Supporting Information

Detecting kinase activities from single cell lysate using concentration-enhanced mobility shift assay

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Supplementary Methods

Microchip operation. The poly(dimethysiloxane) (PDMS) microchip was fabricated as described previously[1]. Before the experiment, the PDMS device channels were passivated with 1% bovine serum albumin (BSA) for 1 hour to reduce nonspecific binding of the sample to channel walls. After that, the channels were flushed with deionized water 3 times and filled with buffer solution (5 mM Tris-HCl, pH 7) until the samples were ready to be loaded. Samples were loaded into each of the five inlet reservoirs and drawn into the microchannel by applying a brief suction at the outlet reservoir. The liquid height difference between the inlet reservoir and the empty outlet reservoir caused a well-controlled gravitational flow of sample solution from inlet to outlet, without any need for external pump. Electrodes were inserted into the inlet and buffer reservoirs on the chip and connected to a power supply (Stanford Research Systems, Sunnyvale, CA). To initiate the concentration-enhanced mobility shift assay 50 V was applied at the inlet reservoirs while grounding the side channels.

Peptide synthesis. 5-FAM labeled peptide substrates for PKA (Kemptide[2], LRRASLG) and Akt (Crosstide[3], GRPRTSSFAEG and GRPRTSSFAEG-NH_2) were obtained from AnaSpec (Freemont, CA). 5-FAM labeled peptide substrate for MK2 (MK2tide[4], AHLQRQLSIA) was synthesized at Selleck Chemicals (Houston, TX). Fluorescent kinase substrate peptides modified with charged amino acids, 5-FAM-EELGRTGRRNSI for PKA and FITC-EEKKLNRTLSVA for MK2 were synthesized at Selleck Chemicals and NeoBioscience (Cambridge, MA) respectively. Nonfluorescent peptide spacers (nGE: G-E-(n-1)G, nG2E: G-E-G-E-(n-2)G) were synthesized at Selleck Chemicals (Peptide sequences are in Table S1).

Buffer recipes. Buffer A: 25 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT,

0.01% Triton X-100, 200 μg/mL BSA. Buffer B: 5 mM Tris-HCl (pH 7), 100 μg/mL BSA. Buffer C: 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 15 mM $MgCl_2$, 5 mM β-glycerolphosphate, 1 mM EGTA, 0.2 mM $Na_3VO_{4,}$, 0.2 mM DTT, 1 mM ATP, 200 μg/mL BSA. Buffer D: 1% Triton X-100, 50 mM β-glycerolphosphate, 10 mM sodium pyrophosphate, 30 mM NaF, 1 mM benzamidine, 2 mM EGTA, 100 μM $Na_3VO_{4,}$, 1 mM DTT, 10 μL/mL protease inhibitors (protease inhibitor cocktail III, Calbiochem), 10 μL/mL phosphatase inhibitors (phosphatase inhibitor cocktail 1, Calbiochem)

Measurement instrument and image analysis. An inverted epifluorescence microscope IX 71 (Olympus, Center Valley, PA) equipped with a cooled CCD camera (SensiCam, Cooke Corp., Romulus, MI) was used for fluorescence imaging. A mechanical shutter which only opens for 100 ms every 5 s when images are taken was used to prevent photobleaching of the fluorescent molecules. The images were analyzed using the NIH ImageJ software. Flat-field correction was performed by dividing a reference image of the device taken before each experiment. Concentrations of phosphorylated and unphosphorylated peptide substrates were assumed to be directly proportional to the focused peak height as demonstrated in previous work[1,5]. Phosphorylation ratio was calculated by dividing the peak height of the phosphorylated substrate by the sum of the peak heights of the phosphorylated and unphosphorylated substrates. Dose response curves and statistical analysis was obtained using Origin 7 software (OriginLab Corp., Northampton, MA).

Substrate cross-reactivity measurements. To measure the pair-wise cross-reactivity between each substrate and kinase, 1 μM of each substrate was separately reacted with a fixed final concentration (100 ng/mL) of recombinant kinase in Buffer A for 1 hour at room temperature.

The phosphorylation fraction was measured on the concentration-enhanced mobility shift assay platform as described previously.

Preparation of bulk cell lysate. HepG2 cells (a human hepatoblastoma cell line) were obtained from ATCC (Manassas, VA). HepG2 cells were seeded on 10-cm tissue culture-treated polystyrene plates at 1×10^5 cells/cm^2 in Eagle's minimum essential medium (EMEM; ATCC) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 100 U/ml penicillin (Sigma, St. Louis, MO), and 100 μg/ml streptomycin (Sigma) and were incubated at 37°C in 5% CO2. One day after seeding, medium was changed to fresh EMEM without FBS. One day after medium change, HepG2 cells reached confluence and were stimulated with 500 ng/mL insulin (Sigma) in EMEM (to activate Akt) or 25 μM forskolin (Sigma) in EMEM without FBS (to activate PKA), or left untreated (EMEM without FBS). For both conditions (activated and unactivated), cells were placed on ice 30 minutes following stimulation and culture medium was removed. Cells were lysed with lysis buffer (Buffer D, containing protease and phosphatase inhibitors) for 20 minutes at 4°C followed by scrapping. Lysates were clarified by centrifugation at 16,000g for 15 minutes at 4°C. Clarified lysates were analyzed using a bicinchonicic assay (Pierce, Rockford, IL) to determine the total cellular protein concentration. Stock lysates were divided into single-use aliquot and stored at -80°C.

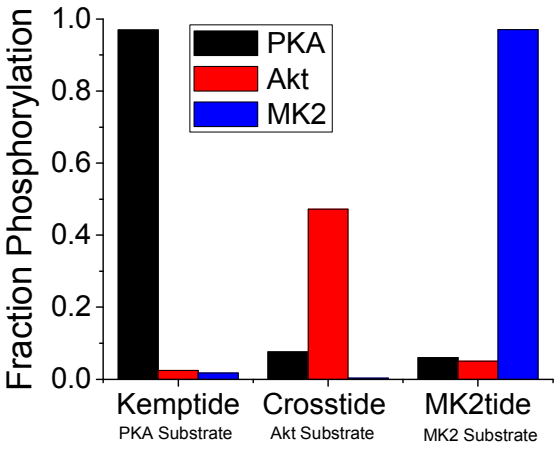
Single adherent cell culture and isolation. Single adherent HepG2 cells are isolated and grown in custom made nL volume cell culture chambers defined by non-cytotoxic silicone gasket wells on coverglass. The gaskets were composed of a stack of two thin PDMS sheets (250 μm thick, Silicone Specialty Products). An array of through holes (7×7, 2.8 mm pitch) was made by punching through the PDMS sheets with a 500 μm OD biopsy punch (Harris). The hole punching process was automated by mounting the biopsy punch on a CNC drilling machine

(Sherline) and using an open-source software (Modrilla) to control the exact coordinates where the holes will be punched. The automated process took <3 minutes to punch 49 holes. To prepare the microwells for cell culture, the perforated silicone sheet was treated with oxygen plasma (Harrick) for one minute and bonded permanently onto a piece of acid washed coverglass slip (22 mm×22 mm). The oxygen plasma treatment sterilized the PDMS gasket and increased its wettability to facilitate cell seeding in the later step. The devices were immediately immersed in 1× Phosphate Buffered Saline (PBS) after bonding and placed in a vacuum dessicator for 10 minutes to remove any trapped bubbles. Devices were kept in 1× PBS until ready to use. To seed single cells in the microwells, the devices were first immersed in 1mL of complete cell culture media in a 35 mm tissue culture polystyrene plate. Freshly trypsinized HepG2 single cell suspension was diluted to 1250 cells / mL in 1 mL complete culture media and added to the tissue culture plate. The cell suspension was mixed by gently pipetting up and down and allowed to settle overnight at 37 °C in 5% CO2. Cells settled randomly into the microwells following a Poisson distribution. The microwells were imaged under a microscope and the number of cells in each well was recorded. On average 30% of the 49 wells contained single cells under these conditions. A histogram showing the cell distribution in each of the wells is included in Supplementary Figure 3.

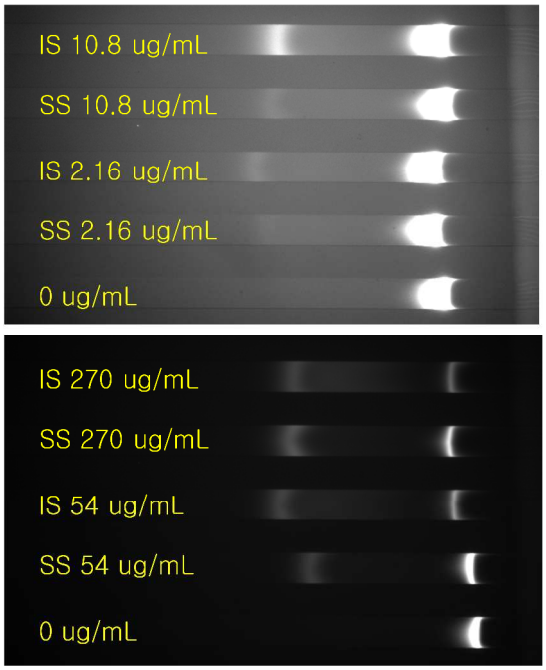
Single cell lysis and kinase assay. Ultrasonication is a proven technique for cell lysis and has been shown to be able to lyse cells in microfluidic devices in less than 10 s[6]. In this paper, single cells confined in nL size microwells were ultrasonically lysed so that the released intracellular kinases can catalyze phosphorylation of peptide substrates that were pre-added to the microwells just before lysis. To perform cell lysis in reaction buffer, the cells in microwells were first washed once with ice-cold PBS and followed by ice-cold Tris-Buffered Saline (TBS). The

microwells were then overflowed with kinase reaction buffer (Buffer C with 2 μM fluorescent substrate, 0.01% Triton X-100, 10 μg/mL protease and phosphatase inhibitors, and inhibitors of off-target kinases(0.4 μM PKI-tide, 5 μM GF109203X) containing the substrates. Next, the wells were sealed with a piece of Kapton tape and the excess substrate was squeezed out. The sealed device was then immediately placed in an ultrasonic water bath (VWR 150D Aquasonic) and sonicated at full power for 30 s to lyse the cells within the microwells. Cell lysis was confirmed by imaging under the microscope. The kinase reaction was then allowed to proceed in the sealed microwells for 90 minutes in a 37 °C incubator.

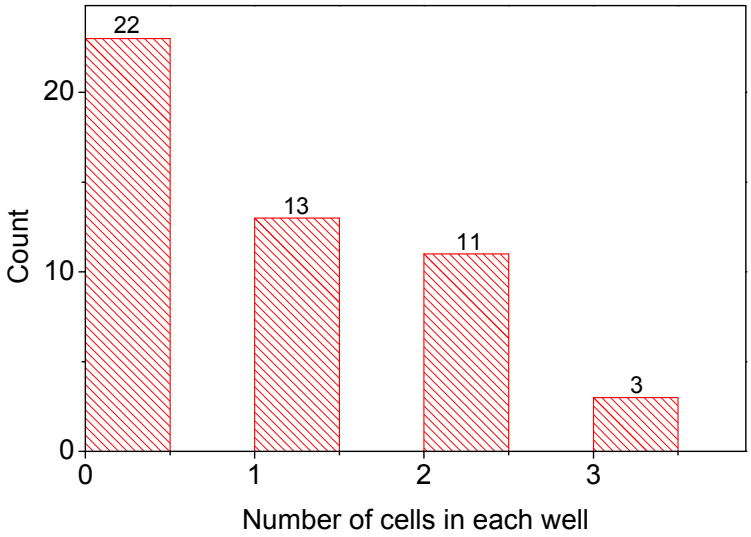
The kinase reaction product from the sealed 40 nL chambers were retrieved by diluting them into a much larger volume (12 μL). This dilution step stops the kinase reaction and facilitates sample handling. To perform the dilution step, another piece of PDMS (1 mm thick) with 2 mm diameter holes (2 mm biopsy punch) was aligned and reversibly bonded on top of the Kapton tape side of the device to form dilution chambers. 5 μL of the dilution buffer (Buffer B) was pipetted into each dilution chamber. The device was then placed in a dry 35 mm polystyrene dish for imaging under an inverted microscope. While looking under the microscope, the Kapton tape above the microwells was punctured using a hypodermic needle (27G1/2, BD) so that the microwell contents can be mixed with the dilution buffer. While the reaction product could diffuse into the dilution buffer, the process could be slow. Mixing was enhanced by agitating the device above an ultrasonic bath operating at low power for 30 s. The mixture in each dilution chamber was pipetted into a 96 well PCR plate and further topped up to 12 μL with dilution buffer before being stored at -80 °C for later use. 10 μL of this final sample was used in the concentration-enhanced mobility shift assay.



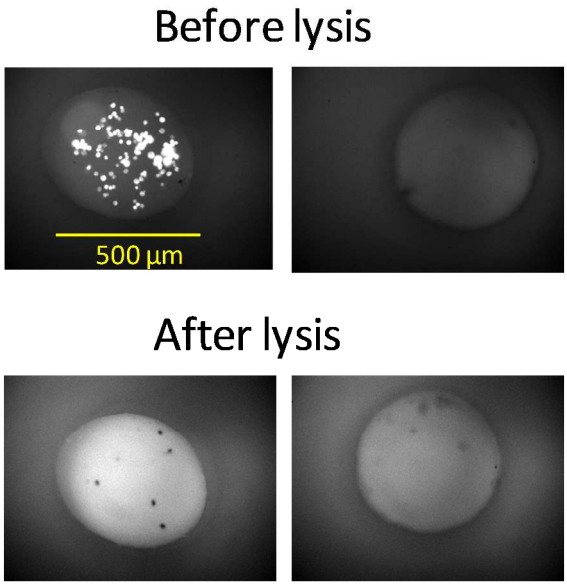
Supplementary Figure 1: Analysis of the pair-wise cross-reactivity between three kinases (PKA, Akt , MK2) and their substrates (Kemptide, Crosstide, MK2tide).



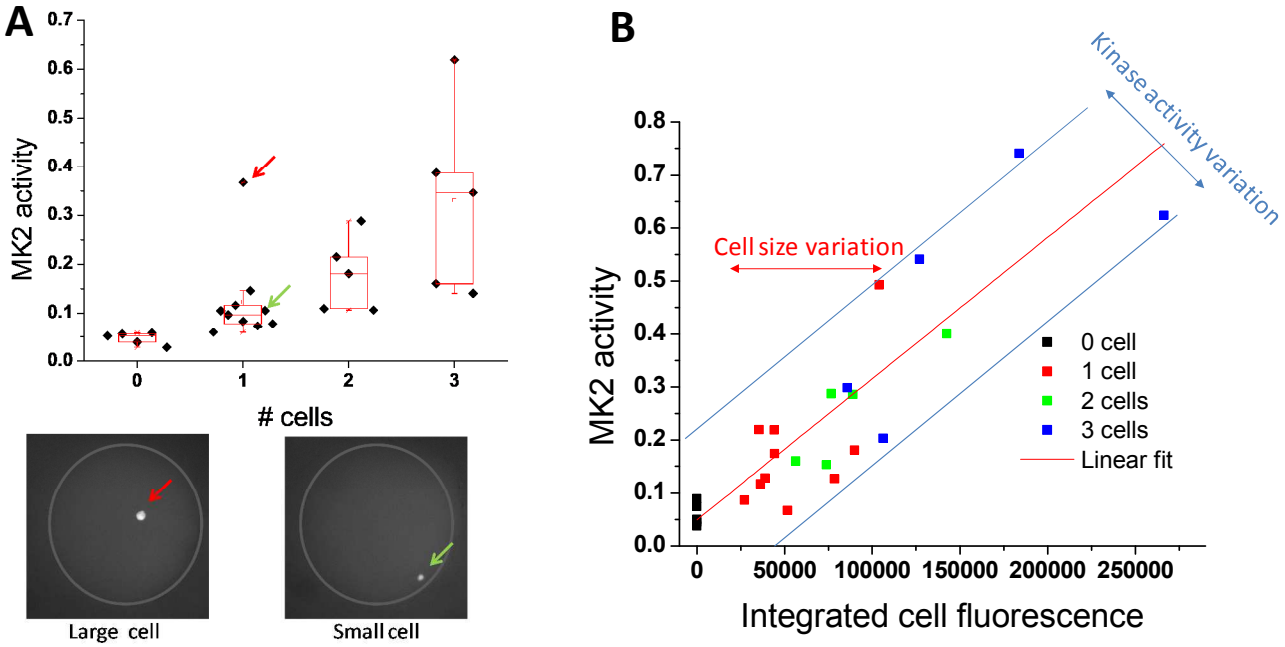
Supplementary Figure 2: Representative results showing concentration-enhanced mobility shift assay with cell lysate for detection of Akt activity in serum starved (SS) and 5 minutes 500 ng/mL insulin stimulated (IS) HepG2 cell lysate.



Supplementary Figure 3: Histogram showing the distribution of number of cells in each well. This cell distribution is obtained when 1250 cells are seeded in a 35 mm diameter petri dish. The diameter of the wells is 500 µm.



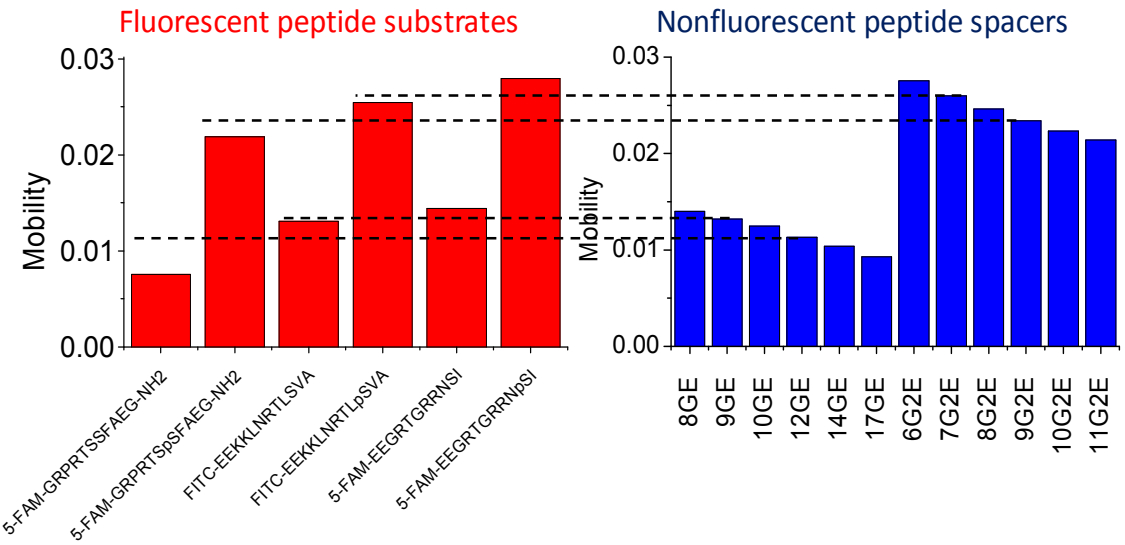
Supplementary Figure 4: Diagram showing Cell Tracker Orange labeled HepG2 cells are completely lysed after 30 s ultrasonic treatment, intracellular contents including the cytoplasmic live cell dye are released and confined in the microwells,



Supplementary Figure 5: a) Scatter plot shows the total kinase activities (substrate phosphorylation ratio) in microwells containing zero, one, two and three cells. Total kinase activity increases with the number of cells. Outlier data points correspond to large cells. b) Linear relationship between kinase activity (substrate phosphorylation ratio) and cell size using cell fluorescence as a proxy (R^2=0.77).

Peptide	Sequence
8GE	GEGGGGGGG
9GE	GEGGGGGGGG
10GE	GEGGGGGGGGG
12GE	GEGGGGGGGGGGG
14GE	GEGGGGGGGGGGGGG
17GE	GEGGGGGGGGGGGGGGGG
6G2E	GEGEGGGG
7G2E	GEGEGGGGG
8G2E	GEGEGGGGGG
9G2E	GEGEGGGGGGG
10G2E	GEGEGGGGGGGG
11G2E	GEGEGGGGGGGGG

Supplementary Table 1: Sequences of nonfluorescent peptide spacers



Supplementary Figure 6: Predicted mobilities of the fluorophore-tagged peptide substrates and synthetic peptide spacers according to the Offord Model. To resolve a pair of analytes of interest, a synthetic peptide with an intermediate mobility can be chosen as a spacer.

Reference

1. Cheow, L. F.; Han, J. Continuous signal enhancement for sensitive aptamer affinity probe electrophoresis assay using electrokinetic concentration. *Analytical chemistry* **2011,** *83* (18), 7086-7093.

2. Kemp, B. E.; Graves, D. J.; Benjamini, E. L. I. E.; Krebs, E. G. Role of multiple basic residues in determining the substrate specificity of cyclic AMP-dependent protein kinase. *Journal of Biological Chemistry* **1977,** *252* (14), 4888-4894.

3. Cross, D. A.; Alessi, D. R.; Cohen, P.; Andjelkovich, M.; Hemmings, B. A. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* **1995,** *378* (6559), 785-789.

4. Manke, I. A.; Nguyen, A.; Lim, D.; Stewart, M. Q.; Elia, A. E.; Yaffe, M. B. MAPKAP Kinase-2 Is a Cell Cycle Checkpoint Kinase that Regulates the G2/M Transition and S Phase Progression in Response to UV Irradiation. *Molecular cell* **2005,** *17* (1), 37-48.

5. Munson, M. S.; Meacham, J. M.; Ross, D.; Locascio, L. E. Development of aptamer-based affinity assays using temperature gradient focusing: Minimization of the limit of detection. *Electrophoresis* **2008,** *29* (16), 3456-3465.

6. Wu, C.; Lillehoj, P. B.; Sabet, L.; Wang, P.; Ho, C.-M. Ultrasonication on a microfluidic chip to lyse single and multiple Pseudo-nitzschia for marine biotoxin analysis. *Biotechnology journal* **2011,** *6* (2), 150-155.