AN INTEGRATED MICROFLUIDIC PROBE FOR MULTIPLEXED SINGLE CELL KINASE KINASE ACTIVITY MEASUREMENT

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ABSTRACT

We present an integrated microfluidic probe that enables the measurement of multiple kinase activities in selected phenotypically distinct single cells from large cell populations on standard tissue culture platforms. The contents of a cell are captured without disturbing its extracellular context by creating a small lysis zone at the probe tip by hydrodynamic confinement. Pneumatic micro-valves are then used to separate and mix the captured lysate into different assay mixtures in separate small volume chambers for a fluorimetric assay. We demonstrate here the ability to simultaneously measure the activity of three kinases: Akt, MAPKAPK2, PKA and a loading control enzyme, GAPDH, from a single cell. This single cell assay platform enables the correlation of cellular phenotype to intracellular biochemical state at the single cell level and hence can provide a clearer understanding of cell behavior in heterogeneous cell populations.

KEYWORDS

Single Cell Assay, Kinase Assay, Microfluidic Probe, Multiplexing

INTRODUCTION

Cellular heterogeneity may result from intrinsic stochastic fluctuations or due to cell-to-cell variations in a cell's history and extracellular microenvironment [1, 2]. Heterogeneous subpopulations of cells can drive disease or development outcomes [1-3] but are overlooked in traditional biochemical assays which, due to limited sensitivity, measure ensemble averages of 10^3-10^6 cells. In the context of attempts to unravel cellular signaling networks, where measurement of multiple signaling nodes, usually proteins, becomes essential, heterogeneity leads to the emergence of a blurred picture where even distinguishing correlated and anti-correlated nodes becomes difficult [3]. Single cell measurements of multiple protein activities when correlated with the cellular phenotype measurements can help resolve these ambiguities.

Recent traditional and microfluidic tools [4] have focused on studying genetic heterogeneity in single cells harnessing the sensitivity resulting nucleic acid amplification techniques. However such techniques fail to capture important non-genetic – translational, post-translational and extracellular environment regulated – sources of heterogeneity which create unique proteomes in different cells which may drive the diverse phenotypes observed. Other single cell proteomic techniques using flow cytometry or imaging depend on the use of phospho-specific antibodies [5] and thus measure phosphorylation as a surrogate for protein activity – a correlation which may not be perfect especially in the presence of multiple post-translational modifications [6]. Genetically encoded reporters can be used to measure protein activity in live single cells but require genetic manipulation of cells and are difficult to multiplex which limits their use. Direct measurement of protein activity from single cells remains difficult due to

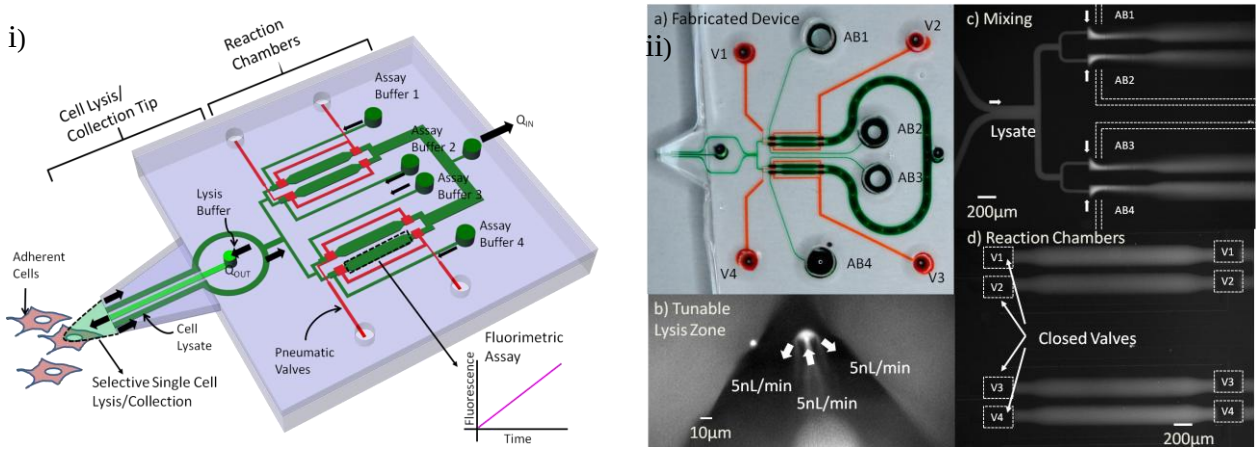

Figure 1: i) The integrated microfluidic probe consists of two modules: cell lysis/collection tip to selectively lyse and collect single cell contents and a set of four reaction chambers in which the lysate can be mixed with different assay reagents and held for observation to measure activities of different kinases. ii) a) Fabricated two layer PDMS device: top 5mm layer with channels and chambers (green) and lower 30µm layer which forms the base of the tip and contains the valve lines (red). b) Formation of lysis zone at tip c) Mixing of cell lysate with different assay buffers d) Reaction chambers filled and valves closed.

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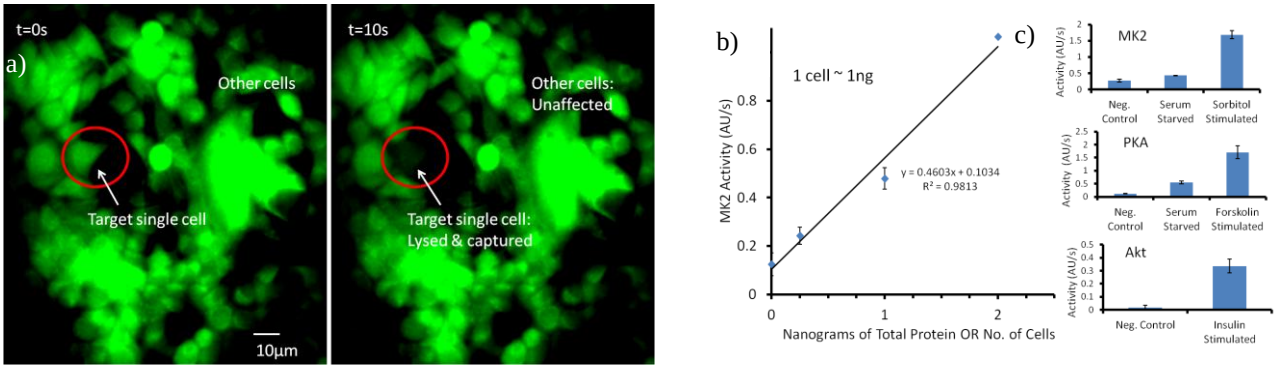

Figure 2: a) A selected single MCF-7 cell is lysed and captured using the microfluidic probe by flowing out a lysis buffer (with 1% Triton-X 100) and simultaneously flowing the cell lysate back into the device. Due to the limited lysis zone, other contacting and nearby cells even on a tissue culture dish grown to about ~95% confluence remain unaffected. b) Calibration of sensitivity and repeatability of MK2 activity assay in microfluidic probe using cell lysate obtained from bulk lysis of sorbitol-stimulated HepG2 cells which are expected to contain ~1ng/cell of total protein. c) Measurement of activities of the 3 kinases, MK2, PKA and Akt from 1 ng of lysate obtained from cells under different stimulation conditions is demonstrated.)

limited assay sensitivity. Single cell activity measurement by microinjection of substrates has been demonstrated but can suffer from non-specific intracellular reactions [6].

We reported earlier [7] the development of a microfluidic probe which captures the contents of a single cell and measures a single kinase activity therein. Simultaneous measurement of multiple kinase activities and higher measurement throughput, desirable for understanding cellular signaling, are achieved here by developing the next generation of this microfluidic probe.

PRINCIPLE

The device (Figure 1a) reported here obtains ~10-fold higher sensitivity and 4-fold higher throughput than its predecessor [7] and the ability to measure four different targets including a loading control. It interfaces with standard tissue culture plates and collects contents of visually selected single cells of interest from adherent cell populations as described earlier [7] by creating a small, tunable lysis zone at its tip using hydrodynamic confinement of a lysis buffer [8]. It can probe multiple cells and the lysate from each cell can be mixed with assay reagents containing fluorogenic substrates [9] and flowed into a different chamber. It can also measure multiple kinase activities from a single cell by dividing the single cell lysate into parts and mixing them each with different assay reagents in different chambers. The flow is directed using pneumatic micro-valves which are then also used to isolate the chambers for observation. The limited dilution of the cell lysate in this process yields high kinase assay sensitivity which is sufficient to probe many proteins from single cells.

EXPERIMENT

The fabricated PDMS device and its complete operation are shown in Figure 1b. The device consists of three layers: a bare glass slide which forms the base, a thin membrane (~30µm) layer which forms the base of the tip and enables the probe to reach close to adherent cells and also contains the pneumatic valves, and a thick (~5mm) layer which contains the channels, chambers and flow and control connections. Out-flow of the lysis buffer and in-flow of the lysate/reaction mixture are driven using two syringe pumps and the pneumatic valves are controlled using a custom-built air pressure controller. The chip is mounted on a micromanipulator which enables precisely locating the tip next to the selected single cell before the lysis and capture process is initiated by driving the lysis buffer flow. After capture of the reaction mixtures in the separate chambers, the valves are closed and the chip is transferred to a fluorescence microscope stage for observation.

RESULTS AND DISCUSSION

Selective lysis and capture of a single cell from a fluorescently labeled population of adherent cells of a human breast carcinoma cell line (MCF-7) is shown in Figure 2a. Even on the ~95% confluent tissue culture plate used, the selected single cell is lysed and captured without disturbing any neighboring cells thus ensuring that contents only of the selected cell are loaded on to the chip.

A calibration of the MAPKAPK2 kinase assay sensitivity on the device using bulk hepatocellular carcinoma (HepG2) cell lysate is shown in Figure 2b. These cells are known to contain about 1ng of protein in each cell. This calibration curve establishes the limit of detection of the assay to be at least 4-fold below that needed to measure single cell kinase activity. Also shown in Figure 2c is the ability to distinguish the effects of stimulation using insulin, sorbitol and forskolin on the activity of three different kinases Akt, MAPKAPK2 and PKA respectively, in 1ng of bulk cell lysate.

Single cell measurement of the kinase MAPKAPK2 in serum-starved cells and upon stimulation using sorbitol is

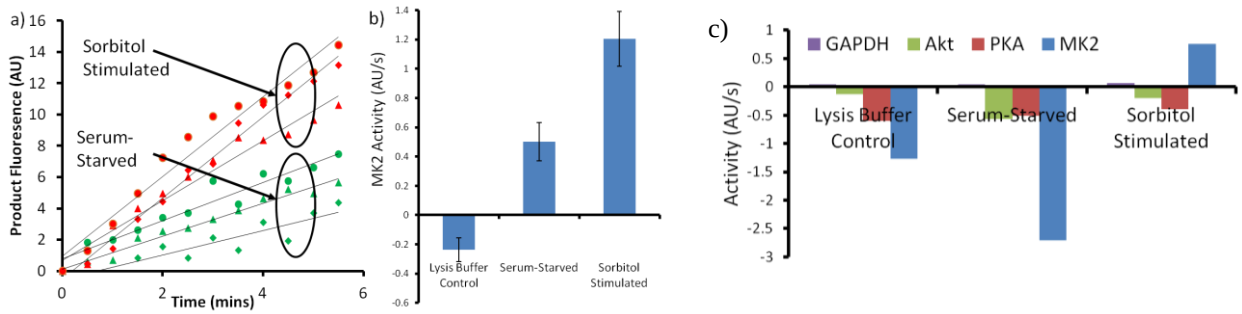

Figure 3: a) Activation of MK2 in single serum-starved HepG2 cells upon sorbitol stimulation is studied. Osmotic shock due to sorbitol is seen to increase MK2 activity about 3-fold. The MK2 activity measured in sorbitol-stimulated single cells corresponds roughly (~2X) to that measured from equivalent amount of bulk cell lysate (Fig 2b). Product fluorescence vs. time curves are shown in a) while their slopes are plotted in b). c) Simultaneous measurement of the three kinases, PKA, MK2 and Akt and a loading control enzyme, GAPDH, in a single HepG2 cell is demonstrated and the effect of Sorbitol stimulation of cells in increasing MK2 activity selectively is seen.

shown in Figure 3a. Single cell kinase activity can be clearly distinguished from the negative control in which a plate with only tissue culture medium was probed. The measured single cell activity from stimulated cells corresponds roughly to that measured from equivalent amounts of stimulated bulk cell lysate. The average fold-activation (about 3-fold) of the kinase observed in single cells mimics measured that seen in serum-starved and stimulated bulk cell lysates in this case.

The ability to simultaneously measure the activities of three kinases, Akt, MK2 and PKA, along with the loading control enzyme, GAPDH from a single cell is demonstrate in Figure 3b. Again the kinase and GAPDH activities in a single cell are clearly distinguished from that in the negative control. The selective activation of the hyperosmotic-stress activated kinase MAPKAPK2 is clearly seen upon sorbitol stimulation of serum-starved cells while the Akt, PKA and GAPDH levels do not change significantly as a result of this treatment. This shows the sensitivity and specificity of this multiplexed kinase activity measurement technique.

CONCLUSION

We have developed and demonstrated an integrated microfluidic probe that enables multiplexed single cell kinase activity measurements directly from adherent cell populations on a tissue culture plate and thus presents an opportunity to correlate cellular phenotype of single cells with their biochemical state. This single cell detection platform is agnostic to specific sensing chemistry, so other biochemical assays (enzymatic or binding or PCR) can also be implemented with minimal modification.

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