. FN*: 10 μg ml^{-1}. UV: 365 nm; 150 s exposure at 20 mW cm^{-2}. **b)** The cross-section view of a microwell of the *in situ* scWB device fabricated with UV-crosslinking. **c)** The cross-section view of a microwell of the *in situ* scWB device fabricated without UV-crosslinking. In **b)** and **c)**, the left panels are false-color confocal fluorescence micrographs (orthogonal view of the stack images along the vertical direction). The right panels are fluorescence intensity profiles averaged over the horizontal direction along the vertical direction of the yellow box. Microwell: 50 μm in diameter.

Characterization of the fibronectin coating on the *in situ* scWB devices

To scrutinize the distribution of the fibronectin (FN) proteins in the PA gel of the *in situ* scWB devices, we measured the FN layer thickness (h) and the microwell fluorescence intensity ($I_{microwell}$) using the rhodamine-labeled FN (FN*). We performed the measurement with devices of various microwell diameters (D) and FN* concentrations (c) for a thorough evaluation.

Table S1. Average[a] FN* layer thickness (h) and CV on the *in situ* scWB devices of different microwell diameters and FN* concentrations.

	D = 50 μm, c = 1 μg ml^{-1}	D = 50 μm, c = 10 μg ml^{-1}	D = 50 μm, c = 100 μg ml^{-1}	D = 100 μm, c = 10 μg ml^{-1}
h (μm)	24	25	19	18
CV (%)	6.7	5.9	1.9	4.0

[a] Each data point was obtained by averaging over 3 locations across a device.

Table S2. Total microwell numeber (n), average microwell fluorescence intensity[a] ($I_{microwell}$) and CV on the *in situ* scWB devices of different microwell diameters and FN* concentrations.

	D = 50 μm, c = 1 μg ml^{-1}	D = 50 μm, c = 10 μg ml^{-1}	D = 50 μm, c = 100 μg ml^{-1}	D = 100 μm, c = 10 μg ml^{-1}
n	2074	1921	2176	2261
$I_{microwell}$	331	1948	11572	3055
CV (%)	18.6	16.1	16.4	20.0

[a] The microwell fluorescence intensity was calculated by averaging the fluorescence intensity over a square of 25 μm x 25 μm in the center of the microwell.

Cell viability assessment

To evaluate the viability of the cells after overnight culture on the *in situ* scWB device, we performed the viability assay to HeLa cells. Due to the potential toxicity or perturbation of the chemicals (Calcein AM / Ethdium homodimer-1) used in the assay, we performed the end-point assay in this study. Briefly, HeLa cells cultured on different devices were stained with the live/dead kit and assessed for viability at the beginning (1 h) and end (12 h) of the culture session. The viability was then compared between the two time points for FN-patterned gels (*in situ* scWB devices), unpatterned gels (normal scWB devices, negative control) and FN-patterned glass slides (positive control) (Figure S2a). The normalized viability after overnight culture indicates no significant difference for FN-patterned gels (Welch's *t*-test, $p = 0.86$) and FN-patterned glass slides (Welch's *t*-test, $p = 0.87$) (Figure S2b), suggesting the capability of the *in situ* scWB device in maintaining a cell-compatible environment for short-term cell culture. Interestingly, the negative control (cells cultured on the unpatterned PA gels) also presents a comparable viability between 1-h and 12-h culture (Welch's *t*-test, $p = 0.65$). We attribute this high viability to (1) the limited duration in the short-term experiments, under which circumstances the cells' tolerance to harsh environments dominates. (2) the possibility that the dead cells are dislodged from the microwells through culture media circulation and wash steps. In fact, we notice that some dead cells are located outside microwells on the surface of the gel and the total cell number in the microwells on an unpatterned scWB device is smaller after culture versus before culture. Despite the comparable viability in the negative control, the cells are unable to form the robust adhesion to the microwells and thus fail to spread (Figure S2a) as they normally do *in vivo* and *in vitro*. For cells cultured on the FN-patterned scWB and FN-patterned glass slide, the conditioned media (media extracted from the 2-day cell culture) was mixed at a ratio of 2:1 to the fresh media for the cell culture. The conditioned media was used in our experiments to facilitate the rapid cell spreading at the low-density cell culture (~2500 cm^{-2}), and was observed to contribute to the low viability (patterned scWB: 71% ± 6%, patterned glass: 73% ± 14%) before culture.

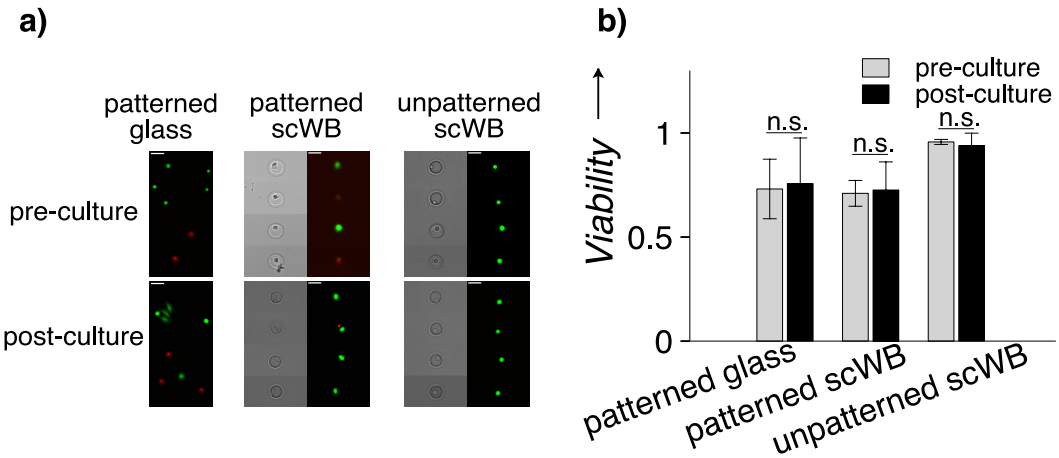


Figure S2. Viability assays performed to cells cultured overnight on FN-coated glass, *in situ* scWB devices and unmodified scWB devices indicates that cells maintain viable after overnight culture under all of the conditions. **a)** Representative bright-field and false-color fluorescence micrographs. Live and dead cells are indicated in green and red from the Calcein AM / Ethidium homodimer-1 live/dead stain. Scale bars: 50 μm. **b)** Viability quantitated by the ratio of the live cell number to the total cell number. Welch's *t*-test significance level: n.s., p >0.05.

Table S3. Statistical analysis of the HeLa cell differences in the circularity[a] and projected area that were cultured overnight on the *in situ* scWB devices with various FN concentrations (*c*).

	c = 0 μg ml^{-1}	c = 1 μg ml^{-1}	c = 10 μg ml^{-1}	c = 100 μg ml^{-1}
cell number (*n*)	143	232	159	116
Mann-Whitney test[b] for circularity (*p_circularity*)	n/a	3.5×10^{-14}	1.1×10^{-20}	7.3×10^{-28}
Mann-Whitney test for projected area (*p_area*)	n/a	0.5	8.7×10^{-9}	9.3×10^{-15}

[a] The cell circularity was defined as 4π x (projected area / perimeter^2). [b] The Mann-Whitney test was performed between the negative control (c = 0 μg ml^{-1}) and various FN concentrations.

Osmotic stress-induced kinase phosphorylation measured in bulk

To evaluate the osmotic stress responses of kinase phosphorylation in HeLa cells, we performed conventional western blot with HeLa cells at the population level prior to the single-cell measurement. We used sucrose as the osmotic inducer because it is low in conductivity and thus would not cause severe Joule heating during electrophoresis. Other common ionic osmotic inducers such as sodium chloride could also be used if the constant temperature can be maintained via good ventilation. We chose to measure the phosphorylation of ERK1/2 (p-ERK) and p38 (p-p38) because they are the important kinases in two representative MAPK (mitogen-activated protein kinase) pathways that were widely reported to be involved in osmotic stress-induced signaling.[8]

We measured the p-ERK and p-p38 levels under iso- (300 mOsm), hypo- (200 mOsm) and hyper-tonic (500 mOsm) conditions with various stress durations (Figure S3a). We observe that for both ERK and p38, hypo-osmotic stress-induced phosphorylation is negligible compared to the phosphorylation induced by the hyper-osmotic stress. Hyper-osmotic stress-induced phosphorylation can be detected at a time scale of minutes, and plateaus around 15 minutes of stress. The elevated phosphorylation maintains through the 60-min hyper-osmotic stimulation, with a slight decrease observed for p-p38 (Figure S3b). Compared with the reported hyper-osmotic stress-induced phosphorylation (~5-10 fold change),[9] the higher fold change of p-ERK and p-p38 here (~20 fold) likely stems from the unreliable reading of the detection system at the lower signal intensity. Nevertheless, for both ERK and p38, the marked phosphorylation change is observed induced by hypertonic sucrose between 15 and 60 min in HeLa cells.

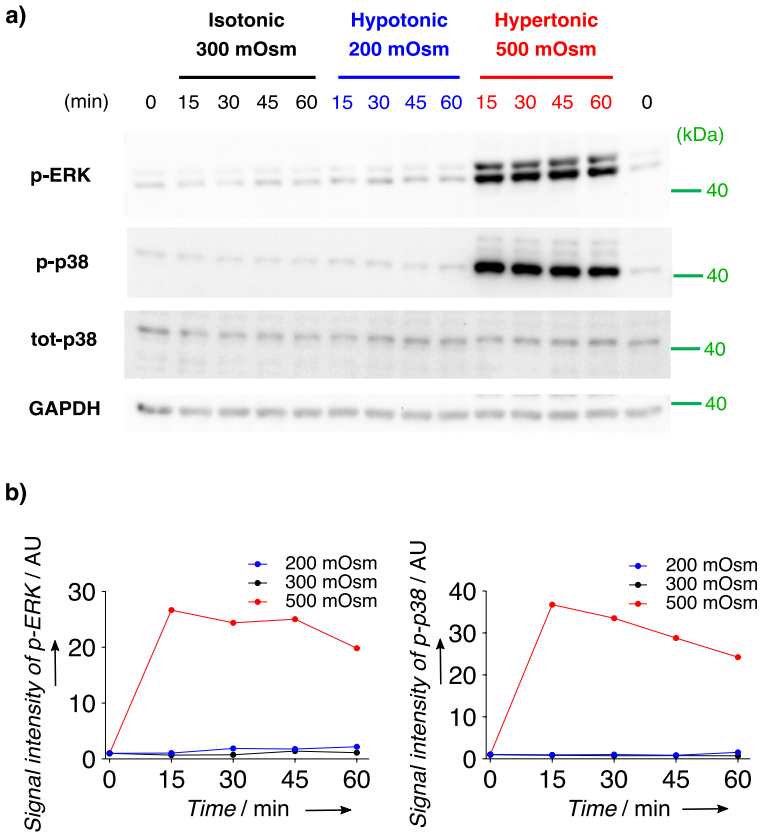


Figure S3. Hyper-osmotic stress-induced phosphorylation of ERK1/2 (ERK) and p38 can be detected at the population level. Phosphorylation changes were measured under the iso- (300 mOsm), hypo- (200 mOsm) and hyper-tonic (500 mOsm) conditions at various stress durations. **a)** Western blotting results of phosphorylated ERK (p-ERK), phosphorylated p38 (p-p38), total p38 (tot-p38), and GAPDH. **b)** Changes of p-ERK and p-p38 with stress duration under the iso- , hypo- and hyper-tonic conditions. The blot signals were normalized to the values of the control sample (no stress condition).

Independent replicates of normal scWB measurements

To confirm the observations in scWB of trypsinized cells from conventional 2D cell culture with stimulation (normal scWB), we reproduced the osmotic stress measurements for p-ERK and p-p38, with >100 cells in each experimental group. To focus on the phosphorylation change induced by the hyper-osmotic stress, we compared the phosphorylated proteins (p-ERK and p-p38) under the iso- and hyper-tonic conditions, using β-tubulin signals (a housekeeping protein that is unresponsive to osmotic stimulation, as indicated in Figure S3) to normalize the values. We observe no phosphorylation increase of ERK in the median level of the group under the hyper-tonic condition for all of the three independent scWB measurements. In contrast, there is a decrease of p-ERK under the hyper-tonic condition in two experiments (Figure S4, top left and top right), in agreement with the sampling experiments. Similarly, the decrease of p-p38 under the hyper-tonic condition is observed in two of the three measurements (Figure S4, bottom left and bottom middle). Although the increase of p-p38 under the hyper-tonic condition is detected in one of the three measurements (Figure S4, bottom right), the increase level (~2.6 fold) is less than the level measured in *in situ* scWB.

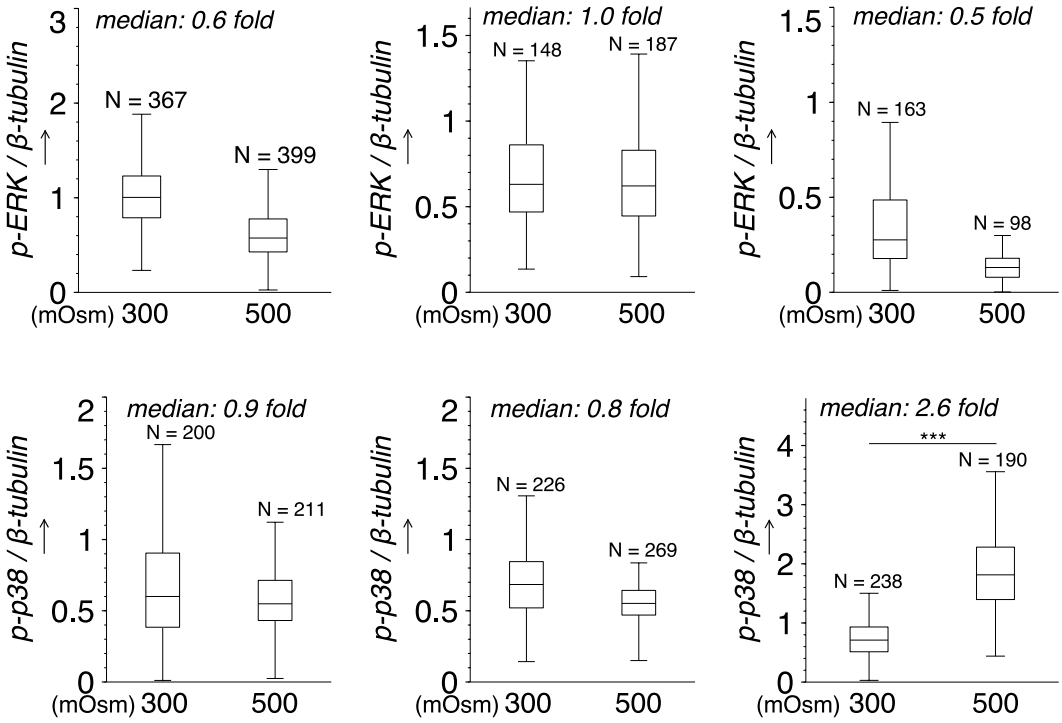


Figure S4. Three independent measurements of the hyper-osmotic stress-induced p-ERK and p-p38 using the normal scWB. The signals of p-ERK and p-p38 were normalized to the signals of β-tubulin from the same cell. Boxes represent the first and third quartiles of analyte distributions. Black lines indicate the median values. Whiskers are 1.5 fold of interquartile range. Mann–Whitney significance levels : ***, p <0.001. Sample size n is indicated for each group. The fold of the phosphorylation change induced by the hyper-osmotic solution was calculated using the median values of a population.

Disruptive sample preparation in single-cell measurement of p-ERK and p-p38

To pinpoint the factors that contribute to the undetected phosphorylation of ERK and p38 in scWB of trypsinized cells from conventional 2D cell culture with stimulation (normal scWB), we performed the sampling experiments (Figure S5a). Specifically, we sampled cells from each of the critical preparation steps in the normal scWB assay, and measured the phosphorylation levels using the slab gel western blot. The three critical steps in the normal scWB are trypsinization, centrifugation and cell settling. The time outside the culture media and the chemical or mechanical disturbances involved in these steps may trigger inadvertent phosphorylation and hence obscure the osmotic stress responses.

We compared the phosphorylation levels under the iso- and hyper-tonic conditions in each sampling (Figure S5b, Figure S5c), and observe that for both ERK and p38, after a 3-min trypsinization step, the phosphorylation difference between the iso- and hyper-tonic treatment is reduced compared to the control sampling, mainly due to the triggered phosphorylation in the iso-tonic samples. P-ERK levels keep decreasing throughout the 3-min centrifugation and the 30-min cell-settling steps in both iso- and hyper-tonic samples, whereas p-p38 levels start to decrease during the cell-settling step. Eventually the phosphorylation levels for both ERK and p38 are disrupted after the sample preparation. Taken together, the sampling experiments indicate that trypsinization and the out-of-culture settling in sample preparation of scWB distort the hyper-osmotic stress-induced phosphorylation.

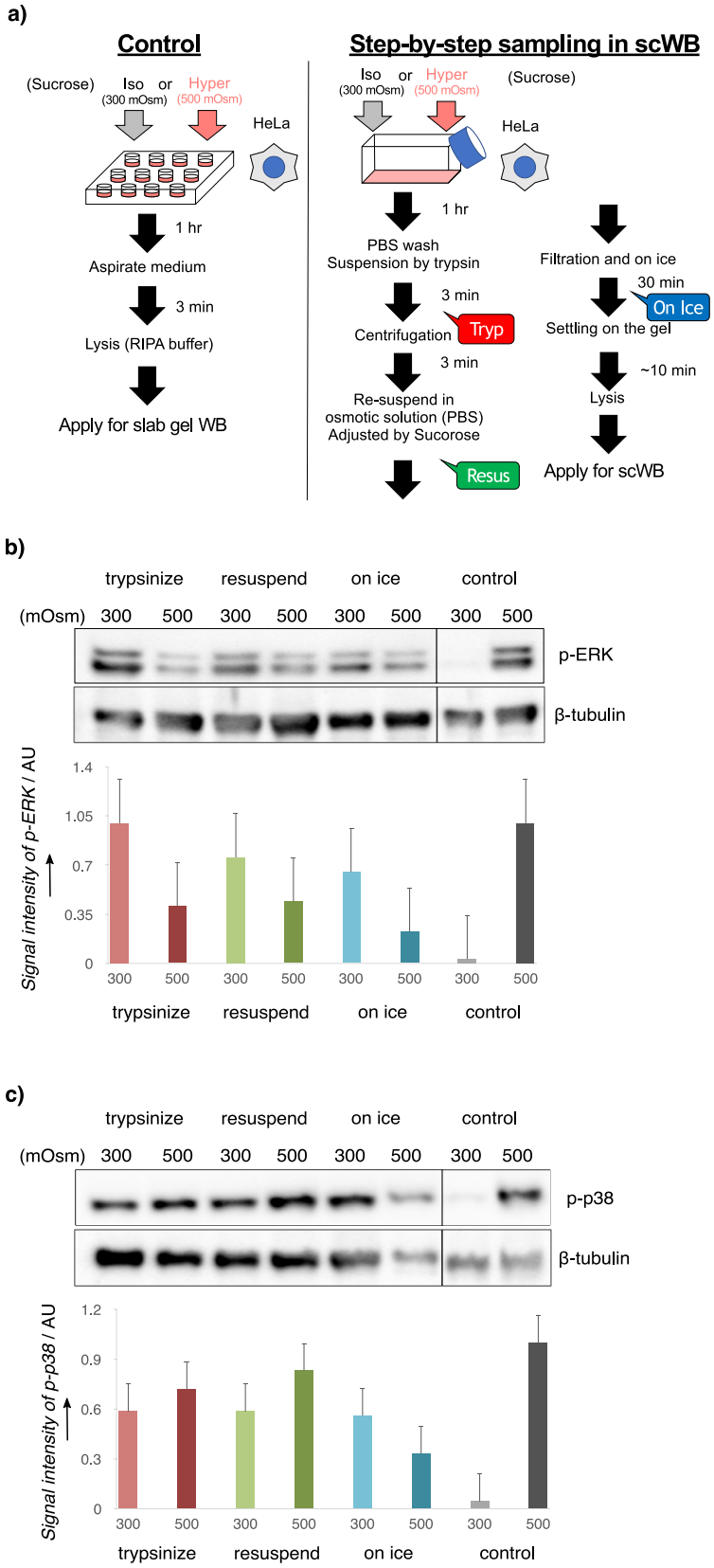


Figure S5. Western blot analysis performed on the samples extracted from each sample-preparation step of the single-cell western blot experiments suggests the disruption of the sample preparation on phosphorylation measurement in the normal scWB assay. **a)** The schematic of the sampling analysis. **b)** The quantitated results of p-ERK from 3 technical replicates. **c)** The quantitated results of p-p38 from 3 technical replicates. Error bars: standard deviation.

Independent replicates of *in situ* scWB measurements

To confirm the observations in *in situ* scWB, we reproduced the osmotic stress measurements for p-ERK and p-p38, with >100 cells in each experimental group. As in the normal scWB experiments, we again compared the phosphorylated proteins (p-ERK and p-p38) under the iso- and hyper-tonic conditions, using β-tubulin signals to normalize the values. For both ERK and p38, we observe a significant (Mann-Whitney test, p <0.001) phosphorylation increase in the median level under the hyper-tonic condition for all of the three independent *in situ* scWB measurements. The hyper-osmotic stress-induced increases of p-ERK and p-p38 are comparable to the reported values in the literature.[8]

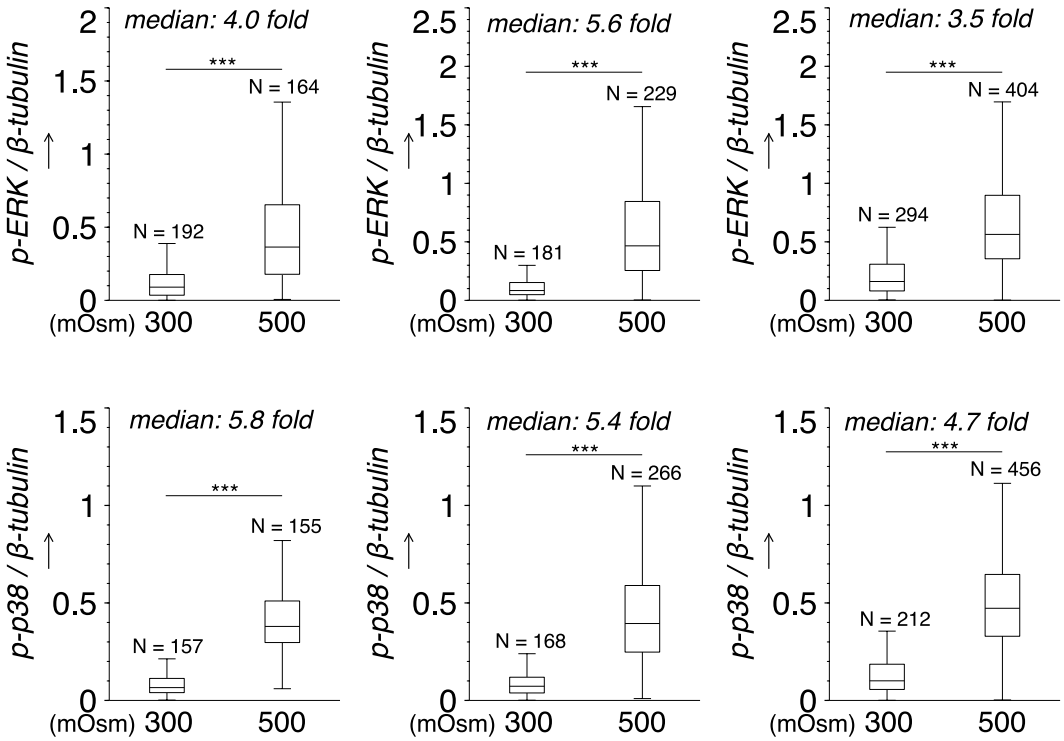


Figure S6. Three independent measurements of hyper-osmotic stress-induced p-ERK and p-p38 using *in situ* scWB. The signals of p-ERK and p-p38 were normalized to the signals of β-tubulin from the same cell. Boxes represent the first and third quartiles of analyte distributions. Black lines indicate the median values. Whiskers are 1.5 fold of interquartile range. Mann–Whitney significance levels : ***, p <0.001. Sample size n is indicated for each group. The fold of the phosphorylation change induced by the hyper-osmotic solution was calculated using the median values of a population.

Phosphorylation of ERK induced at different osmolarities

The hyper-osmotic stress responses of p-ERK were also measured at other (than 500 mOsm) osmolarities. We observe that a 60-min stimulation with 400 mOsm sucrose does not induce a significant increase (Mann-Whitney test, p >0.05) in median p-ERK, whereas a higher increase (6.0 fold) of median p-ERK is induced by a 60-min stimulation with 600 mOsm sucrose (Figure S7). Using *in situ* scWB, we can differentiate the sensitivity levels of the phosphorylation change in response to different hyper-osmotic stresses.

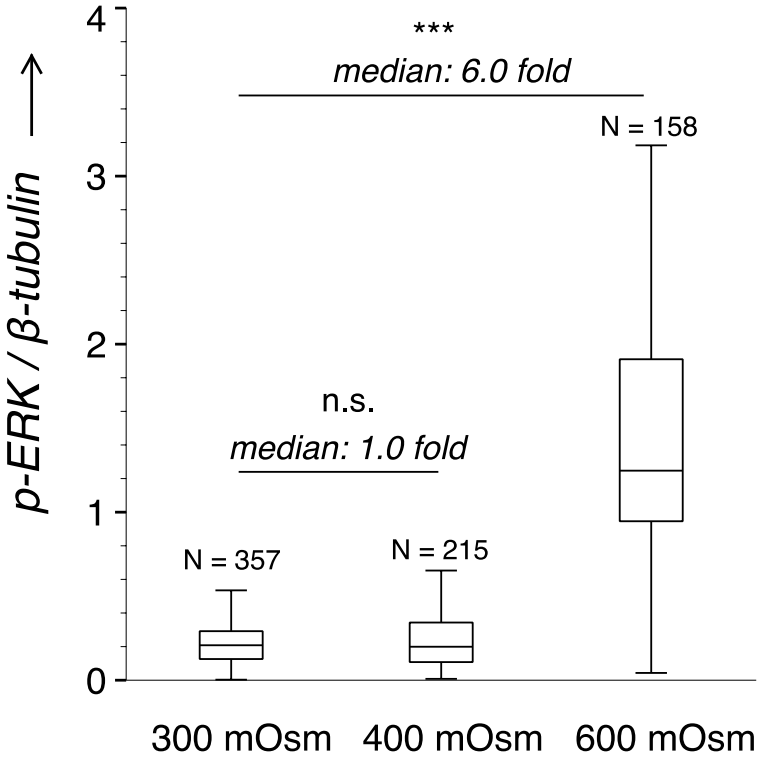


Figure S7. Changes of p-ERK under various hyper-osmotic conditions indicate the sensitivity of *in situ* scWB in measuring challenging proteoforms of single adherent cells. The signals of p-ERK were normalized to the signals of β-tubulin from the same cell. Boxes represent the first and third quartiles of analyte distributions. Black lines indicate the median values. Whiskers are 1.5 fold of interquartile range. Mann–Whitney significance levels : n.s., p >0.05. ***, p <0.001. The sample size n is indicated for each group. The fold of the phosphorylation change induced by the hyper-osmotic solution was calculated using the median values of a population.

Confocal micrographs of *in situ* scWB devices fabricated without fibronectin

To confirm that the fluorescence signals observed at the surface of the *in situ* scWB devices are from the rhodamine-labeled fibronectin (FN*), not from the artefacts (e.g., the refraction index mismatch at the interfaces), we fabricated the *in situ* scWB devices with no FN*, performed immunoprobing using AlexaFluor 647-labeled antibody (anti-FN), and examined the fluorescence at the rhodamine and AlexaFluor647 channels. For both FN* and anti-FN channels, the fluorescence intensity profile along the microwell height direction exhibits the background level of fluorescence, suggesting that the fluorescence observed at the surface of the *in situ* scWB devices can be accounted for the FN* layer.

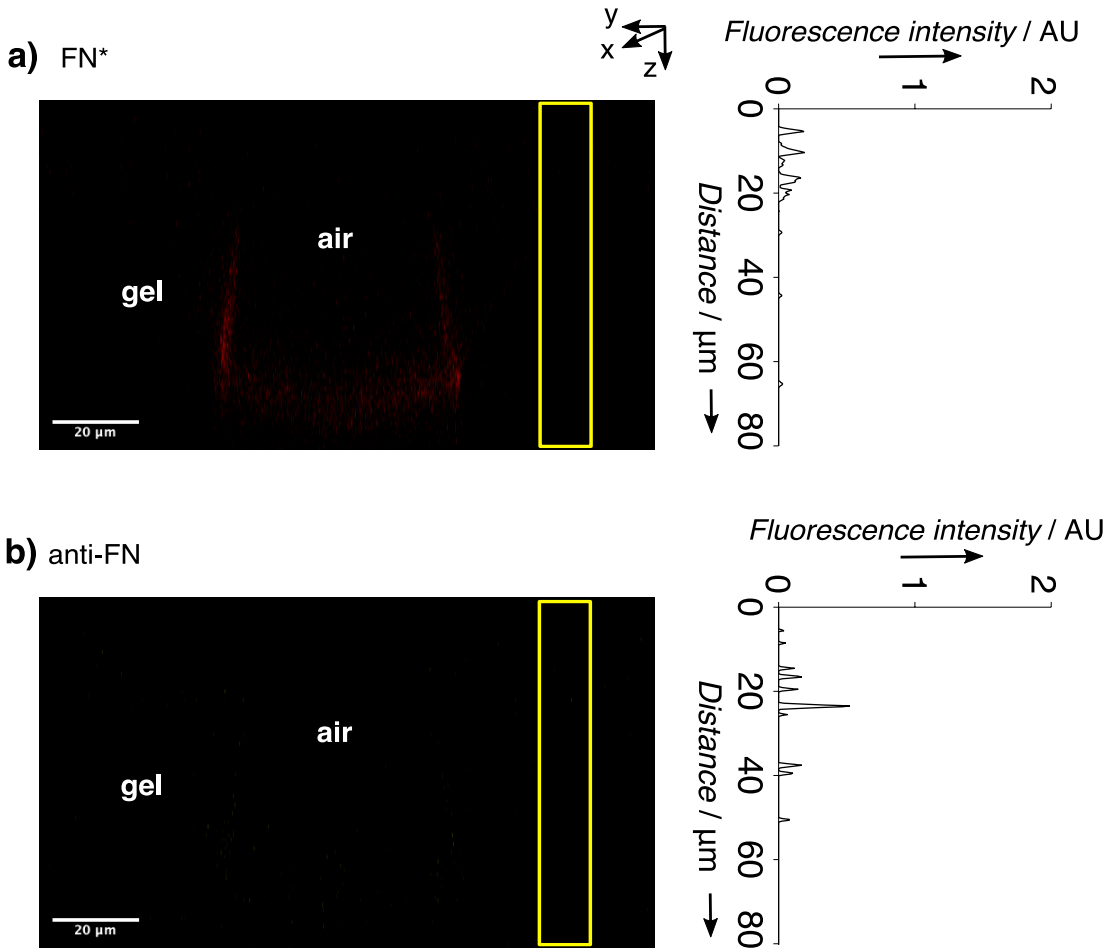


Figure S8. The rhodamine-labeled fibronectin (FN*) layer contributes to the fluorescence at the surface of the *in situ* scWB devices. **a)** The confocal micrograph of a microwell (50 μm in diameter) in the PA gel from an *in situ* scWB device (no FN* applied) acquired from the rhodamine channel. Left: the orthogonal view (y-z plane) from the z-stack micrographs (false-colored). Right: the fluorescence intensity profile (along z-axis) of the yellow box in the gel of the device. **b)** The confocal micrograph of the microwell (immunoprobed with AlexFluor 647-labeled FN antibody (anti-FN)) acquired from the AlexFluor 647 channel. Left: the orthogonal view (y-z plane) from the z-stack micrographs (false-colored). Right: the fluorescence intensity profile (along z-axis) of the yellow box in the gel of the device. Microwell depth: ~45 μm.

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Author Contributions

Y. Z. and A. E. H. designed the project. Y. Z. developed, fabricated and characterized the *in situ* scWB devices. Y. Z. (*in situ* scWB) and I. N. (scWB of trypsinized cells from conventional 2D cell culture with stimulation (normal scWB) and conventional western blot) performed the osmotic stress experiments. I. N. designed and performed the scWB sampling experiments. I. N. and Y. Z. wrote the Matlab scripts and performed the data quantification and statistical analysis. All authors interpreted the results and wrote the manuscript.