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Total Internal Reflection Fluorescence Flow Cytometry

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Abstract

Total internal reflection fluorescence microscopy (TIRFM) has been widely used to explore biological events that are close to the cell membrane by illuminating fluorescent molecules using the evanescent wave. However, TIRFM is typically limited to the examination of a low number of cells and the results do not reveal potential heterogeneity in the cell population. In this report, we develop an analytical tool referred to as total internal reflection fluorescence flow cytometry (TIRF-FC) to examine the region of the cell membrane with a throughput of ~100–150 cells/s and single cell resolution. We use an elastomeric valve that is partially closed to force flowing cells in contact with the glass surface where the evanescent field resides. We demonstrate that TIRF-FC is able to detect the differences in the subcellular location of an intracellular fluorescent protein. Proper data processing and analysis allows TIRF-FC to be quantitative. With the high throughput, TIRF-FC will be a very useful tool for generating information on cell populations with events and dynamics close to the cell surface.

INTRODUCTION

The cell membrane is the location where most biological signals are sent or received by a cell. Key events such as organelle and protein trafficking take place close to the cell surface and the visualization of these processes has been largely relying on imaging tools such as Total Internal Reflection Fluorescence Microscopy (TIRFM)^{1–7}. In a typical TIRFM setup, evanescent waves that are generated by totally reflected incident light at a glass-water interface penetrate into an adherent cell on the glass surface with a depth less than 200 nm. Only the features and events at the plasma membrane and the cytoplasmic region close to the plasma membrane are illuminated and visualized using the evanescent field. In this way, TIRFM eliminates potential fluorescence background from much of the cytosolic region that may obscure the events close to the cell surface. In spite of the advantages, like most imaging tools TIRFM is limited to the investigation of a small number of cells due to the small size of the illumination and imaging frames. Although useful biological information can be generated using the technique, whether such data are representative of the large cell population remains questionable, especially when heterogeneous cell populations such as those obtained from primary materials are involved. On the other hand, conventional flow cytometry does not differentiate fluorescence emission

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

from different subcellular locations of a cell⁸. High throughput techniques that can provide information on the cell surface features for a cell population with single cell resolution have been lacking.

Here we report a microfluidics-based tool, referred to as total internal reflection fluorescence flow cytometry (TIRF-FC), to examine cells in a flow for their surface features with a throughput ~100–150 cells/s. A microfluidic device with an elastomeric valve creates a constriction to drive flowing cells into an evanescent field which excites only fluorescent species in the region of the cell membrane. Our results show that TIRF-FC is sensitive to the differences in the intracellular localizations of fluorescent molecules. Thousands of cells can be examined with high throughput without requiring adhesion of cells on the substrate. We envision that TIRF-FC can be a powerful tool to study intracellular dynamics and to screen heterogeneous cell populations when biological events at the cell surface are of interest.

EXPERIMENTAL SECTION

Microchip fabrication

The device was fabricated using multilayer soft lithography^{9, 10}. The microscale patterns were first created using a computer-aided design software (FreeHand MX, Macromedia) and then printed out on high-resolution (5080 dpi) transparencies as photomasks in photolithography. The control layer master (photoresist/3 inch silicon wafer) was made using a negative photoresist SU-8 2025 (Microchem) with a thickness of ~25 μm (measured by a Sloan Dektak3 ST profilometer). The fluidic layer master was made using a positive photoresist AZ 9260 (Clariant) with a thickness of ~14 μm . The fluidic layer master was then baked at 120 $^{\circ}\text{C}$ for 2 min to generate a rounded cross section for the channel. The thickness at the center of the rounded fluidic channel was measured to be ~18 μm after baking. Both control and fluidic layers of the device were molded with PDMS of the same composition (GE Silicones RTV 615, MG Chemicals, mass ratio of A:B=10:1). The fluidic layer had a thickness of 35 μm (formed by spinning liquid PDMS prepolymer at 4000 rpm for 30s) which left the thickness of the PDMS membrane between the fluidic channel and the control channel ~17 μm . The control layer had a thickness of ~0.5 cm. The two layers were bonded together upon contact after oxidizing the two PDMS surfaces using a plasma cleaner (Harrick). The combined PDMS layers were then bonded to a 45 mm $\times$ 50 mm (No.1, Fisher Scientific) glass slip (~170 μm thick) using the same oxidation method. The fluidic channels were conditioned with 1% Pluronic F-127 (Invitrogen) for 1 h before experiment to avoid cell adsorption to the surfaces.

Cell samples

Syk-deficient DT40 B cells stably expressing SykEGFP (SykEGFP-DT40-Syk⁻) or SykEGFP-NLS (SykEGFP-NLS-DT40-Syk⁻) were produced as described previously^{11, 12}. SykEGFP-NLS contains the nuclear localization signal (NLS) from simian virus 40 large T antigen at the C-terminus. Both DT40 cell lines were cultured for at least 15 passages in complete medium (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1% chicken serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 IU/ml penicillin G, and 100 $\mu\text{g}/\text{ml}$ streptomycin) before experiments in the TIRF-FC devices. Cells were harvested and suspended in phosphate-buffered saline (PBS). To label cells with DiOC₁₈(3) (Invitrogen), 5×10^6 DT40 cells in 1 ml were mixed with DiOC₁₈(3) at final concentrations of 0.5, 1, 2, and 4 μM and incubated for 10 min. All cell samples were centrifuged at $300 \times g$ for 5 min, washed twice in PBS and re-suspended at a final cell density of 10^7 cells/ml in PBS before the experiment. Chinese hamster ovary cells (CHO-K1) was cultured according to the protocol applied in our previous study¹⁰. The CHO cell density was 10^7 cells/ml in PBS before the experiment.

Optical setup

The experimental setup is shown in Figure S1 (Supporting Information). An air-cooled 100mW argon ion laser (Spectra-Physics) was applied as the light source for laser-induced fluorescence. The laser beam at 488 nm was spectrally selected by a prism (Thorlabs) and filtered by a 10LF10-488 bandpass filter (Newport) before its intensity was adjusted by neutral density filters (Newport). The laser beam was expanded 5 times in diameter by lens L1 ($f=15$ mm, Thorlabs) and L2 ($f=75$ mm, Thorlabs) before it was focused by a lens L3 ($f=400$ mm, Thorlabs) and entered the laser port B of an invert fluorescence microscope (IX-71, Olympus). The laser was then filtered by a dichroic beamsplitter (505DCLP, Chroma Technology) and focused on the back focal plane (BFP) of a high numerical aperture TIRF objective (PlanApo, oil, 60X, NA=1.45, Olympus). The specimens in the microfluidic channel were illuminated by paraxial rays with Koehler illumination. The diameter of the circular illuminated area was approximately 90 μm . The intensity of the 488 nm light that was incident on the coverslip was ~ 2.1 mW. We were able to switch the mode of illumination between widefield epi-fluorescence and TIRF by either shifting the position of lens L3 vertically or pivoting M1. The fluorescence light from specimen was collected by the same objective. After passing through the dichroic filter and emitter (D535/40 emission filter, Chroma Technology), the emitted light was collected either by a photomultiplier tube (R9220, Hamamatsu) biased at 730 V or a CCD camera (ORCA-285, Hamamatsu). The 28 mm-diameter side-on photomultiplier tube was able to collect the light from the entire illuminated area when the flow cytometry data were taken.

Microchip operation

The microfluidic device was mounted on an inverted fluorescence microscope with an oil immersion 60X TIRFM objective (Figure S1 in Supporting Information). The 3 inlets were connected with two syringe pumps (PHD infusion pump, Harvard Apparatus) through plastic tubing. The volumetric flow rates were set at 200 $\mu\text{l/h}$ for the sample inlet and 400 $\mu\text{l/h}$ for each of two side inlets to obtain hydrodynamic focusing. The width of the sample stream under the valve was around 20 μm (the rounded shape of the cross section of the fluidic channel helped decrease the sample stream width). The control channel was filled with deionized water to prevent air from leaking into the fluidic channel. The microfluidic elastomeric valve was actuated by pressure provided by a nitrogen cylinder through a fast-response solenoid valve (ASCO Scientific) (Figure 1). The solenoid valve was controlled by a valve control circuit (described in detail below) which quickly open-closed the valve in case of adsorption of cells to prevent clogging.

Valve control

The elastomeric valve was kept partially closed during the operation of our microfluidic device in order to push flowing cells into contact with the glass surface. However, clogging and accumulation of cells could occur, depending on the cell size and deformability. In order to solve the problem, we designed a circuit to control the valve so that it could actuate (open and then go back to its partially closed state again) when a fluorescence intensity higher than a threshold is detected (usually caused by an adsorbed fluorescent cell). This design greatly alleviated the clogging problem so that we could continuously screen at least ~ 4000 cells in a run. The valve was actuated for ~ 10 times during a typical run with ~ 4000 DT40 B cells flowing through. It was actuated for less than 5 times when CHO cells of a similar sample size were tested (CHO cells appeared to have higher deformability than DT40 B cells).

The fluorescence signal collected by the PMT was processed by a low noise preamplifier (SR570, Standard Research Systems) with the cutoff frequency and the sensitivity set at 10K Hz and 100 $\mu\text{A/V}$, respectively. The current signal from the preamplifier was transformed to voltage VF which was processed by the valve control circuit. The valve control circuit was composed of an active low pass filter, a comparator, and a reed relay. The configuration of the

circuit is shown in Figure 2a. V_F was firstly filtered and amplified by a circuit with resistors R_1/R_2 , a capacitor and an operational amplifier (LM358P, National Semiconductor). Only low frequency signal was allowed to pass in order to prevent flowing cells of high fluorescence intensity from triggering the valve actuation. The cutoff frequency is defined as

$$f_c = \frac{1}{2\pi R_2 C} \quad (1)$$

Where R_2 has a resistance of 9820 Ω and C has a capacitance of 10 μF so that f_c is 1.62 Hz. The processed signal V_P has a gain of 20 determined by

$$\text{gain} = -\frac{R_2}{R_1} \quad (2)$$

where R_1 was 490 Ω . By comparing with V_{ref} (3.02 V), the comparator (LM 339N, Motorola) operated the reed relay (R56-1D.5-6D, NTE Electronics) to control the solenoid valve and hence the microfluidic elastomeric valve. The clogging event with a fluorescent cell has a median to high level signal with low frequency, which triggered the valve to open and consequently remove the clogging cell. The valve returned to its original state (partially closed) immediately after removing the clog. The response time for the valve is less than 2 ms. The actuation of the valve causes a negative signal in the fluorescence intensity data (Figure 2b), due to the reflection in the circuit when it responded.

Signal processing

V_F from the preamplifier was input into a PCI data acquisition card (PCI-6254, National Instruments) operated by LabView software (National Instruments). When the device was in operation, the fluorescence intensity recorded in voltage by LabView at 10^6 Hz showed a series of spikes with each of them corresponding to one cell flowing through the detection window. The data were processed by programs written in MATLAB to determine the width and height of each spike. The width of the spikes ranged from 0.15 to 40 ms. The spikes having dimensions within certain boundaries would be counted as valid data (e.g. the artifact spikes generated by the cell adsorption and the valve actuation would be discarded in this process). Then the data were sorted into histograms of the fluorescence for a cell population. The fluorescence intensity (the height of the spikes) ranging from 1 mV to 10 V was converted to 4 decade logarithmic voltage scale and then 256 scale channels, due to the small sample size of 3000–4000 cells in each histogram. The throughput of cells is 100–150 cells per second through the laser detection spot. More information on the data processing is provided in the Supporting Information Figure S2.

RESULTS AND DISCUSSION

As shown in Figure 1, a multilayer microfluidic device with an elastomeric valve was used in the study⁹. Hydrodynamic focusing aligned cells in a single file at the center of the channel in order for them to flow under the elastomeric valve. The valve was partially closed during the operation in order to form a flexible constriction that forced flowing cells to contact the glass substrate due to their deformation. An objective-based TIRF setup was implemented to introduce the evanescent field on the glass surface for cell illumination. A deformed flowing cell had a part of its surface illuminated by the evanescent field and the fluorescent signal was collected by a photomultiplier tube (no fluorescent signal was detected when the valve was not pressurized). Most of cells were able to pass under the partially closed valve due to the deformability of both the PDMS membrane and the cells. We designed a control circuit to

actuate the elastomeric valve (open-close) whenever both the fluorescence intensity and the residence time were higher than designated thresholds, in order to remove fluorescent cells that adhered to the valve surface. This practice was necessary and effective for preventing the clogging of the device. Typically our devices were able to continuously screen ~4000 cells with a throughput of ~100–150 cells/s without being clogged.

We tested the performance of the TIRF-FC using chicken B cells and Chinese hamster ovary (CHO) cells. Chicken B cells with enhanced green fluorescent protein (EGFP) tagged spleen tyrosine kinase (Syk) expression (SykEGFP-DT40-Syk⁺) were used for testing the effects of the actuation pressure of the elastomeric valve on the fluorescence intensity from cells. Syk is an important protein-tyrosine kinase involved in signaling pathways that are critical for cell growth and development^{13–15}. SykEGFP locates in the entire cytosol and nucleus in this cell line and the fluorescent signal from the cells was detected by our TIRF-FC apparatus. A histogram of log (fluorescence intensity) could be obtained for a cell population of ~3000 cells. In Figure 3a, we show that higher fluorescence intensity was detected when the actuation pressure of the valve was increased from 20 psi to 30 psi. The mean fluorescence intensity at 20 psi was 71 (in channels), while it increased to 94 at 25 psi and 121 at 30 psi. Higher actuation pressure in the control channel pushed the PDMS membrane (between the control channel and the fluidic channel) closer to the glass substrate and this made cells deform more during the passage under the valve. The more substantial deformation led to larger cell surface area in contact with the glass surface and stronger TIRF signal. We also tested CHO cells (D~14.6 μm with a standard deviation of 2.2 μm) that are larger than chicken B cells (D~10.8 μm with a standard deviation of 1.3 μm). CHO cells were stained with various concentrations of DiOC₁₈(3). As shown in Figure 3b, with 25 psi as the actuation pressure of the valve, the fluorescence histograms taken from CHO cells with various degrees of labeling can be differentiated as the mean fluorescence intensity channel increased gradually from 97 with 0.5 μM DiOC₁₈(3) staining to 195 with 4 μM DiOC₁₈(3) staining.

TIRF-FC is capable of determining whether fluorescent molecules are in the proximity of the cellular membrane. We compared chicken B cells transfected with plasmids coding for SykEGFP and SykEGFP-NLS¹¹. As shown in Figure 4a, Syk-EGFP is expressed in the entire cytosol and the fluorescent fusion protein can be seen using TIRFM imaging. In contrast, SykEGFP-NLS, which contains a nuclear localizing sequence (NLS) and localizes primarily to the nucleus, cannot be detected using TIRFM. As shown in Figure 4b and 4c, our microfluidic tool was able to differentiate the two cases. Cells carrying SykEGFP-NLS essentially did not generate any fluorescence signal. This confirms that TIRF-FC is effective for observing the differences in the subcellular location of intracellular molecules when the amount of molecules in the periphery of the membrane varies. The dynamics in a protein's subcellular location, or protein translocations, are important events involved in a large number of protein activation processes^{16–18}. The recognition of the change in the protein subcellular location is therefore an important trait of TIRF-FC.

The mechanical properties and the operational parameters of the elastomeric valve affect the results and their quantitative analysis. For example, the actuation pressure and the mechanics of the valve affect the degree of the PDMS membrane deformation and consequently these change the contact surface area and the relative position (i.e. whether the cell passes under the center of the valve) of a cell when it flows under the valve. However, such effects can be largely eliminated by proper processing and calibration of the fluorescence data. In Figure 5, we carry out a simple procedure to convert the fluorescence intensity data in Figure 3(a) (taken at different actuation pressures) into the fluorescence density data. For each spike generated by a flowing cell, we extract information on its height (H) and bottom width (W) (details in Supporting Information Figure S2). We use $H \times W$ to estimate the amount of fluorescent molecules detected (we ignore any constant in this analysis). We also assume that the bottom

width W of the spike gives a rough measure of the linear dimension of the contact surface. Then we use $W \times W$ to estimate the contact surface area for each flowing cell. Based on these assumptions, the area density of the fluorescent species detected can be obtained for each cell by calculating the value of H/W of the spike generated by the cell. Because the three histograms in Figure 3(a) were taken using the same cell type, in Figure 5 we show that the histograms of the fluorescence density are very close to each other despite that they were taken under different actuation pressures. This suggests that with appropriate calibration the operational parameters may have little influence on the area fluorescence density on the cell membrane obtained by TIRF-FC. In principle, the data calibration can be further improved by having more accurate information on the cell size (e.g. by light scattering) and adding correction to compensate the differences in the velocity of the cells while they pass through the detection point (i.e. large cells squeeze through the valve more slowly than small cells and create boost to their detected fluorescence). We believe that proper data processing and analysis will allow TIRF-FC to be a quantitative tool for studying the dynamics at the membrane and its proximity.

CONCLUSIONS

We demonstrate microfluidics-based TIRF-FC for carrying out high-throughput examination of single cells with evanescent field illumination. The technique provides information on a cell population compared to examining a few cells using imaging. TIRF-FC retains the trait of detecting events at the periphery of the cell surface. Furthermore, our technique does not require adhesion between the cells and the surface. We envision that this technique will provide insight into the dynamics and heterogeneity in a cell population when biological processes involving events close to the cell membrane occur. Future development of the tool will benefit from adding parameters of detection (e.g. light scattering) and improved protocols for calibrating/analyzing the data.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

(a) A schematic illustration of the microfluidic device for screening fluorescent species at the cell surface (not to scale). The partially-closed elastomeric valve renders a flowing cell in contact with the glass cover slip where there is an evanescent field created by an incident laser beam (488 nm). (b) The layout of the microfluidic chip and the TIRF illumination of flowing cells. The control channel (gray) is on top of the fluidic channel (black) with a polydimethylsiloxane (PDMS) membrane in between. The depth is 25 μm for the control channel. The rounded fluidic channel has depth of $\sim 18 \mu\text{m}$ at the center. The dimensions are labeled ($W1=300 \mu\text{m}$, $W2=200 \mu\text{m}$, $W3=50 \mu\text{m}$, and $L1=1.5 \text{ mm}$). The inset fluorescent image (right) shows the fluorescent trail left by fluorescent cells (SyKEGFP-DT40-Syk⁻) when they flowed through the center of the channel under hydrodynamic focusing and with the valve partially closed by 25 psi pressure.

Figure 2.

(a) The configuration of the valve control circuit. (b) Fluorescence intensity data recorded by LabView revealing one clogging event and the subsequent removal of the clog with the valve actuation (circled in the figure).

Figure 3.

The percentile histograms of log (Fluorescence Intensity) from SykEGFP-DT40-Syk⁻ cells and DiOC₁₈(3) stained CHO cells detected using TIRF-FC. All histograms were normalized to eliminate the effect of sample size. The percentile frequency on the y axis is the frequency divided by the total number of observations. 3000–4000 cells were involved in each histogram. (a) Histograms of SykEGFP-DT40-Syk⁻ cells with 20 psi, 25 psi and 30 psi actuation pressure in the elastomeric valve. (b) Histograms of CHO cells stained by 0.5 μ M, 1 μ M, 2 μ M, 4 μ M DiOC₁₈(3) for 10 min. The actuation pressure of the valve was maintained at 25 psi in all cases.

(a) **(b)** **(c)** **Figure 4.**

(a) Phase contrast (PC), epifluorescent (EPI), overlay and TIRF images of SykEGFPDT40-Syk⁻ and SykEGFP-NLS-DT40-Syk⁻ cells. The images were taken after cells settled down onto a cover slip. (b) Fluorescence intensity recorded when SykEGFP-DT40-Syk⁻ cells flowed through the detection point for 10 seconds. (c) Fluorescence intensity recorded when SykEGFPNLS-DT40-Syk⁻ cells (with the same density as SykEGFP-DT40-Syk⁻ cells) flowed through the detection point for 10 seconds.

Figure 5.

The reanalysis of the data from Figure 3(a) by creating the histograms of the area fluorescence density. The area fluorescence density of a cell was estimated by calculating H/W with H and W being the height and the bottom width of a spike generated by the cell, respectively.