RECORDING SIGNAL TRANSDUCTION DYNAMICS WITH UNPRECEDENTED TEMPORAL RESOLUTION

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ABSTRACT

The binding of ligands to receptors initiates signal transduction. Our understanding of the early signal transduction events is limited by the poor temporal resolution of current analytical methods. To address this challenge we have developed a microfluidic strategy for capturing these processes with millisecond temporal resolution. A single cell continuous flow processing method based on deterministic lateral displacement principles was developed and used for cell triggering with microsecond precision. Using precisely defined ligand incubation periods the sub-second receptor tyrosine kinase autophosphorylation dynamics were investigated.

KEYWORD

Microfluidics, Deterministic Lateral Displacement, Signal Transduction, Single Cells, IGF-1 Receptor

INTRODUCTION

The interface between the cell surface and its microenvironment represents the communication front for information processing, signaling and the emergent behavior of the biological system. These events are initiated and cascade following the interaction of ligands with their host receptors. These molecular interactions are of critical importance for understanding a tremendous spectrum of diseases and are prime targets for therapeutic intervention. Early signal transduction events include conformational and autocatalytic modifications, such as tyrosine auto-phosphorylation. This functionality is fundamental to the onset of many signaling pathways. In this work, we focus on type I insulin-like growth factor receptor (IGF-1 receptor), a receptor tyrosine kinase. The IGF-1 receptor plays an important role in both normal and abnormal growth. It is particularly important in anchorage-independent growth. Alterations in the auto-phosphorylation balance may therefore lead ultimately to malignancy [1, 2]. However, the sequence and mechanism of the conformational and biochemical alterations occurring during signal transduction are poorly understood. Methods which enable near-instantaneous receptor stimulation and reaction arrest are required to investigate the sub-second molecular mechanisms underpinning signal transduction.

Current time course methods involve the addition of macroscale volumes for receptor stimulation followed by the rapid addition of other macroscale volumes to arrest processes and capture molecular states. With this approach the stimulation and arrest of the cells' receptors are slow, limiting the temporal resolution of the experiments. In contrast, at microscopic scales flows are combined in the laminar state with diffusion-limited mixing such that stimulation and arrest only occurs at the interface between flows (*i.e.* only a small sub-fraction of cells are stimulated) [3]. This abstract details the design and characterization of a microfluidic strategy for coating single cells with ligands and subsequent signal transduction arrest with microsecond precision. The technique was used to investigate the sub-second dynamics of ligand-mediated IGF-IR auto-phosphorylation.

EXPERIMENT

To capture signal transduction transitions we have developed a deterministic lateral displacement system based on a pinched flow fractionation [4] architecture for deflection of cells across the virtual interface between laminar streams for near-instantaneous ligand delivery and reaction arrest. The method involves differential volumetric flows converging at a microchannel constriction that we refer to as a stream thinning element (STE) (Fig. 1) [4]. Transport of

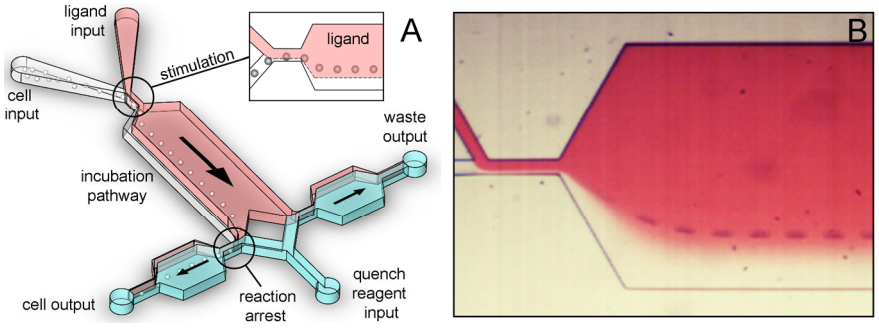

Figure 1. Diagram of the deterministic lateral displacement system with a STE for stimulation, incubation path and channel bifurcation with a STE for reaction arrest (A). Single cell deflection into a dye-doped ligand stream (B).

a continuous stream of cells through the STE causes the cells to impinge on the surface for deflection into a parallel stream containing the ligand. Here, the STE compresses the cell containing stream width below the cell radius for deflection into the ligand-doped stream. Subsequent channel expansion can be used to amplify the displacement, submerging the cells in the ligand-doped flow. Following an incubation period, the process is repeated for reaction arrest.

Microfluidic circuits were replicated in PDMS, packaged by plasma bonding and fluidically interconnected using a plug and play strategy for flow actuation with a syringe pump. Systems were designed with 25-µm-wide and 200-µm-long STE structures and an expansion channel 250 or 500 µm in width (*i.e.* a 10-fold or 20-fold expansion). The STE structure is repeated downstream for reaction arrest at distances which define the duration of treatment (*e.g.* 100 ms). A flow ratio of 7:1 (stimulant Q:cell Q and reaction arrest buffer Q:cell Q) was used for deflection producing a cell stream width of 3.1 microns, significantly smaller than the 7.9 µm (SD ± 1.1 µm) HeLa S3 cell radius. The maximum cell stimulation and reaction arrest mean velocities within the STEs were 100 and 400 mm/s, respectively. At 500 mm/s there is minimal impact on membrane integrity and cell viability (Fig. 2(A)). However, the velocity limits are defined by the reaction arrest STE, where the addition of the reaction arrest buffer increases the STE velocity 4-fold. The velocity limit is determined by inertial effects, which generate a vortex at ≥500 mm/s. This flow pattern prevents reaction arrest at defined periods. (Fig. 2(B)). To avoid this problem a reaction arrest STE velocity of 400 mm/s was used necessitating a stimulation STE velocity of 100 mm/s. At this velocity 95.4% (SD ± 3.5%) of the cells remained intact during transit through the device (Fig. 2 (A)) and were viable as determined by subsequent culture.

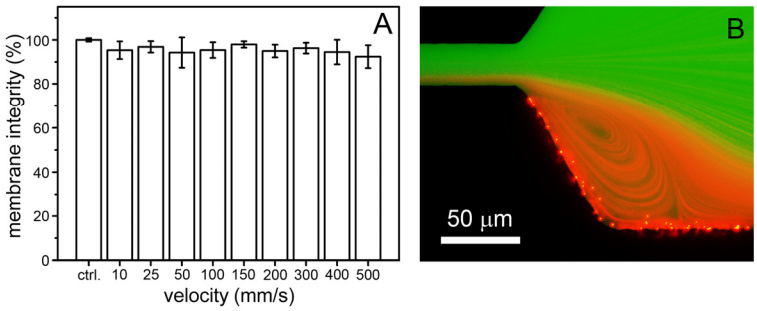

Figure 2. STE velocity impact on membrane integrity (A). Vortex produced by inertial effects using a STE velocity of 500 mm/s (B).

Using 100 mm/s stimulation and 400 mm/s reaction arrest velocity conditions we have designed different microfluidic stimulation–quench systems for capturing early signal transduction events using defined incubation periods (0.1, 0.5 and 2 seconds). The temporal resolution was measured using a high speed camera (EoSens mini2, Mikrotron GmbH). The STE transport time or τ_{switch} is defined as the period from first dipping the cell into the ligand stream, transport along the STE, and finally complete submersion in the ligand-doped flow. The τ_{switch} was 2.24 ± 0.15 ms at the stimulation STE and estimated to be ¼ this value at the reaction arrest STE (Fig. 3(A,B)). On closer inspection, cell–wall interactions were observed to impart a rolling motion to the cells which acts to envelope the cell with ligands. With a 'no-slip' boundary condition and zero diffusion distance assumption, the τ_{switch} of this self-mixing process was 103 ± 30 µs at the stimulation STE (Fig. 3 (C,D)). The higher velocity at the reaction arrest STE prevented the observation of possible cell rolling with the current camera.

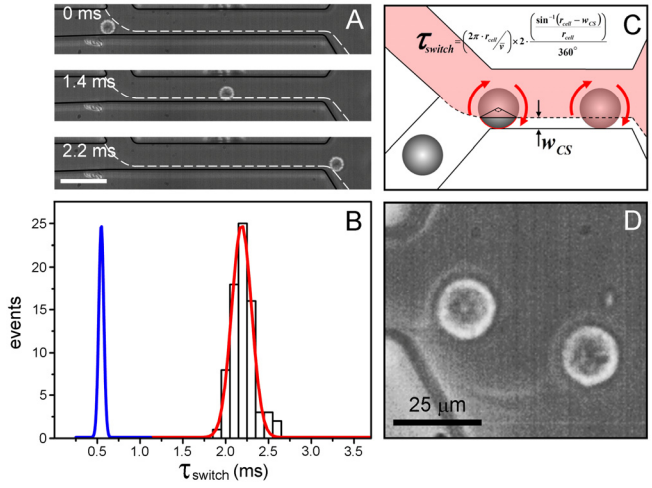

Figure 3. Cell transported through the STE (A). The τ_{switch} for cell stimulation (red) and reaction arrest (blue) (B). Cell rolling in the STE which produces a self-mixing phenomena. The rolling τ_{switch} is determined by the cell stream width (w_{cs}) and the cell radius (r_{cell})(C). Disruption of a dye-doped laminar stream by rolling single cells (D).

The temporal precision of the incubation phase is affected by cell size heterogeneity; different-sized cells follow different trajectories and within the parabolic, Poiseuille flow different cell velocities result which leads to size-dependent ligand incubation periods. This was managed by the use of a wider (500 µm) expansion channel to create a flat-fronted Hele-Shaw velocity profile in combination with vertical cell focusing at the channel mid-height using a lift flow. The velocity and incubation coefficient of variation were reduced from 9.9 % to 3.7% (Fig. 4).

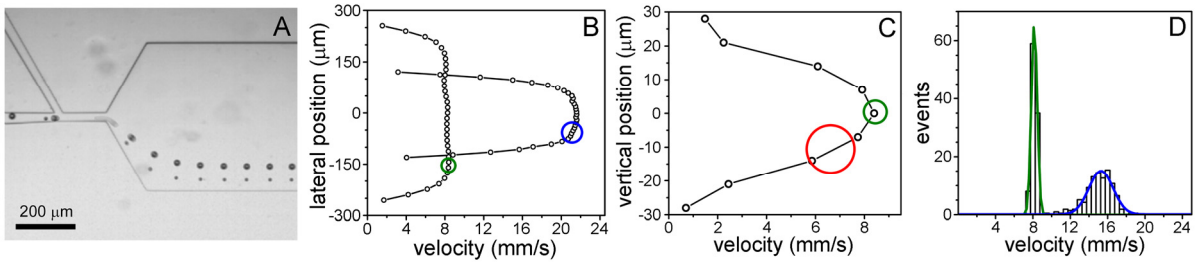

Figure 4. Size-dependent trajectories (A). Lateral (B) and vertical (C) flow profiles measured by µPIV. Blue, green and red circles indicate the cell positions. Cell velocity distribution in the original (blue) and optimized (green) systems (D).

The system has been used to investigate the onset of IGF-1 receptor auto-phosphorylation. Cells were stimulated using a saturating 100 ng/mL IGF-doped flow, and following incubation the phosphorylation states were captured by deflection into a fixation buffer stream in readiness for off-chip phospho-IGFR immunostaining (Fig. 5(A)). Tyrosine residue 1131 within the kinase activation center was investigated. Within 100 milliseconds Tyr1131 was phosphorylated at levels (mean intensity 45.3, SD ± 14.0) equivalent to 0.50 and 2.00 s treatments and also the positive control (5 minute treatment, Fig. 5(B)). Auto-phosphorylation therefore occurs within 100 ms of ligand binding. Indicative of the stochastic nature of signaling, the negative controls had varied phosphorylation levels. Nevertheless, the negative control values (mean intensity 19.78, SD ± 20.59) were significantly lower ($p < 0.001$) than the values from IGF-treated cells.

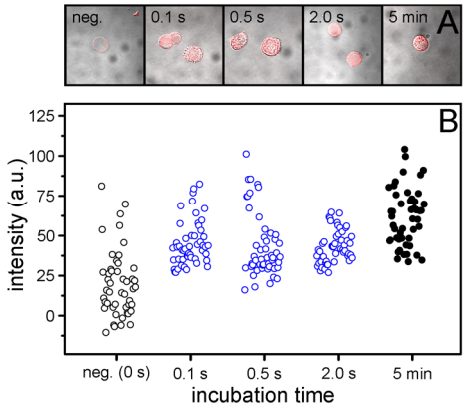

Figure 5. Overlaid bright field and fluorescent microscopy images of single cells stained for pTyr1131 (A). IGF-1 receptor Tyr1131 auto-phosphorylation transitions. (B).

In summary, this abstract presents a novel method for capturing ultra-fast cell surface events. The microfluidic method involves microsecond switching precision, enabling events to be determined with millisecond resolution. To demonstrate the potential of this technology we are the first to report receptor tyrosine kinase autophosphorylation within 100 milliseconds of ligand stimulation. The technique offers unique insights into whole cell signal transduction kinetics which can be used to elucidate molecular mechanisms and define targets for rationally-designed therapeutics.

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