LAB-IN-A-TRENCH PLATFORM FOR REAL-TIME MONITORING OF CELL SURFACE PROTEIN EXPRESSION

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ABSTRACT

In this work we for the first time demonstrate real-time monitoring of the expression of membrane proteins in native, live cells, free of hydrodynamic stress at single cell resolution. This micro-optofluidic mechanism is uniquely enabled by the intricate interplay of gravity induced sedimentation with laminar flow, fast diffusion and short optical path length on our lab-in-a-trench platform[1].

INTRODUCTION

Microfluidics has the potential to significantly change the way modern biology is performed. The often cited advantages of microfluidic devices are the ability to work with lower cost, higher speed, smaller sample and reagent volumes and the possibility of parallel operation. They also hold the promise of integrating multi-step laboratory protocols on a single chip (i.e., lab-on-a-chip) [2, 3]; thus clearing the way towards true "sample to answer" devices. For some bioanalytical measurements, however, the most important consequences of successfully implementing lab-on-a-chip will be enhanced assay reproducibility and more quantitative results [4] when compared to classical analytical procedures. Much of the research efforts since the 1990s have focused on developing a wide range of "stand alone" micro fluidic components for performing diverse bio-analytical functions such as capillary electrophoresis (CE) [5], polymerase chain reaction (PCR) [6], dynamic cell culture [7], cell lysis [8, 9], immunoassays [10, 11].

Cell based assays are an essential tool in biological and clinical research, as well as in the Pharma and Biotech industry. High-throughput cell-based screening platforms are especially critical in the pharmaceutical industry for target validation [12]. In the past decade, the main focus of cell culture technology has been on developing high-throughput cell-based assays capable of providing valuable information on potential drug targets as well as advancing cell biology [13]. A popular form of high throughput is parallel analysis with various culture conditions (i.e. different concentration or different stimuli) or the analysis of different target cells. A range of concentrations can be readily established on the chip through the use of microfluidic gradient generators, which has motivated the construction of systems for the investigation of the cellular response to different reagent concentrations [14, 15]. Multiple reagent concentrations have also been combined with multiple cell types on a single device to form two-dimensional cell culture arrays for combinatorial analysis [16-18]. Additionally 2-dimensional cell culture arrays with different stimuli [19] and with different reporter cells [20] have also been demonstrated.

Here we explore the advantages of micro-scale cell culture and handling beyond the often cited reductions in liquid volumes and processing times. We have already implemented a freely programmable sequence of basic unit operations such as particle sedimentation, retention, lysis, exposure to reagents, and washing for cell or particle based assays in our lab-in-a-trench platform [1]. We further explored this unique combination of micro-optofluidic parameter space to achieve the unprecedented recording of videos featuring the expression of native cell-surface proteins in real time. Such assays can also be parallelized, e.g. in an array of 64 fluidically separated modules, each offering an 8-fold replication of a single experiment (Fig. 1).

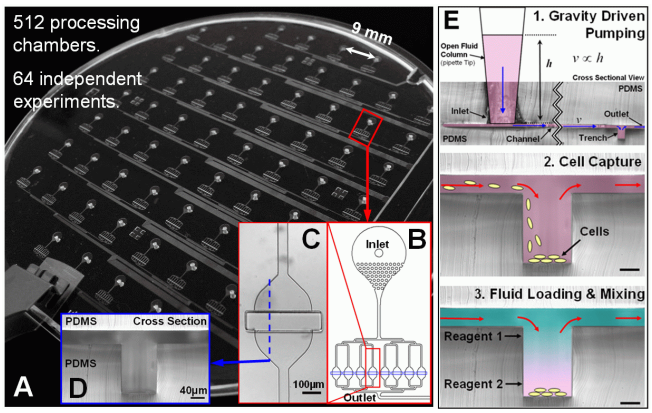

Figure 1: The array (A) consists of 64 modules (B).Each module is further divided in 8 "lab-in-a-trench" units (C,D) running in parallel. Flow is generated and controlled by the height of the open fluid column in a pipette tip (E).

WORKING PRINCIPLE

The microfluidic trench structure allows efficient capture and retention of cells or particles through gravity based sedimentation. The laminar flow conditions assure a vanishing flow field throughout the trench, thus permanently retaining particles and minimizing hydrodynamic stress. Furthermore, its small depth of 150 µm allows continuous loading, mixing and refreshment of reagents by mere diffusion from a controlled flow through the supply channel on the top of the trench (Fig. 2A). For the surface-sensitive cell assays investigated in the paper, we also utilized the short vertical path length through the shallow trench to provide high signal-to-noise ratio between the fluorescent antibodies bound and thus up-concentrated on the surface of the retained cells, and the diluted antibodies in the background liquid (Fig. 2). The unique combination within this micro-optofluidic parameter space

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permits an unprecedented recording of videos featuring the expression of cell-surface proteins in real time.

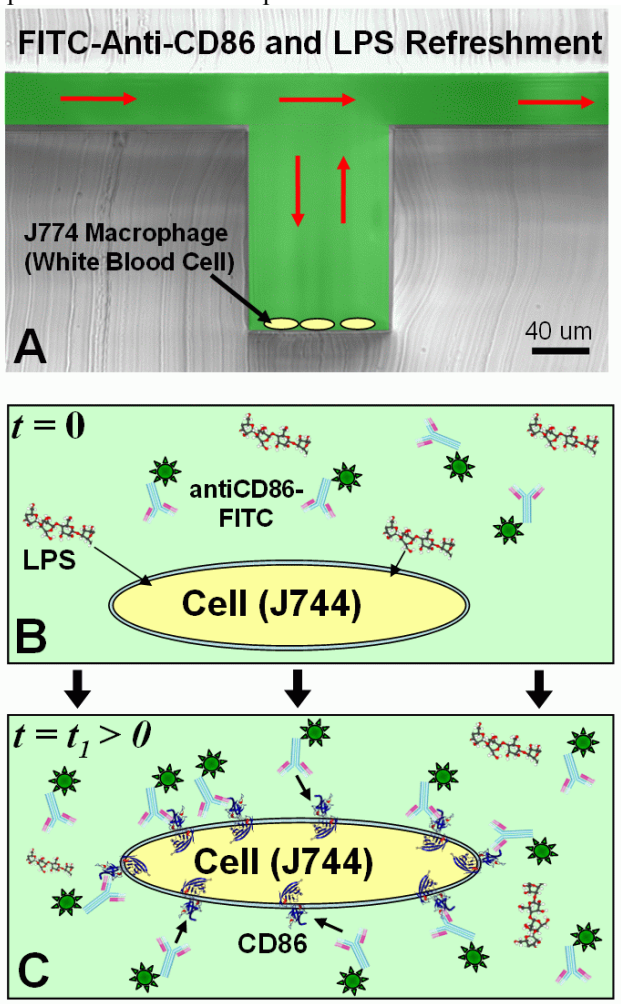

Figure 2: Mechanism of real time membrane protein transduction. A: Cells loaded at the bottom of the trench structure are protected from the direct flow and shear stress. B: Macrophage cells are exposed to medium containing the stimulation agent LPS and a low concentration of fluorescently labelled antibodies. C: LPS activated macrophages express CD86 co-stimulatory proteins on the cell surface. As the cell surface concentration of CD86 increases, so does the concentration of bound FITC-anti CD86, this way increasing the fluorescence on the cell surface. Continuous perfusion flow ensures a constant, low concentration of dissolved FITC-anti CD86 antibodies and therefore negligible background noise.

MATERIALS AND METHODS

Fabrication: The microfluidic device was fabricated using standard soft lithography replica moulding techniques. A mould was created through a double-layer process first using negative photoresist, SU8-3050 (Microchem U.S.A.) and then SU8-2150 (Microchem U.S.A.) both layers were deposited onto a clean silicon wafer using a spin coater (P6700 Specialty Coating Systems, Inc., U.S.A.). The first layer photo resist (5 mL) was spread onto the wafer at 500 RPM for 10 s, and the rotation rate was then ramped at an acceleration of 350 RPM/s to 4000 RPM, at which rate the sample was spun for 30 s to form a 40 µm layer. The wafer was then soft baked at 95 ºC for 15 min, then UV-exposed for 30 s at 9.5 mW/cm2 using a Karl-Süss KSM MJB-55W mask aligner. The wafer was post-exposure baked for 1 min at 65 ºC and 62 min at 95 ºC, allowed to cool to room temperature, developed in Microposit EC Solvent (Chestech Ltd., UK) developer and sonicated (Branson 5510, Kell-Storm, USA) for 4 min, and blown dry with nitrogen. The second layer photo resist (5 mL) SU8-3025 (Microchem U.S.A.) was spread onto the patterned wafer at 500 RPM for 10 s, and the rotation rate was then ramped at an acceleration of 350 RPM/s to 4,000 RPM, at which rate the sample was spun for 3 sec. The wafer was then soft baked at 95 ºC for 10 min. Then another layer of photo resist (5 mL) SU8-2150 (Microchem U.S.A.) was spread onto the wafer at 500 RPM for 5 s, and the rotation rate was then ramped at an acceleration of 200 RPM/s to 2,500 RPM, at which rate the sample was spun for 30 s to form a 150 µm layer. The wafer was then soft baked at 65 ºC for 6 min. and then at 105 ºC for 15 min, then aligned and UV-exposed for 70 s at 9.5 mW/cm2. The wafer was post-exposure baked for 6 min at 65 ºC and 12 min at 95 ºC, allowed to cool to room temperature, developed in Microposit EC Solvent (Chestech Ltd., UK) developer for 12 min, and blown dry with nitrogen. The two layer SU-8 mould was then coated with perfluorosilane in a vacuum chamber for reducing the PDMS adhesion. PDMS prepolymer (Sylgard 184, Dow Corning) was prepared at 10:1 (w/w) ratio, degassed in a vacuum chamber for 30 min, then spin coated (500 RPM for 30 sec.) on the SU8 mould and cured in a 100 ºC oven for 5 min. producing an ~500 µm thick fluidic layer. The PDMS was then carefully peeled off the mould. As the upper seal a second non patterned wafer was also coated with a 2 mm thick layer of PDMS prepolymer and cured at 80 ºC for 4 hrs. Fluid inlets and outlets were punched into the 2 mm thick layer upper layer. A flat-tip needle was used for making the inlets (1.3 mm OD) and outlet (3 mm OD) holes. The device was assembled by placing the fluidic layer (with the fluidic structures exposed on the upper side) on a 500 µm thick 4 inch glass wafer for support. Then the assembly was treated with O_2 plasma (PDC-002 Harrick Scientific Corp. USA) for 2 min., after which the upper PDMS layer (containing the inlets and outlets) was manually aligned and bonded at 60 ºC for 2 hrs. The entire device was degassed overnight to remove bubbles that may form between the glass and PDMS.

Cell Culture: J774 macrophage cells were cultured on and off chip using a CO_2 independent medium supplemented with 10% bovine serum albumin, L-Glutamine and penicillin/streptomycin. For the on-chip cell culture and stimulation experiments the entire device was incubated at 37 ºC in an inverted fluorescence microscope (Olympus IX81) fitted with an incubation chamber (Solent Scientific, UK).

Cell Stimulation and Real-time Staining: For the surface overexpression of CD86 protein, macrophages were

stimulated on chip with lipopolysaccharide (LPS) at 200 ng/ml by adding it into the perfusion culture medium, and maintaining a perfusion flow velocity of approximately 500 µm/s at the chamber inlet. For the real-time staining of the CD86 surface molecule we added FITC-labelled anti-CD86 antibody (2331-FUN-1, DB Biosciences) at a 1/100 dilution from stock into the same perfusion medium as the LPS. Bright field and green fluorescent images were acquired by mounting the chip on an inverted fluorescence microscope (Olympus IX81) and acquiring images every 5 min for 10 hours.

EXPERIMENTAL RESLUTS

In our proof of concept experiments, FITC-labelled anti-CD86 antibodies were used at a 1/100 dilution of neat. During an antigen-dependent inflammatory response of the adaptive immune system, the co-stimulatory molecule CD86 is over-expressed on the surface of activated, antigen-presenting macrophage cells in order to induce an effective T-cell response. We stimulated the over-expression of CD86 on the membrane of J774 macrophage cells by treatment with LPS (200 ng/ml) while a negative control was performed in LPS-free culture medium. As the LPS-stimulated macrophages began to express CD86 on their surface, dissolved antibodies bound specifically to newly emerging CD86 cell surface proteins. This coupling generated an intense, surface-specific fluorescent signal which could be resolved at a single-cell level (Figs. 3-5). Detection antibodies consumed in the surface reactions were continuously replenished by diffusion from the feed channel at the top. Figures 3-5 show that the micron-scale (vertical) optical path efficiently suppresses the fluorescence signal originating from fluorescent antibodies in solution, thus lowering the LOD to physiologically relevant levels.

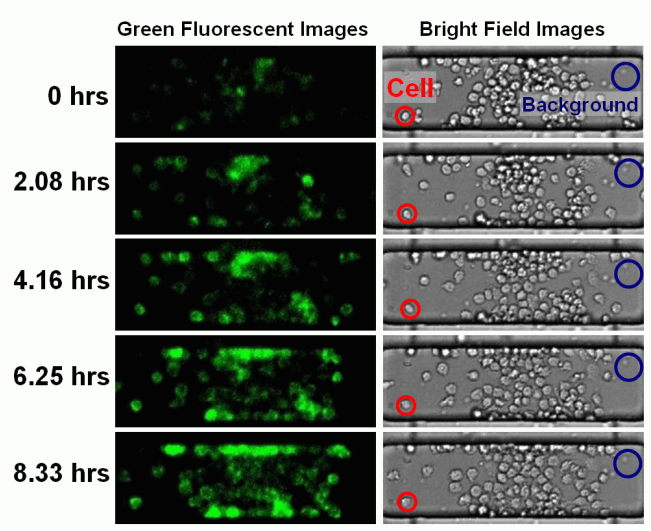

Figure 3: Time lapse imaging of macrophage cells (J774) stimulated with LPS (200 ng/ml).

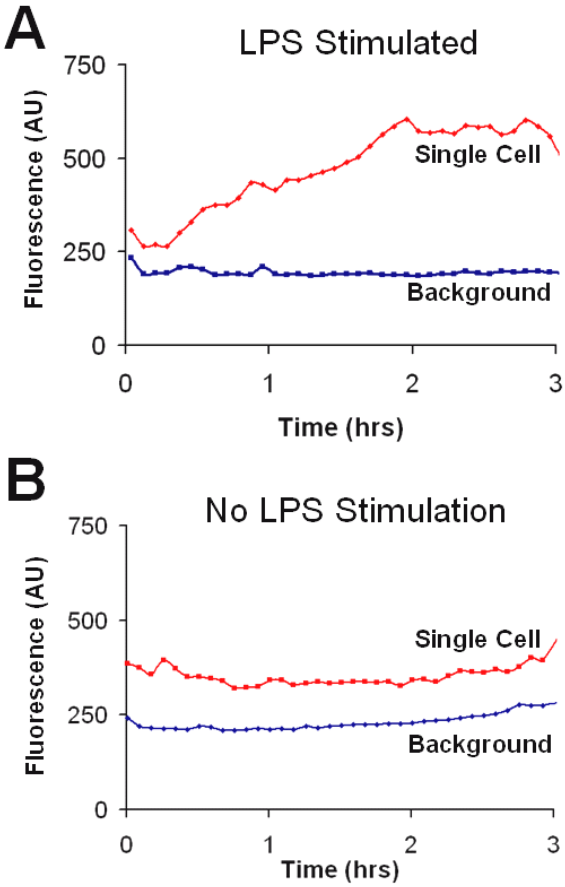

Figure 4: Real time kinetic quantification of CD86 protein expression on LPS stimulated (A) and non-stimulated (B) single cells. The signal intensities were obtained from the average fluorescence within regions of interest in the time lapse images, as indicated in Fig. 3.

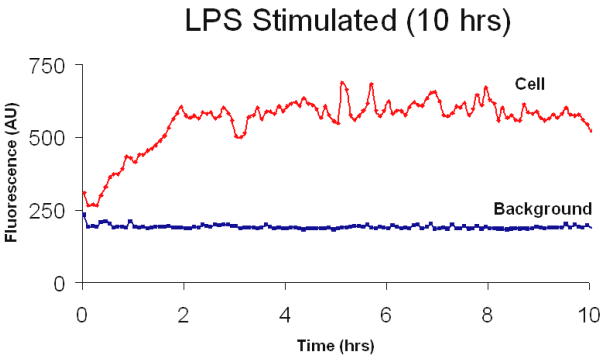

Figure 5: Real time kinetic quantification of CD86 protein expression on a single cell CD86 stimulated with LPS for 10 hrs.

SUMMARY & OUTLOOK

We for the first time present a novel lab-in-a-trench platform for real-time monitoring of interactions between surface-expressed proteins and dissolved antibodies at single-cell resolution by virtue of a novel micro-optofluidic technique. As a pilot application, we successfully visualized and quantified the LPS stimulation of macrophages in real-time, which cannot be achieved with existing tools. We are currently exploring the multiplexing of specifically labelled antibodies and the use of this technique for the direct investigation of dynamic cell-to-cell signalling via receptor proteins.

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