IN-CELL WESTERN[TM] ON DIGITAL MICROFLUIDICS FOR ANALYSIS OF SIGNALING PATHWAYS IN SINGLE CELLS

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ABSTRACT

We introduce a new method for carrying out In-Cell Western[TM] assays relying on digital microfluidics (DMF) for droplet-based fluid handling and microarray scanning for high resolution image analysis. This technique is fast, automated, reagent efficient, and capable of quantifying protein phosphorylation at the single-cell level. We propose that this technique will have similar utility as flow cytometry, but with the important capacity to evaluate and visualize adherent cells *in situ*.

KEYWORDS

In-Cell Western, Digital microfluidics, immunocytochemistry, electrowetting-on-dielectric, ELISA

INTRODUCTION

The In-Cell Western[TM] (ICW) is an emerging immunocytochemistry technique (trademarked by LI-COR®) used to quantify protein targets in intact cells, and has proven to be a powerful method for studying cellular signaling pathways via phospho-proteins targets.[1] Compared to conventional Western immunoblotting, the ICW requires less extensive sample preparation (no blotting required) and has high-throughput capacity (performed in multiwell plate format). Although the ICW is a promising technique, it suffers from a key limitation: the inability to resolve signals from individual cells. Typically, the average signal intensity of each well is acquired using a specialized ICW scanning instrument (100-200 µm resolution). In addition, the ICW is often labour-intensive and reagent consuming, requiring numerous manual pipetting steps in well plates. To address these drawbacks, we developed a unique new method for carrying out ICW assays that incorporates digital microfluidics and microarray imaging for the quantitative analysis of signaling pathways in single cells.
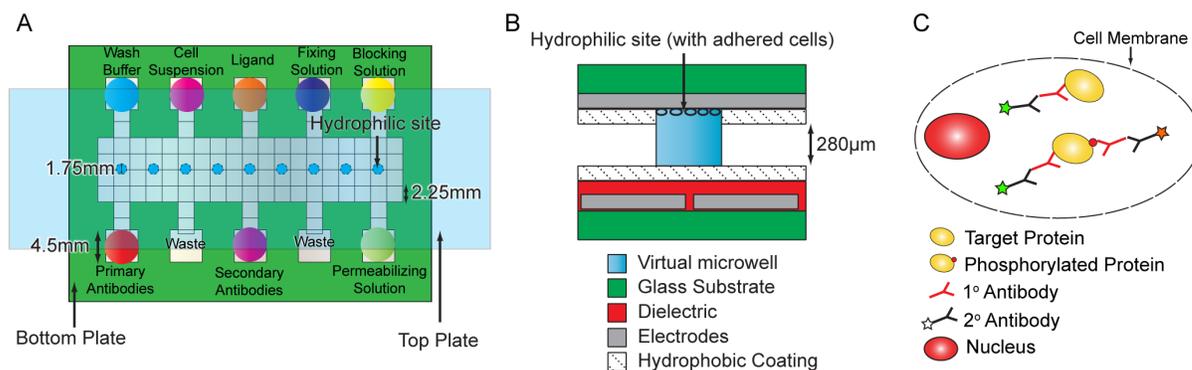

Figure 1: Device and method. (A) Top view of DMF device with key dimensions. (B) Side-view of DMF device, not to scale. (C) In-Cell Western[TM] assay scheme to quantify protein phosphorylation.

Digital microfluidics (DMF) is a technology that enables the electrostatic manipulation of discrete droplets (pL to µL) on an array of insulated electrodes.[2] By applying pre-programmed sequences of potentials to these electrodes, automated droplet handling can be realized with the advantage of low-reagent consumption, individual sample addressability, and parallelization. These salient features have motivated the development of DMF devices for various applications, including the culture and analysis of adherent primary cells.[3] The new methods described here rely on microfabricated hydrophilic sites in the device top-plate (Figure 1A) for rapid and precise metering of reagents to cells grown in self-contained droplets known as virtual microwells (Figure 1B).[4] Because the microfabricated top-plate (bearing adhered cells) is in the format of a typical microscope slide (3" by 1"), the stained cells can be analyzed using a standard microarray scanner (5-10 µm resolution), ubiquitously found in molecular biology laboratories. Using DMF and a microarray scanner, we successfully developed a modified version of an ICW capable of determining the ratio of phosphorylated-protein to total protein (phos-protein/total protein) in single cells using specific primary (1°) and labeled secondary (2°) antibodies (Figure 1C).

EXPERIMENTAL

Device fabrication and operation

Electrode bearing bottom-plates were fabricated as described previously.[3] Hydrophilic sites were generated on the indium tin oxide (ITO) coated top-plate by a fluorocarbon lift-off technique. Briefly, photoresist patterns were formed by photolithography, followed by spin-coating with Teflon-AF (1% wt/wt in FC40, 3000 RPM, 30 sec), baking on a hot-plate (10 min, 165 °C), then immersion in acetone until liftoff occurred (~ 5-10 sec). Devices were assembled with top-plate and bottom-plate separated by a spacer formed from four pieces of double-sided tape (total spacer thickness 280 μm) (Figure 1B). To drive droplet movement, an AC sine wave (~150 V_{RMS}, 10 KHz) was applied between the top-plate (ground) and sequential electrodes on the bottom-plate via the exposed contact pads.

DMF cell culture and In-Cell Western optimization

Digital microfluidics was used to automate the various droplet handling steps required for an ICW assay, including cell seeding, ligand stimulation, incubation, fixing, permeabilization, antibody staining, and washing. Briefly, NIH 3T3 fibroblasts of various concentrations (10^3 to 10^6 cells/mL) were delivered to the virtual microwells (1.75 mm diameter) (Figure 1A, B). The device was inverted and incubated at 37^oC for 24 hours, allowing cells to settle (by gravity) and adhere to the hydrophilic site. Subsequently, the adhered cells were fixed (paraformaldehyde), permeabilized (Tween-20), blocked (bovine serum albumin or non-fat dried milk), and stained (1° Rabbit Anti-GAPDH and 2° Anti-Rabbit IgG-Cy3). After the final rinse, the DMF device top-plate was analyzed in a microarray scanner (Axon GenePix 4000B).

On-device stimulation and analysis

NIH 3T3 fibroblasts were seeded in virtual microwells at 10^5 cells/mL and stimulated with platelet-derived growth factor (PDGF-BB) at various concentrations and durations. After stimulation, fixing, permeabilization, and blocking, Akt phosphorylation was probed with two 1° antibodies: a phospho-specific antibody (Rabbit Anti-phospho-Akt) and a normalization antibody (Mouse Anti-Akt) that recognized the Akt regardless of phosphorylation status. These 1° antibodies were subsequently tagged with spectrally distinct 2° antibodies (Anti-Mouse IgG-AF647 and Anti-Rabbit IgG-AF555); the resulting microarray images were analyzed using ImageJ.
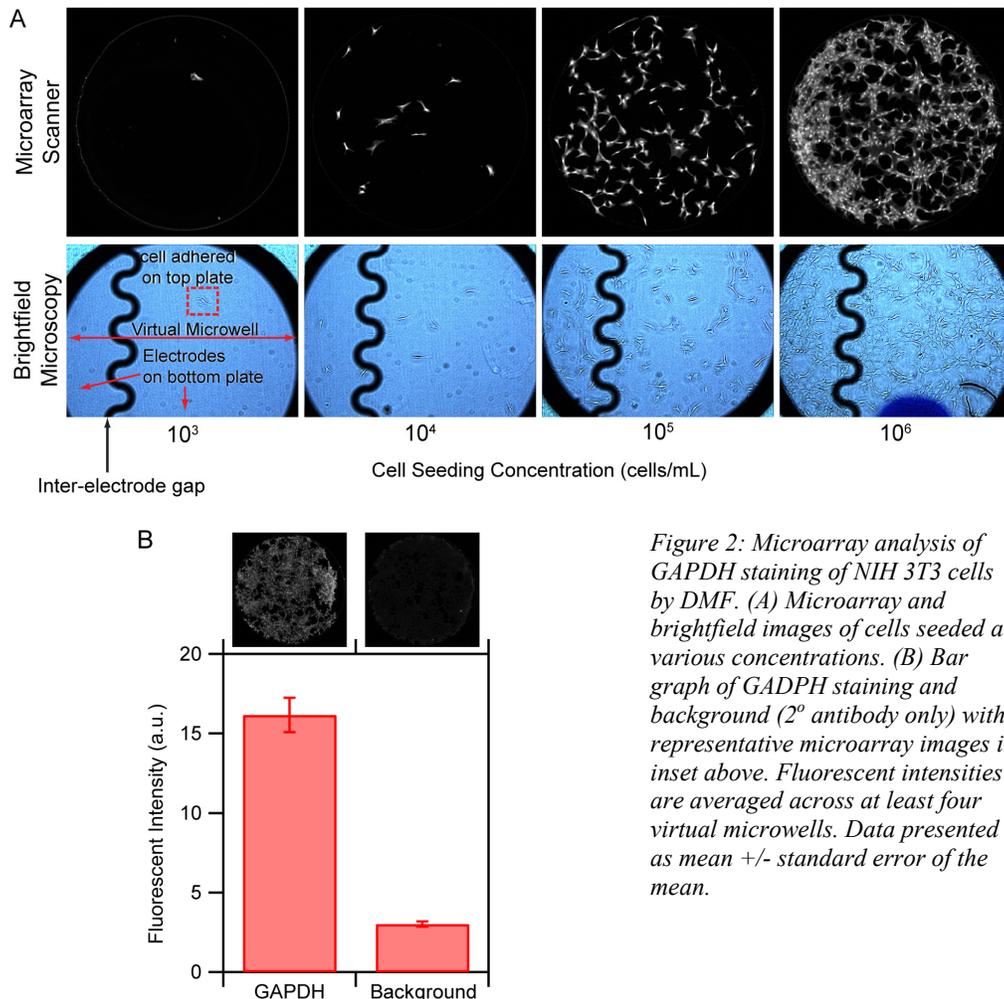

Figure 2: Microarray analysis of GAPDH staining of NIH 3T3 cells by DMF. (A) Microarray and brightfield images of cells seeded at various concentrations. (B) Bar graph of GADPH staining and background (2° antibody only) with representative microarray images in inset above. Fluorescent intensities are averaged across at least four virtual microwells. Data presented as mean +/- standard error of the mean.

RESULTS AND DISCUSSION

As a first test, a constitutively expressed protein, GADPH, was stained and evaluated on-chip for various cell seeding concentrations (Figure 2A). The results demonstrated that ~10^5 cells/mL is ideal (not overly crowded) for simple single-cell image analysis, seeding about 100 cells in each virtual microwell (area of 2.4 mm^2). In addition, these preliminary tests allowed us to optimize the ICW protocol to ensure that a sufficient signal to noise can be achieved. As shown in Figure 2B, the GADPH signal was significantly (at least 5x) higher than background signal (2° antibody only). We expect this sensitivity to improve further in single cell analysis since the background noise outside of the cells will be ignored.

Using this optimized method, we investigated the phosphorylation of Akt protein (a regulator of cell survival) as a function of exposure to platelet-derived growth factor (PDGF-BB) in NIH 3T3 fibroblasts. As shown in Figure 3A (which is presented in a style similar to that of flow cytometry data), upon stimulation by PDGF-BB for 7.5 minutes, a majority of the cells increased in Akt activation. Likewise, as shown in Figure 3B, the Akt activation trend as a function of time was consistent with what is expected from the literature.[5] Finally, we evaluated the concentration dependence of PDGF-BB on Akt activation (Figure 3C). These results show that Akt is activated with PDGF-BB stimulation in a time and PDGF-BB concentration dependent manner and there exist a negative feedback loop mechanism that modulates Akt for higher concentration and duration of stimulation.

The new DMF method developed here significantly reduced reagent consumption. A typical ICW well-plate assay requires 150 µL fixing solution, 3 mL wash buffer, 150 µL blocking solution, and 100 µL antibody solution. In contrast, the DMF method requires 7 µL fixing solution, 42 µL wash buffer, 7 µL blocking solution, and 14 µL antibody solution. This represents a 50-fold reduction in reagent consumption in the DMF method, from 3.4 mL to 70 µL. In addition, 150x more cells are used for an ICW well-plate assay compared to DMF (15,000 cells vs. 100 cells). Although we are interrogating fewer cells in the DMF method, we are able to perform single cell analysis and gain insight in the cell population distribution.
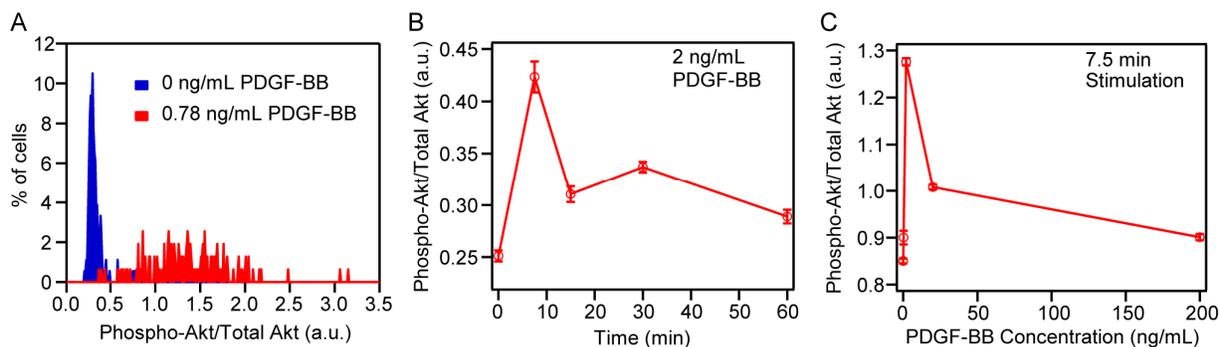

Figure 3: NIH 3T3 cells stimulated by PDGF-BB ligand. (A) Single-cell distribution of non-stimulated (blue) and stimulated cells (red) (n>157). (B) Time-dependent profile of Akt phosphorylation after ligand stimulation (n>128). (C) Akt phosphorylation as a function of ligand concentration (n>193). Data in (B) and (C) presented as mean +/- standard error of the mean.

CONCLUSION

We have developed a novel quantitative ICW assay relying on microarray imaging and DMF. This technique is fast, automated, reagent efficient, and capable of quantifying proteins at the single-cell level. We propose that this represents an important new tool for single-cell analysis of molecular signaling in adherent cells *in situ*.

ACKNOWLEDGMENTS

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