AN INTEGRATED MICROFLUIDIC PROBE FOR CONCENTRATION-ENHANCED SELECTIVE SINGLE CELL KINASE ACTIVITY MEASUREMENT

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ABSTRACT

We present an integrated microfluidic probe that captures the contents of selected single cells from a cell population adherent on a standard tissue culture platform and directly measures specific protein kinase activities in the captured single cell lysate. A kinetic fluorimetric kinase assay in a small integrated chamber isolated by micro-valves and an end-point concentration-enhanced mobility-shift kinase assay in an integrated nanofluidic concentrator are demonstrated. We demonstrate the use of the probe by measuring MAPKAPK2 and AKT kinase activity in single human hepatocellular carcinoma (HepG2) cells.

KEYWORDS: Single Cell Assay, Kinase Assay, Concentrator, Microfluidic Probe

INTRODUCTION

Traditional cellular assays measure average properties of large numbers (usually 10^3-10^6) of cells, missing differences (e.g. drug responses), between individual cells in supposedly homogenous populations. This has consequences for treatment of diseases such as cancer [1]. Recent microfluidic and traditional tools [2] have studied genetic differences between single cells harnessing the power of nucleic acid amplification techniques. However such techniques fail to capture important non-genetic – translational and post-translational – sources of heterogeneity which create unique proteomes in different cells [1]. Other single cell proteomic techniques using flow cytometry [3] or imaging depend on phospho-specific antibodies to measure phosphorylation state of proteins as a surrogate for activity. Direct measurement of protein activities from single cells remains difficult due to limited sensitivity in activity assays which results from the low abundance of the target proteins in single cells (<1pg) and relatively high reaction volumes (>1µL) needed.

In addition, difficulties in interfacing microfluidics with adherent cells in standard tissue culture platforms has led to the use of cell suspensions in microfluidic single cell assays [2]. Suspending adherent cells however modifies their biochemical state and makes it impossible to correlate the cellular phenotypes such as migration behavior that appear in adherent cells only with biochemical assays such as kinase activity assays. Microinjection of kinase substrates into single adherent cells followed by a capillary electrophoresis based assay [4] has been demonstrated before but the in-cell reaction mode used constrains usable assay chemistries and thus specificity and sensitivity.

PRINCIPLE

The integrated device (Figure 1) reported here interfaces directly with standard tissue culture plates using a microfluidic probe [5] that creates a limited, tunable lysis zone at its tip by simultaneously dispensing and collecting lysis agents. This is used to lyse and collect contents of visually selected single cells of interest from adherent cell populations.

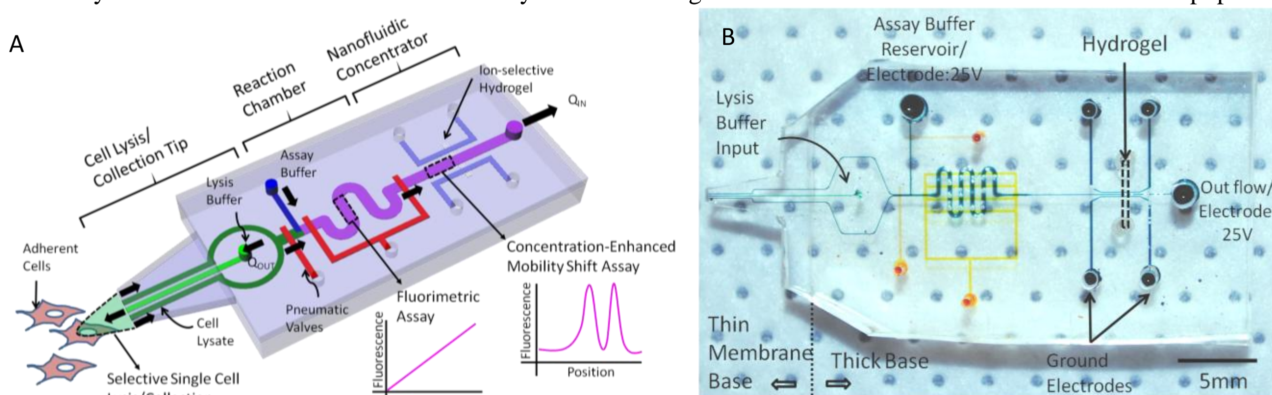

Figure 1: A. The integrated microfluidic probe with 3 modules: cell lysis/collection tip to selectively lyse and collect single cells, a reaction chamber to mix and hold assay reagents and a nanofluidic concentrator to concentrate product peptides and perform a mobility-shift assay for phosphorylated/unphosphorylated substrates. B. Fabricated PDMS device showing the different layers: top piece with two layers: the valve lines (orange) and the channels (blue/green), bonded to bottom piece with two layers: a thin membrane base in the tip region and a thicker base which holds the UV-cured ion-selective hydrogel.

The captured lysate is mixed with assay reagents and the reaction mixture is flowed into a small reaction chamber which is then isolated for observation using pneumatically actuated micro-valves. A kinetic fluorimetric assay using fluorogenic chemosensor kinase substrates that show increased fluorescence upon phosphorylation [6] is performed in this isolated chamber. The small reaction volume in which the lysate from a single cell is captured (~1nL) increases the protein concentration and enables direct measurement of kinase activity from single cells.

Alternatively, the integrated ion-selective hydrogel-based nanofluidic concentrator [7] is used to trap or concentrate the proteins or reaction products in the mixture further to yield very high sensitivity in a concentration-enhanced kinase assay [8], sufficient to probe proteins from single cells. Here, an end point concentration-enhanced mobility shift kinase assay has been performed using the difference in electrophoretic mobilities of the unphosphorylated and phosphorylated fluorescently-tagged substrate peptides which enables their simultaneous separation and concentration into separate bands in the electric field gradient zone created due to ion-concentration polarization by a voltage applied across an ion-selective hydrogel membrane.

EXPERIMENTAL

The fabricated PDMS device is shown in Figure 1B. It consists of two plasma-bonded monolithic pieces each containing two layers. The top piece contains the pneumatic control lines and flow channels. The bottom piece consists of a thin membrane (~20μm thick) in the tip region, which enables it to reach close to adherent cells on the tissue culture plate and a thicker base (5mm) in the rest of the chip which holds the UV-cured ion-selective hydrogel and provides a stable base to the chip.

After making flow and pneumatic connections and priming with buffer solutions, the chip is mounted on a micromanipulator and lowered into a tissue culture plate placed on a microscope stage. The tip is aligned to a target cell and lowered to touch the plate near it. The lysis buffer outflow and the capture inflow are applied to create the small lysis zone (Figure 2A.i). This enables selective single cell lysis (Figure 2B) without affecting the rest of the cells.

The captured lysate then flows to a T-junction and mixes at a fixed ration with a reaction buffer containing kinase assay reagents (kinase substrate, inhibitors for non-target kinases etc) (Figure 2A.ii) and flows into a reaction chamber which is then closed using the pneumatic valves (Figure 2A.iii). For the fluorimetric assay, this isolated chamber is then observed over time. For the concentration-enhanced mobility shift assay, the chamber is opened after a fixed reaction time and the mixture is flowed into the integrated concentrator while a voltage is applied across the ion-selective membrane.

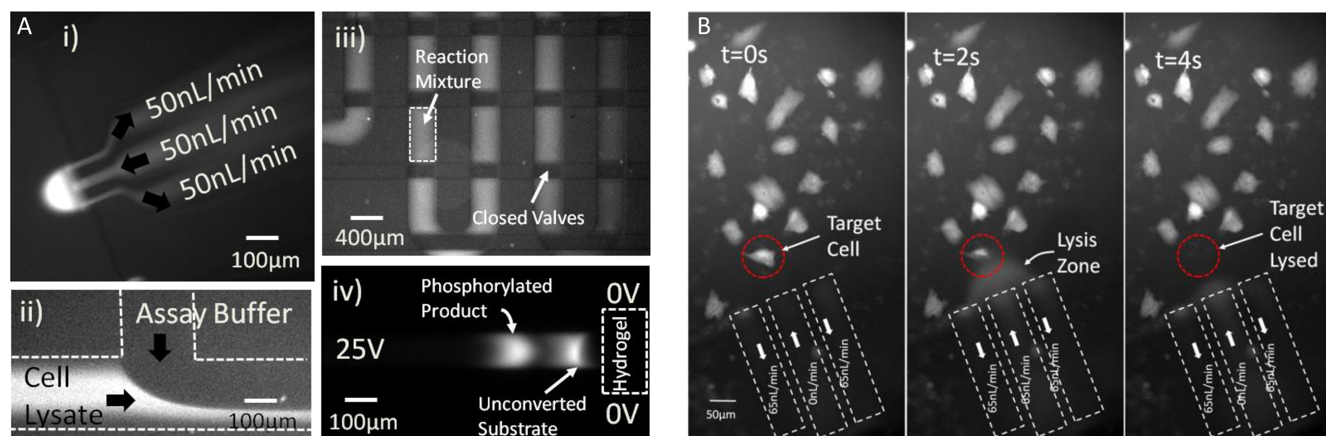

Figure 2: A. Device operation: a) a stable tunable lysis zone outside the tip as lysis buffer is flowed out/back in b) collected cell lysate mixes with the kinase-specific substrate in assay buffer c) valves are closed to capture the reaction mixture which is observed over time or d) flowed into the concentrator for a mobility shift assay. B. A selected cell from a green-fluorescent protein expressing adherent MCF-7 population is lysed using the microfluidic probe with a lysis buffer containing 1% Triton-X 100 while the surrounding cells remain unaffected by this process. The lysis zone can be seen in fluorescence from a tracer added to the lysis buffer.

RESULTS AND DISCUSSION

Measurement of the activity of the stress-activated kinase, MAPKAPK2 in single sorbitol-stimulated, adherent HepG2 cells using a direct, kinetic fluorimetric assay in the isolated chamber is shown in Figure 3A along with negative controls that sampled tissue culture medium only and a calibration control that sampled a bulk cell lysate of known total protein concentration. Note that the measurement from the three different cells all show similar positive slope due to the reaction while the negative controls show a negative slope due to photo-bleaching. Also the measured single cell activity corresponds well to that in comparable amounts (~1ng/cell) of bulk HepG2 cell lysate which was sampled using the probe and measured under identical conditions.

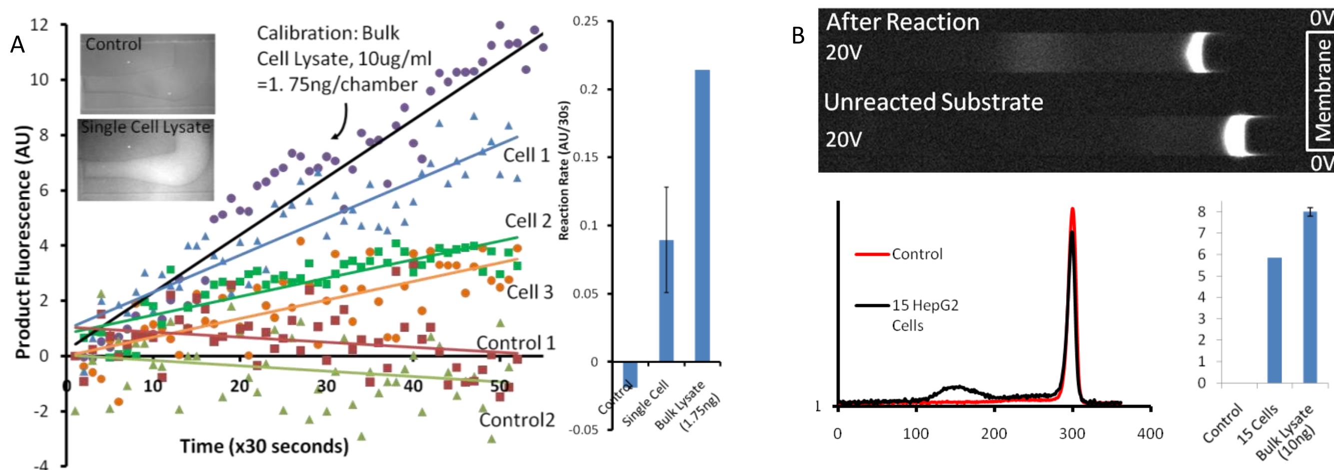

Figure 3: A. Single cell MK2 Assay – Single HepG2 cells were lysed and the captured lysate was reacted with a fluorogenic MK2 substrate. Measured single cell activity corresponds well to the activity measured from a comparable amount (~1.75ng) of bulk cell lysate as these cells have about ~1ng/cell of total protein. B. Concentration-enhanced mobility shift Akt assay - A small group (~15) of HepG2 cells was lysed and the captured lysate was reacted with a fluorescent Akt substrate Products were diluted out and loaded into a multiplexed concentrator chip along with a negative control (unconverted substrate). A 5.8% phosphorylation was measured which compares well to corresponding amount of bulk lysate.

Measurement of activity of the kinase Akt, that plays a role in multiple cellular processes ranging from proliferation to migration, from a small (~15) group of adherent cells using a fluorescently tagged substrate was also performed after extracting the reaction products from the chamber and running an end-point concentration-enhanced mobility shift assay (Figure 3B) in a separate multiplexed concentrator chip in which unreacted substrate is run simultaneously in a separate channel as a control. The fluorescence profile from both channels is plotted and used to find the percentage of phosphorylated product in the reaction mixture. Again, a phosphorylation rate similar to comparable amounts of bulk HepG2 lysate was measured.

CONCLUSION

We have developed and demonstrated an integrated microfluidic probe that enables 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.

ACKNOWLEDGEMENTS

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