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Microfluidic Concentration-Enhanced Cellular Kinase Activity Assay

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In cell signaling pathways, information is transmitted via specific protein−substrate binding interactions, typically leading to modification of the substrate modulating a next biochemical or biophysical step in the signaling cascade. The ability to experimentally monitor the enzymatic activity of each kinase within multipathway signaling networks is critical for understanding complicated network dynamics. Many technologies are available for measuring and monitoring the signaling processes in regulatory pathways.[1] For affinity-based technologies (kinase assays), the availability of specific antibodies (or equivalent) toward the given target has always been the main hurdle for general application of the technique. Also reaction times required to turn over enough substrates for detection can be long, typically a few hours. While mass spectrometry (MS) is a versatile measurement tool for proteins with the ability of discerning even subtle post-translational modifications, throughput is generally low, with the requirement of a relatively large sample size (10^5-10^7 cells). As a result, measurement of signals within regulatory pathways is largely done by averaging over many cells, which may or may not be at the same stages of intracellular signal processing. It is widely accepted, therefore, that such a population-average measurement provides only a "blurred" picture of inner workings of the complex pathways.[2−4] Although there are few reports of measurement technologies capable of assaying kinase activities in single cells, these rely on injection of specialized kinase substrates directly into living cells[5] or require imaging[6] or flow cytometry[7] approaches and thus are not compatible with other types of assays. Here, we describe a novel microfluidics approach for assaying kinase activity in samples from small numbers of cells using standard cell culture and lysis methods compatible with other types of lysate-based assays (e.g., real-time polymerase chain reaction) and integrateable into existing lab-on-a-chip approaches.

Various types of enzyme activity assays have been implemented in microfluidic chips previously.[8−10] However measurement of low abundance kinase activities has not yet been reported, mainly due to the low kinase turnover rate. Recently, we have developed a novel nanofluidic biomolecule concentration device which can be used to collect and trap proteins contained in a sample into a much smaller volume, increasing the local concentration significantly.[11,12] Using the concentration-enhancing device, we also reported a method of increasing both kinetics and sensitivity of immunoassays,[13] as well as the trace level enzyme (trypsin) activity assay.[14] So far these assays have been demonstrated only in a standard buffer system, not in a complex, real physiological sample as required for routine applications.

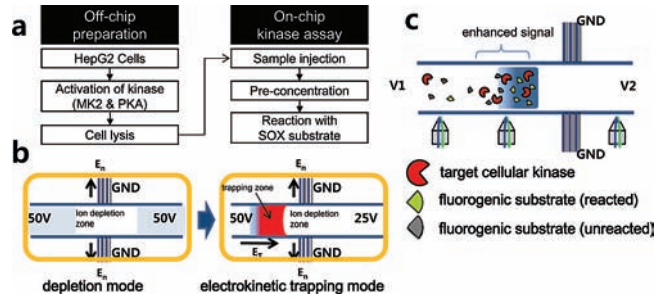

Figure 1. Schematic representation of the concentration-enhanced cell kinase assays using micro/nanofluidic preconcentration chip. (a) Outline schemes with off-chip preparation and on-chip kinase assay. (b) Operation mode. (c) Assay schemes of PDMS preconcentration chip.

Herein, we report concentration-enhanced cell kinase assays in a micro/nanofluidic platform *directly from cell lysates*, to enable scientific studies on cell signaling pathways at the single-cell level. We demonstrate the concentration-enhanced enzyme assay with two important cellular kinases, MK2 and PKA, from human HepG2 cells (Table S1) only with lysates from a few cells.

The key operations as well as the poly(dimethylsiloxane) (PDMS) nanofluidic preconcentrator chip are shown in Figure 1. The micro/nanofluidic preconcentration chip was composed of two microchannels with a planar ion-selective Nafion membrane across the microchannels.[12] The middle channel is where the ion depletion zone is created, which is used to trap proteins contained within cell culture lysate from HepG2 cells, as well as a kinase-activated fluorogenic chemosensor (Sox substrate[15]). The outer channel, which surrounds the sample channel on the left and right in the middle section of the device (See Figure S1), is also filled with a 1X PBS buffer solution. By using a Nafion nanojunction that shows a high ionic permselectivity[12] we can achieve stable concentration operations even in a complex physiological sample (Table S1) with a relatively high ionic strength. Detailed experimental procedures can be found in the Supporting Information (Figures S1−S3).

HepG2 cell lysate was prepared to have an overall protein concentration of ∼125 μg/mL. The cells used in this experiment have ∼1 ng of protein per cell, so this lysate represents ∼125 cells/μL. We diluted 0.5 μL of such lysates into 4.5 μL of the premixed biosensor cocktail (with ATP, inhibitor cocktails, kinase buffer and 10 mM Sox substrates). The resulting 5 μL lysate−Sox substrate mix (containing cellular proteins from roughly 65 cells) was then loaded into the sample reservoir of the device (Sample 1/1′ and 2/2′ in Figure 2). Also as a positive control, recombinant enzyme (MK2 (125 ng/mL) and PKA (60 ng/mL)) was mixed in 1X PBS buffer and used in the assay (Sample 3/3′ in Figure 2). Then, reactions to turn over Sox substrates (to render them fluorescent) by kinases were monitored, both with (conditions 1′−3′) and without (conditions 1−3) concentration enhancement. All the kinase

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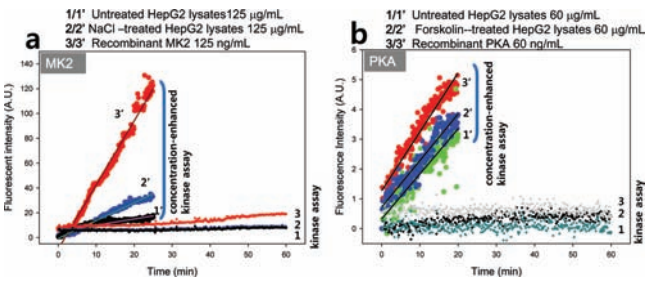

Figure 2. Performance of concentration-enhanced kinase activity assay. (a) Fluorescence enhancement of untreated HepG2 lysates (1), NaCl-stimulated HepG2 lysates (2), and recombinant active MK2 enzyme (3) in an MK2 kinase assay with concentration enhancement using nanofluidic network. Significant increases in MK2 phosphorylation of the Sox substrate and resultant fluorescence signal are observed using the 20-min on-site preconcentration method (1′, 2′, 3′) compared to unconcentrated assays (1, 2, 3). (b) Fluorescence enhancement of untreated HepG2 lysates (1′), Forskolin-stimulated HepG2 lysates (2′), and recombinant active PKA enzyme (3′) in a PKA kinase assay with concentration enhancement using nanofluidic network compared to unconcentrated assays (1, 2, 3).

activities were checked with the reaction in the standard microplate reader before the assays in the microfluidic chip (Figure S4).

We observed no fluorescence signal changes (with or without concentration enhancement) for our negative control samples (contains lysate buffer but not HepG2 cell lysate). Fluorescence intensities of turned-over substrates increase linearly with assay time. It was shown, for both MK2 and PKA, that the sensitivity of the activity measurement has been improved significantly due to the concentration enhancement. For example, comparison between condition 2′ and 2 in Figure 2a shows an ∼11-fold increase in turnover rate (rate of increase in signal) of MK2. This allowed us to clearly differentiate conditions 1′(1) and 2′(2) within 7 min (Figure S5), which was not possible in the unconcentrated assay, where traces for conditions 1 and 2 are shown to overlap even after 1 h of reaction time (Figure 2a). In the experiment with PKA (Figure 2b), similar enhancement was observed. Without concentration enhancement, no PKA signal was observed in untreated HepG2 lysates, Forskolin-treated HepG2 lysates, and recombinant PKA even after 60 min. But, with the concentration enhancement, the Sox substrate phosphorylation by PKA was significantly increased. The activated HepG2 lysate sample (with 30-min Forskolin treatment, Figure 2b, data 2′) demonstrated an ∼11-fold increase in fluorogenic substrate phosphorylation, compared with the non-concentrated assay (Figure 2b, data 2).

We repeated the same experiment with diluted cell lysates (down to 1.9 μg/mL) to test the ultimate sensitivity of the assay. Table 1 shows MK2 kinase reaction velocity as a function of lysate or kinase concentration; these results demonstrate that the reaction rate and the sensitivity of low-concentration kinase activity measurement can be dramatically increased by implementing a 20-min concentration. Without concentration, the MK2 kinase reaction velocity at 125 μg/mL lysate concentration (125 ng/mL for recombinant kinase) was 0.045, 0.052, and 0.185 AU/min for untreated lysates, NaCl-treated lysates, and recombinant MK2, respectively. With concentration, these values increased to 0.49, 1.43, and 5.16 AU/min, respectively, which is an ∼25-fold increase in reaction velocity. Furthermore, with concentration, NaCl-treated MK2 cell lysates can be measured down to 1.9 μg/mL, which is at least a 65-fold enhancement in the limit of detection. Detection from ∼10 μg/mL lysate, which is a conservative estimate of our current detection limit, represents a kinase assay from ∼5 cells. This clearly demonstrates the applicability of our assay for single-cell level study of cell regulatory pathways.

Table 1. MK2-Sox Reaction Velocity As a Function of Cell Lysate (Recombinant Kinase) Concentration (AU/min)

		μg/mL (ng/mL for recombinant MK2)			
	lysate protein concentration	1.9	7.8	31.5	125
with concn	recombinant MK2	0.33	2.15	3.9	5.16
	activated lysate	0.18	0.49	1.06	1.43
	untreated lysate	0.05	0.21	0.41	0.49
without concn	recombinant MK2	0	0	0	0.185
	activated lysate	0	0	0	0.052
	untreated lysate	0	0	0	0.045

The sensitivity enhancement was more prominent in the detection of diluted lysates. We suspect that this is caused by the additional interference from inhibiting enzymes in higher-concentration cell lysate samples. If inhibiting enzymes are concentrated along with the target kinases above a certain level in the concentration plug, they can (nonspecifically) interact with the target kinases, reducing their turnover rate. Presumably, this would be less of a problem in detections using lower cell number samples.

In conclusion, we have demonstrated a novel device for *in vitro* concentration-enhanced cell kinase assays, with at least a 25-fold increase in reaction velocity and 65-fold enhancement in sensitivity. Also, we shortened the assay time from ∼1 h to 10−20 min and decreased the amount of sample needed down to ∼5 μL (from ∼200 μL using standard methods). This device, with its simplicity and efficient capability for assaying kinase activity in physiologically complex and relevant samples, could be a generic and powerful tool for diagnostics and systems biology research. If optimized, this scheme could lead to the quantitative measurement of cellular kinase activities potentially at or near single-cell level, which would provide an unblurred picture of the inner workings of cell regulatory pathways.

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Supporting Information Available: Detailed experimental procedure, cell kinase activity data using microplate reader, and complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Albeck, J. G.; MacBeath, G.; White, F. M.; Sorger, P. K.; Lauffenburger, D. A.; Gaudet, S. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 803–812.
(2) Lahav, G.; Rosenfeld, N.; Sigal, A.; Geva-Zatorsky, N.; Levine, A. J.; Elowitz, M. B.; Alon, U. *Nat. Genet.* **2004**, *36*, 147–150.
(3) Nair, V. D.; Yuen, T.; Olanow, C. W.; Sealfon, S. C. *J. Biol. Chem.* **2004**, *279*, 27494–27501.
(4) Nelson, D. E.; et al. *Science* **2004**, *306*, 704–708.
(5) Meredith, G. D.; Sims, C. E.; Soughayer, J. S.; Allbritton, N. L. *Nat. Biotechnol.* **2000**, *18*, 309–312.
(6) Irish, J. M.; Hovland, R.; Krutzik, P. O.; Perez, O. D.; Bruserud, Ø.; Gjertsen, B. T.; Nolan, G. P. *Cell* **2004**, *118*, 217–228.
(7) Taylor, R. J.; Falconnet, D.; Niemist Ä ¶, A.; Ramsey, S. A.; Prinz, S.; Shmulevich, I.; Galitski, T.; Hansen, C. L. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 3758–3763.
(8) Hadd, A. G.; Raymond, D. E.; Halliwell, J. W.; Jacobson, S. C.; Ramsey, J. M. *Anal. Chem.* **1997**, *69*, 3407–3412.
(9) Wang, J.; Chatrathi, M. P.; Tian, B. *Anal. Chem.* **2001**, 1296–1300.
(10) Seong, G. H.; Heo, J.; Crooks, R. M. *Anal. Chem.* **2003**, *75*, 3161–3167.
(11) Wang, Y. C.; Stevens, A. L.; Han, J. *Anal. Chem.* **2005**, *77*, 4293–4299.
(12) Lee, J. H.; Song, Y.-A.; Han, J. *Lab Chip* **2008**, *8*, 596–601.
(13) Wang, Y.-C.; Han, J. *Lab Chip* **2008**, *8*, 392–394.
(14) Lee, J. H.; Song, Y.-A.; Tannenbaum, S. R.; Han, J. *Anal. Chem.* **2008**, *80*, 3198–3204.
(15) Shults, M. D.; Janes, K. A.; Lauffenburger, D. A.; Imperiali, B. *Nat. Methods* **2005**, *2*, 277–283.

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