Quantitative measurement of quantum dot uptake at the cell population level using microfluidic evanescent-wave-based flow cytometry

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Quantitative measurement of quantum dot uptake at the cell population level using microfluidic evanescent-wave-based flow cytometry

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The intracellular uptake of nanoparticles (NPs) is an important process for molecular and cellular labeling, drug/gene delivery and medical imaging. The vast majority of investigations into NP uptake have been conducted using confocal imaging that is limited to observation of a small number of cells. Such data may not yield quantitative information about the cell population due to the tiny sample size and the potential heterogeneity. Flow cytometry is the technique of choice for studying cell populations with single cell resolution. Unfortunately, classic flow cytometry detects fluorescence from whole cells and does not shed light on subcellular dynamics. In this report, we demonstrate the use of microfluidics-based total internal reflection fluorescence flow cytometry (TIRF-FC) for examining initial quantum dot (QD) entry into cells and the associated subcellular movement at the single cell level with a rate of ~200 cells s⁻¹. Our cytometric tool allows extraction of quantitative data from a large cell population and reveals details about the QD transport in the periphery of the cell membrane (~100 nm deep into the cytosol). Our data indicate that the fluorescence density at the membrane vicinity decreases after initial QD dosage due to the decline in the density of QDs in the evanescent field and the transport into the cytosol is very rapid.

Introduction

Nanoparticles (NPs) in various forms (quantum dots, micelles, metallic NPs, dendrimers, viral particles, etc.) have been increasingly explored for molecular and cellular labeling, biosensing, drug and gene delivery, and medical imaging. These NP systems have tailored size, shape and surface chemistry and exhibit unique optical, electrical, magnetic, and pharmacokinetic properties that are critical for their functions.¹⁻⁴ A large number of these applications are based on the intracellular delivery of NPs across the plasma membrane. NPs typically become internalized by cells via either receptor-mediated endocytosis or membrane-mediated pinocytosis.⁵ Despite the large number of reports on these NP vehicles, our knowledge about the mechanistic details of these delivery processes remains incomplete. Current investigations largely rely on fluorescent imaging (particularly confocal fluorescence imaging) to track the subcellular movement and transport of NPs in the cells. Although real-time imaging provides important information on the temporal dynamics of these internalization processes, such data are typically generated based on observation of a small number of cells due to the limited frame size. Data generated by a small sample of cells do not represent well the large cell population, especially when the population is heterogeneous. Thus it remains challenging to reconstruct quantitative and meaningful delivery data at the tissue level based on results yielded by imaging a small number of cells, due to the expected heterogeneity in cell samples and tissues. This limitation particularly hinders the quantitative characterization of NP-based gene/drug internalization, where the delivery efficacy and kinetics into the entire cell population or the tissue is the most relevant. In contrast to imaging, flow cytometry is the most effective and rapid approach for examining a sizable cell population with single cell resolution. Unfortunately, conventional flow cytometry does not reflect subcellular localization of the fluorescent species and thus is ineffective for studying intracellular transport.

In this report, we demonstrate the use of a flow cytometric technique based on evanescent-wave excitation, referred to as total internal reflection fluorescence flow cytometry (TIRF-FC),⁶⁻⁷ for quantitative measurement of the uptake of quantum dots (QDs) into cells at a throughput of ~200 cells s⁻¹. QDs are semiconductor NP fluorescent probes with size-tunable optical properties that are most often used for labeling and tracking of cells and molecules.⁸⁻¹⁰ QDs can also serve as vehicles for peptide/
gene delivery.\textsuperscript{11–15} TIRF-FC detects only fluorescent species in the membrane region excited by an evanescent field (\(\sim 100\) nm in thickness, formed by total internal reflection of a laser beam) while cells rapidly flow through a microfluidic channel and was previously demonstrated by us for subcellular detection of protein dynamics.\textsuperscript{6,7} Here our results show that transactivated transcription (TAT) peptide-conjugated QDs (TAT-QDs) stay at the cell periphery statically until their rapid translocation toward the microtubule organizing center (MTOC). TIRF-FC signal accurately reflects the density of QDs in the evanescent field. The decrease in the TIRF-FC fluorescence density of a cell population over time (after the initial QD dosage) was due to the rapid escape of QDs from the membrane region into the cytosol rather than slow QD movement in the evanescent field layer. We demonstrate TIRF-FC as a very powerful tool for extracting kinetics associated with QD entry and initial transport in the cell periphery, yielding both single cell resolution and population distribution.

\textbf{Results and discussion}

We applied TIRF-FC to examine the endocytosis of TAT-QDs into Chinese hamster ovary (CHO) cells. CHO cells incubated with TAT-QDs were washed and screened by TIRF-FC. As shown in Fig. 1, when a flowing cell passed the detection point at the microfluidic valve (for a brief period of several milliseconds), the cell was deformed and forced to make contact with the glass surface where the evanescent field was located. The evanescent field (with a thickness of roughly \(\sim 100\) nm) illuminated the cell membrane and its immediate vicinity in the cytosol. The fluorescence emitted by QDs in this region was detected by a photomultiplier tube and recorded as a signal from this single cell. The data from a cell population can be compiled into a histogram to reflect the distribution. More details about the device and the data processing are presented in the Experimental section.

TAT-conjugated QDs were made by QD–streptavidin incubated with TAT–biotin. TAT is a cell penetrating peptide derived from human immunodeficiency virus type 1 (HIV-1) and TAT-QDs (\(\sim 20\) nm in diameter) enter cells by macropinocytosis (a specialized and fluid-phase form of endocytosis).\textsuperscript{16,17} Epi-fluorescence images in Fig. 2 show that TAT-QDs were largely present at the outer periphery of the cells after initial incubation (\(\sim 10\) min). QDs started to emerge at the perinuclear region known as the microtubule organizing center (MTOC) within 1 h and were increasingly transported to the MTOC within the period of 4 h. In contrast, streptavidin-coated QDs (without TAT coating) do not enter the cells spontaneously. It was interesting to note that most QDs located either at the cell periphery or MTOC at any given time. This implies that the intracellular transport of QDs (encapsulated in vesicles) between the membrane region and MTOC was rapid. The observed delivery in Fig. 2 is consistent with the literature\textsuperscript{17} and confirms the successful QD modification by TAT. There was no obvious detrimental effect on the cell viability and function due to potential QD cytotoxicity during the experimental period, judged by the continuous intracellular trafficking of QDs. Unfortunately, fluorescence image data in Fig. 2 do not reveal the processes at the plasma membrane vicinity which would require nanometre spatial resolution. Furthermore, it is desirable to examine the cell population instead of a small number of cells.

TIRF-FC data quantitatively characterize the dynamics of TAT-QDs at the membrane region (\(\sim 100\) nm deep) over time for a cell population of \(\sim 5000\) and complement the information in Fig. 2.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{TIRF-FC for studying TAT-QD endocytosis. (a) Illumination of TAT-QDs by the evanescent field (\(\sim 100\) nm from the glass surface). QDs are illuminated at the membrane region by the evanescent field (left) and stop emitting fluorescence when they move out of the evanescent field layer and deep into the cytosol (right). (b) The design of a TIRF-FC chip. Cells carried in the middle stream are focused to the center of the channel and the microfluidic valve, once actuated, forces cells to squash through a subcellular-sized opening. A laser (488 nm) with total internal reflection at the glass coverslip produces the evanescent field that illuminates the cell surface in close contact with the glass surface.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2.png}
\caption{Epifluorescence imaging of TAT-QD endocytosis into CHO cells. Cells were incubated with TAT-QDs for 10 min, washed, and incubated in culture media for an additional time (up to 4 h) in a glass bottom dish.}
\end{figure}
obtained by imaging. Fig. 3a shows TIRF-FC data taken after initial QD dosage (by incubation of cells and QDs for 10 min), cell washing, and further cell incubation (without QDs) for additional times (10 min to 4 h). The histogram data reveal how the population distribution of the fluorescence density at the membrane (fluorescence intensity in the evanescent field divided by the area) changed over time after a fixed amount of QD dosage. The fluorescence density histogram for the cell population gradually shifted to the lower end with longer incubation time (Fig. 3a). In Fig. 3b, we plot the average fluorescence density of the cell population (extracted from 3 separate trials shown in Fig. 3a and S1†) over time by setting the value at time 0 after QD dosage as 100% and the background value (generated by a cell population not incubated with QDs) as 0. The data points were extracted from 3 separate trials of TIRF-FC measurements taken on different days (the histograms are shown in (a) and Fig. S1†).

Fig. 3  TIRF-FC analysis of TAT-QD endocytosis at the cell population level. (a) Representative histogram series of the fluorescence density (fluorescence intensity divided by area) for the cell population at various times (10 min to 4 h) after an initial TAT-QD dosage of 10 min. Each histogram includes data from ~5000 cells. Additional histogram series of 2 other trials are included in Fig. S1†. (b) The population-averaged fluorescence density decreases over time after TAT-QD dosage. We set the average fluorescence density 10 min after dosage as 100% and the background level (without QD dosage) as 0. The data points were extracted from 3 separate trials of TIRF-FC measurements taken on different days (the histograms are shown in (a) and Fig. S1†).

Next, we want to determine the origin of the fluorescence density decline after the QD dosage. There could be two possible mechanisms: first, the decrease could be due to the change in the vertical distance between QDs and the glass surface while the QDs were always in the evanescent field and illuminated throughout the process. The evanescent field intensity \( I \) exponentially decays from the glass surface according to eqn (1) and thus is very sensitive to the distance:\textsuperscript{19,20}

\[
I = I_0 e^{-zd}
\]  

(1)

where \( z \) is the perpendicular distance away from the glass surface, \( I_0 \) is the intensity at the glass surface, the penetration depth \( d \) is a constant determined by the laser incident angle, laser wavelength, and the refractive indexes of glass and water. When QDs move further from the glass within the evanescent field layer, their fluorescence intensity decreases due to the lower evanescent field intensity that they are excited by. Second, the fluorescence density decrease could be due to the escape of QDs from the evanescent field. When the number of QDs in the evanescent layer decreases, the fluorescence density also decreases.

In Fig. 5, using TIRF imaging, we monitored the changes in both the fluorescence density and the QD density (i.e. the number of QDs per unit area) after initial QD internalization over time. Fig. 5a shows that adherent CHO cells gradually spread on the glass surface to yield larger contact area as time lapsed for 1 h. We confirmed that with the laser illumination used, the fluorescent intensity of QDs permanently adsorbed onto the glass surface does not vary by photobleaching (shown in Fig. S2†). For

Fig. 4  No histogram shift for the negative controls. (a) TIRF-FC analysis of CHO cells loaded with fluorogenic dye calcein AM and incubated for various durations (10 min to 4 h). (b) TIRF-FC analysis of DT40 cells incubated with anti-IgM-QD for 10 min and then washed and incubated for 10 and 60 min. Anti-IgM-QD binds to the exterior surface of DT40 cells without internalization.

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The evanescent layer was captured by our detection. However, the resonance intensity of QDs (due to movement inside the membrane vicinity after uptake) was so rapid that no variation in the fluorescence density (which is not affected by the contact area) and the areal density of QDs at the same cell surface. Both parameters decrease over time as shown in Fig. S3 and S4†. The strong linear correlation between the two parameters (R² = 0.91) (Fig. 5b) indicates that the fluorescence density decrease at the membrane vicinity (after the initial QD dosage into cells) was mainly due to the decrease in the density of QDs in the evanescent field layer. Taken together, our TIRF-FC data (in Fig. 3) suggest that the QDs in the evanescent field were either quiescent or rapidly moving into the cytosol. The transport of QDs out of the evanescent field was so rapid that no variation in the fluorescence intensity of QDs (due to movement inside the ~100 nm evanescent layer) was captured by our detection.

Conclusions

We show that TIRF-FC detects the subcellular dynamics of QDs at the membrane vicinity after uptake. TIRF-FC yields data with single cell resolution for a large cell population. Such data are most relevant to uptake kinetics at the population or tissue level which cannot be obtained by imaging a few cells. We found that TIRF-FC quantified the density of TAT-QDs in the evanescent field layer (~100 nm into the cytosol). Such data not only reflect the internalization of QDs into cells but also indicate the intra-cellular transport into the cytosol which is often the onset of QD cellular functions. We envision that this approach can be extended to study the uptake of other NP systems.

Experimental section

Cell samples and QD delivery

Chinese hamster ovary (CHO) cells were subcultured every two days in DMEM medium supplemented with 10% fetal bovine serum, 100 IU ml⁻¹ penicillin G, and 100 μg ml⁻¹ streptomycin. Chicken DT40 B cells were cultured in RPMI 1640 media supplemented with 10% heat-inactivated fetal calf serum, 1% chicken serum, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 100 IU ml⁻¹ penicillin G, and 100 μg ml⁻¹ streptomycin. To prepare TAT peptide-conjugated quantum dots (TAT-QDs), 10 nM QD-streptavidin (Qdot 605 conjugate, Invitrogen) was incubated with TAT–biotin (Anaspec) with a ratio of 20 to 1 for 30 min in cell culture medium. Before QD delivery, CHO cells were subcultured in 6 well plates with a density of 3 × 10⁶ cells per well. 200 μl of QD suspension (10 nM) in DMEM medium was added to the well and incubated with cells at 37 °C for 10 min. The cells were then immediately washed by 2 ml PBS to remove the un-internalized QDs. After further incubation for various times at 37 °C, cells were trypsinized (by adding 150 μl of 0.25% trypsin and incubation for 1 min) to detach from the substrate, and then neutralized with 150 μl DMEM medium supplemented with 10% fetal bovine serum prior to analysis by TIRF-FC.

Microchip design, fabrication and operation

The microfluidic TIRF-FC device is composed of a microfluidic channel with hydrodynamic focusing from two side channels and a two-layer pneumatic valve that can reversibly close half-way to create a subcellular space for cells to squash through (Fig. 1). The control layer and the fluidic layer of the device were fabricated using multilayer soft lithography. Briefly, the micropatterns designed by a computer software (Freehand MX, Macromedia) were printed out on a transparency at 5080 dpi resolution. The feature on the transparency was then transferred to a master on a silicon wafer. The control layer master was made using a negative photoresist SU8 2025 (Microchem) with a thickness of 100 μm, while the fluidic layer master was fabricated with a positive photoresist AZ 9260 (Clariant). The rounded cross-section of the fluidic channel was generated by baking the fluidic layer master at 120 °C for 2 min, resulting in a depth of ~18 μm at the center of the fluidic channel. Pre-cured polydimethylsiloxane
A B = 10 : 1 was poured to the control masters to mold the control layer by curing at 80 °C for 40 min. The pre-cured PDMS of the same composition was spun at 4000 rpm for 30 s to form a 35 μm thick layer on the fluidic master and then cured. The two layers were bonded together before sealing with a pre-cleaned glass slip (45 mm × 50 mm × 170 μm, No. 1, Fisher Scientific) by oxidation of the surfaces with a plasma cleaner (Harrick). The intersection of the control channel and the fluidic channel formed a pneumatic valve. The thickness of the PDMS membrane between the two layers of channels was ~17 μm at the channel center. The fluidic channels were conditioned with 1% Pluronic F-68 (Sigma) for 1 h before experiment to avoid cell adsorption onto the surfaces. During the TIRF-FC measurement, the sample and buffer inlets were connected to syringe pumps (PHD infusion pump, Harvard Apparatus) via plastic tubing. PBS was infused at 8 µl min⁻¹ into each of the two side inlets, while the center sample flow that carried cells was set at 2 µl min⁻¹. At the laser detection point, the sample stream had a focused width of 15.8 μm when 0.2 μm fluorescent microbeads were used for visualization.

**Optics**

The optical setup for TIRF-FC was described previously.⁶ Briefly, a laser beam at 488 nm from an air-cooled 100 mW argon ion laser (Spectra-Physics) was applied as the light source for laser-induced fluorescence. After passing through a pair of optic filters (Thorlabs), the laser beam was expanded 5 times in diameter by a pair of lenses (f = 15 mm and 75 mm, Thorlabs) before it was focused by a lens (f = 400 mm, Thorlabs) and entered the laser port B of the inverted 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 TIRF objective (PlanApo, oil, 60×, NA = 1.45, Olympus). By adjustment of the incident light angle, the specimens in the microfluidic channel or on the glass slip were illuminated by the evanescent field. The diameter of the circular illuminated area was approximately 70 μm. The intensity of incident light on the coverslip was measured to be 2.1 mW. The fluorescence light excited was collected by the same objective and filtered through the dichroic filter and emitter (D605/40, Chroma Technology). In order to record the fluorescence signal, the emission light was collected by a 28 mm diameter side-on photomultiplier (R9220, Hamamatsu) biased at 500 V. A CCD camera (Coolsnap HQ, Photometrics) was used for taking fluorescence images.

**TIRF microscopy imaging**

CHO cells were incubated with TAT-QDs at 37 °C for 10 min before they were washed and seeded in the glass bottom dish (MatTek). The dish was pre-coated with fibronectin (Sigma) at a concentration of 100 μg ml⁻¹ at 37 °C for 1 h to facilitate cell adhesion. The cell-containing dish was placed on the microscope equipped with a warmed platform (Warner Instruments) with temperature set at 37 °C. Phase contrast and TIRF images were taken periodically during the 2 h incubation time. Fluorescence intensity of QDs was quantified using ImageJ software.

**Data processing**

The fluorescence emission from single cells collected by the PMT was transformed into a voltage signal recorded by a PCI data acquisition card (PCI-6254, National Instruments) operated by LabVIEW software (National Instruments). These raw data were further processed offline by MATLAB programs to extract the width and height of each spike. The fluorescence density of each cell was calculated from the ratio of the peak height and width.⁶ We have shown previously that the fluorescence density data are minimally affected by variation in the valve actuation and the cell size.⁶ The fluorescence density data were further calibrated and converted to a 4 decade logarithmic voltage scale and assigned into 256 scale channels. Each histogram included a sample size of ~5000 cells.

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**References**