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Mechanochemical Delivery and Dynamic Tracking of Fluorescent Quantum Dots in the Cytoplasm and Nucleus of Living Cells

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Abstract

Studying molecular dynamics inside living cells is a major but highly rewarding challenge in cell biology. We present a nanoscale mechanochemical method to deliver fluorescent quantum dots (QDs) into living cells, using a membrane-penetrating nanoneedle. We demonstrate the selective delivery of monodispersed QDs into the cytoplasm and the nucleus of living cells and the tracking of the delivered QDs inside the cells. The ability to deliver and track QDs may invite unconventional strategies for studying biological processes and biophysical properties in living cells with spatial and temporal precision, potentially with molecular resolution.

Studying biological processes at the molecular level inside a living cell is a major challenge in cell biology.^{1–3} Recent advances in single-molecule techniques have enabled the understanding of biological mechanisms in unprecedented detail.^{1–5} However, the existing techniques are mostly limited to *in vitro* studies, due to the difficulties in imaging individual molecules in the optically noisy cellular environment and accessing directly the interior of living cells.^{1–5} On the other hand, nanoparticles, such as quantum dots (QDs)^{6–8} and magnetic nanoparticles (MNPs),⁹ have shown great promise in this regard because of their bright and stable fluorescence and/or manipulability and their small size relative to that of individual proteins. For example, QDs have been used to study the dynamics of individual membrane proteins,^{10–14} to measure the motion of individual molecular motors in the cytoplasm,¹⁵ to monitor antigen uptake by dendritic cells,¹⁶ and to study the transport of nerve growth factors¹⁷ and the membrane fusion of synaptic vesicles^{18, 19} in neurons; and MNPs have been used to physically manipulate individual membrane receptors to activate signal transduction of living cells.⁹

The unique advantages of nanoparticles for investigating biological problems at the molecular level in living cells, however, have not been fully realized. The main problems include the relatively large sizes of biomolecule-conjugated nanoparticles and nanoparticle–target molecule complexes,^{11–14} the instability of antibody-mediated targeting,^{11, 13, 14} and the difficulty of delivering nanoparticles into the cytoplasm.^{6, 8, 12, 13, 20–22} For instance, because of the lack of efficient ways of delivering dispersed and single QDs into living cells, except in a few demonstrations,¹⁵ the use of QDs has been limited to visualizing cell membrane molecules^{10–14} and biological processes related to the endocytosis.^{16, 21} Although various approaches involving the use of cell-penetrating peptides,^{21, 22} electroporation,²⁰ ballistic

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Supporting Information Available:

nanoparticle delivery method,²³ and more recently, nanoneedles²⁴ have been explored, QDs internalized into cells are often trapped in the endocytic pathway or form aggregates in the cytoplasm, or in other cases, uncontrollable amounts of nanoparticles are delivered into unspecified locations in the cytoplasm, which introduces undesirable fluorescent background noise and/or precludes subsequent targeted labeling of endogenous molecules and thus single-molecule studies. Overall, such approaches have their unique characteristic advantages and disadvantages depending on specific applications^{22, 25}, and among them, the direct microinjection of QDs²⁰ has shown better performance and has enabled a homogeneous labeling of the entire cytoplasm in a dispersed form without the need for endosomal escape²⁶. However, to take advantage of the full potential of nanoparticles for living cell studies, strategies to deliver well-dispersed single nanoparticles into the cytoplasm or targeted organelles of living cells, independent of cell types and the endocytic pathways and without affecting cell physiology or introducing cellular toxicity, are required.

Here we show a nanoscale direct delivery method where we manipulate a nanotube needle carrying a minute amount of “cargo” (in this case, QDs) to mechanically penetrate the cell membrane and deliver the cargo into living cells. We demonstrate the selective delivery of a small number of well-dispersed, single fluorescent QDs into either the cytoplasm or the nucleus of living mammalian cells. We detect and track the delivered QDs and study their dynamics in the cytoplasm and the nucleus of living cells, revealing the biophysical heterogeneity of the cellular environment. This direct delivery method may allow new strategies to study biological problems inside living cells, complementing existing delivery methods.

For realizing the nanoneedle-based direct delivery of QDs into living cells (Figure 1a), we used a chemically-synthesized boron nitride nanotube to work as the nanoneedle. The nanotube has a high-aspect ratio nanoscale structure with a small diameter (~50 nm); it is mechanically rigid but resilient.²⁷ We affixed the nanotube onto the sharpened tip of a macroscopic needle for easy handling and manipulation. We then coated the nanotube with a thin layer of Au (10–20 nm in thickness); the thin Au layer facilitated the use of surface chemistry for attaching QDs and increased the mechanical strength of the nanoneedle. To attach designated QDs onto the Au-coated nanotube (via disulfide bonds), we developed a general procedure for engineering the surface of the nanoneedle as detailed later. To release the QDs from the nanoneedle we exploited the regulatory mechanism of cells that maintains the redox equilibrium in the cytoplasm, in which most disulfide bonds are reduced into thiol groups ($R-SS-R + 2H^+ + 2e^- \rightarrow R-SH + SH-R$).²⁸⁻³⁰ Thus, upon the entry of the nanoneedle into the cytoplasm, the QDs conjugated on the nanoneedle via disulfide bonds could be released by the reductive cleavage of the disulfide bonds (Figure 1a).

This approach possesses several distinct technical benefits for delivering QDs into cell. With the use of a small-diameter nanotube as a needle, it introduces minimal intrusiveness in penetrating through the cell membrane and accessing the interior of live cells. By exploiting surface chemistry to attach and release QDs, it avoids the use of the carrier solution and the pressure-driven injection device required in microinjection-based delivery and circumvents related problems in using small injection needles (e.g., clogging of injection needles and high-pressure required for injection). By making the nanoneedle compatible with micromanipulators commonly installed in an optical microscope, it can be readily adopted in many laboratories without the need of technically-demanding equipments, such as the scanning probe microscope used in a prior study.²⁴ Furthermore, the whole delivery process, including the positioning of the nanoneedle, the penetration of the cell membrane and the reach of the nanoneedle to different intracellular compartments, can be precisely controlled, monitored and recorded *in situ*, not achieved in prior studies.^{22, 24, 25} While this approach might limit the amount of nanoparticles delivered in a single procedure, the local concentration of the delivered nanoparticles could be sufficiently high in the vicinity of the targeted release site to facilitate

efficient molecular targeting if genetically-encoded nanoparticles were used in certain applications, besides lowering the overall fluorescent background, for instance, from the QDs of no desired interest.

The general procedure for attaching QDs onto the nanoneedle via disulfide bonds (Figure 1b) consists of three steps: forming a NH_2 -terminated self-assembly monolayer (SAM) on the Au-coated nanoneedle by the chemisorption of thiols on gold,³¹ conjugating a linker molecule containing a disulfide bond within its spacer onto the SAM, and attaching streptavidin-conjugated QDs by the binding of streptavidin and biotin (see Supporting Information for the detailed procedure). This approach is potentially extendable for attaching other species, such as DNAs, RNAs, proteins, and nanoparticles of various sizes by tuning the surface functionalization with different molecular building blocks. It would also be possible to simultaneously attach different species with controlled densities, for example, by using mixed SAMs with different terminal functional groups.³¹ However, the subsequent imaging and tracking of such delivered species in a three dimensional cellular environment would remain a challenging issue.

We demonstrated the described delivery strategy by delivering fluorescent QDs into living HeLa cells. Figure 2 shows a typical QD delivery experiment targeting the cytoplasm. We manipulated the nanoneedle by using a common piezoelectric micromanipulator (InjectMan NI 2, Eppendorf) integrated in a Leica inverted epifluorescence microscope. The nanoneedle was manipulated to approach and penetrate the cell membrane along its axial direction ($\sim 45^\circ$ to the surface plane) to the depth of $\sim 1\text{--}3\text{ }\mu\text{m}$ into the cytoplasm. The nanoneedle was maintained in this position for 15–30 minutes to allow the reduction of disulfide bonds and the release of QDs inside the cell. The nanoneedle was then retracted from the cell. The delivery process didn't affect the viability and membrane integrity of the cell (see Supporting Figures S1 and S2). During the QD delivery, we could precisely locate the nanoneedle at the targeted release site in the three-dimensional cellular environment by focusing the tip of the nanoneedle in the bright field (Figure 2a), the fluorescence (Figure 2b), or the combined bright field and fluorescence imaging mode (Figure 2c). The bright fluorescence from the QDs attached on the nanoneedle can serve as an ideal optical beacon for aiding the visualization and so the positioning of the nanoneedle inside the cell, potentially allowing the use of nanoneedles with an even smaller diameter beyond the imaging resolution limit of an optical microscope.

We detected both mobile and stationary QDs within the cytoplasm after the delivery (Figure 2d–f), but not in neighboring cells or the surrounding medium, indicating the exclusive release of QDs from the nanoneedle inside the cell. The mobile QDs roamed the area within the cell boundary but outside the nucleus, showing their confinement in the cytoplasm. Notably, because the out-of-focus background signal of QDs was substantially decreased by delivering only a small number of QDs, we could clearly detect single QDs inside cells even with a simple epifluorescence microscope. The photostability of QDs also allowed continuous observation of QDs for the duration of monitoring (~ 30 minutes). When we did similar experiments without maintaining the nanoneedle inside cells for 15–30 minutes, a time period normally required for the disulfide bond to be cleaved in the reducing environment of the cytoplasm, we didn't observe the release of QDs (data not shown). To further verify that the release of QDs was resulted from the reduction of the disulfide bond, we functionalized nanoneedles using a linker molecule that doesn't contain the disulfide bond in its spacer and did similar delivery experiments. In this case, we didn't observe the release of QDs inside cells (see Supporting Figure S3). The control experiment confirmed that the release of QDs was through the reductive cleavage of the disulfide bond and the original attachment of QDs on the nanoneedle was stable.

The direct visual monitoring of the delivery process allowed the direct delivery of QDs into target areas or compartments of living cells at a desirable time, not readily achievable with

conventional methods. To demonstrate this capability, we specifically targeted the QD delivery to the nucleus of living HeLa cells (Figure 3). As the nucleus has a similar reducing environment,^{28, 29} the delivery strategy based on the reductive cleavage of disulfide bonds is applicable to the nucleus as well. To avoid the possibility of detecting QDs at the top or bottom of the nucleus, we identified the equator of the nuclear envelope in the bright-field mode and imaged the cell on the same focal plane in the fluorescence mode (Figure 3a—c). The size of the passive entry to the nucleus has been reported to be smaller than or around 10 nm in²⁰ which excludes the possibility of diffusive introduction of QDs (~20 nm in diameter) diameter, from the cytoplasm into the nucleus. To further verify the nuclear delivery, we expressed the green fluorescence protein (GFP) on the nuclear envelope of living HeLa cells following the general protocol and performed the nuclear delivery experiment on GFP-expressed cells (Figure 3d—f). The delivered QDs were seen to be confined within the nuclear envelope.

We next questioned whether our method could deliver single QDs into living cells. We measured the fluorescence intensity of some stationary QDs delivered into living HeLa cells over a period of time (Figure 4a, b and Supporting Movies S1 and S2). We measured the fluorescence intensity of stationary QDs by subtracting the integrated pixel intensity of the neighboring area (1×1 μm in size) from the intensity of the area (1×1 μm in size) containing a single QD in each time-stamped image. The time averaged intensity of the neighboring area was used as the reference and set to zero. The time-lapse fluorescence signal of most stationary QDs showed the blinking pattern, typical for an isolated single QD,⁶⁻⁸ and was similar to that acquired from diluted QDs on a glass slide in separate measurements, indicating that the delivered QDs were likely single (or a cluster of at most several QDs) (Figure 4a, b and Supporting Movies S1 and S2). For mobile QDs, however, we couldn't distinguish the fluorescence fluctuation and blinking of the QDs from the fluorescence fluctuation and disappearance caused by the out-of-focus movement of the QDs as they diffused around in the three-dimensional environment of cells.

The ability to deliver a small number of QDs into living cells allowed the tracking of the delivered QDs, even with a simple epifluorescence microscope. To quantify their dynamics, we carried out single-molecule tracking (SMT)³² (Figure 4c; see also Supporting Methods and Supporting Movie S3). The acquired MSD versus time data show three types of characteristic motion of QDs in living cell: free diffusive (in ~70% of monitored QDs), corralled (~20%), and stationary (~10%) (Figure 4d). The diffusion coefficient D for QDs in the cytoplasm ranged from ~0.1 to 4 μm²/s (mean = 0.8 ± 1.0 μm²/s, $n = 20$) (Figure 4e), consistent with those reported for macromolecules of comparable size (~20 nm in diameter) measured by ensemble-averaged methods.³³ However, we might underestimate this mean value of D (0.8 μm²/s) as it is more difficult to detect and track fast-moving QDs than slow ones in the three-dimensional environment of the cytoplasm. The D values are ~4- to 200-fold smaller than that in aqueous solution ($D_0 = 17$ μm²/s),³⁴ due to molecular crowding in the cytoplasm³³; the reported D/D_0 values are ~0.001–0.5 for macromolecules.³³ The immobility of some QDs (~10%) is likely due to their trapping to the intracellular structures, such as cytoskeleton and endoplasmic reticulum; the sizes of single QDs or small QD clusters are comparable to the pore sizes in the cytoplasmic meshwork (~30–100 nm).³³

The recorded dynamics of QDs can also be used to quantify the local biophysical properties of the intracellular environment by the bio-microrheology method.³⁵ According to the Stokes-Einstein relation $D = kT / (6\pi\eta r)$, where k is the Boltzmann's constant, T is the absolute temperature, η is the viscosity, and r is the radius of the particle (QDs), the apparent viscosity η of the region where the QD travels can be calculated from the measured D values. Assuming that r of the QDs is the hydrodynamic radius of QDs (12.8 nm obtained from $D_0 = 17$ μm²/s),³⁴ the apparent “nanoscale” viscosity in the cytoplasm is estimated to span from ~4 to ~200

cP in different regions of the cytoplasm, indicating the high physical heterogeneity of the intracellular environment (see also supporting Figure S4).

In contrast to the QDs in the cytoplasm, most QDs introduced into the nucleus were immobilized after the delivery at the time when fluorescence images were acquired. For some QDs, we observed small movement, as shown in Fig. 5, in a confined domain of $L = \sim 300$ nm, similar to the size of typical nuclear microdomains.^{36, 37} However, we also observed that some delivered QDs were dispersed as far as $\sim 10 \mu\text{m}$ from the site of release in the nucleus (Figure 3). As we could not practically observe the release and movement of QDs in real-time from the nanoneedle due to the bright fluorescence of the QDs attached on the nanoneedle overshadowing the vicinity of the release site (the fluorescence of QDs on the nanoneedle was bright enough to be detected even with the presence of transmission light as seen in Fig. 2c), we assigned a maximum time t of ~ 20 minutes as the time allowed for such diffusion, which was the normal operational time interval needed in practice from the instant of penetrating the nanoneedle into the cell to the moment of performing the fluorescence imaging after withdrawing the nanoneedle from the cell. A simple random walk model $d = \sqrt{2Dt}$ with $d \approx 10 \mu\text{m}$ and $t \leq 20$ min then estimates that the diffusion coefficient of QDs in the nucleus can be larger than $\sim 0.04 \mu\text{m}^2/\text{s}$. This estimated D compares favorably to the D values determined for the transport of mRNA-protein complexes ($0.01\text{--}0.09 \mu\text{m}^2/\text{s}$)^{36, 38} and nuclear proteins ($\sim 0.2\text{--}0.5 \mu\text{m}^2/\text{s}$)³⁹ in the nucleus through simple diffusion. As suggested for the transport of nuclear components by diffusion, QDs might also diffuse over an extended distance ($\sim 10 \mu\text{m}$), most likely through interchromatin domains at a similar rate as in the cytoplasm, but eventually be trapped in nuclear compartments, such as chromatin-dense domains, probably due to their non-membrane structures of intermingled fibres.⁴⁰

The ability to deliver a small number of monodispersed nanoparticles into living cells with spatial and temporal precision may make feasible numerous new strategies for biological studies, which would otherwise be technically challenging or even impossible. For example, in combination with effective molecular targeting strategies^{11, 13, 14} using QDs and MNPs as molecular probes, this method can potentially enable simultaneous observation and manipulation of individual molecules in both the cytoplasm and the nucleus of living cells, and afford a broad range of new biological experiments at the single-molecule level. As the release of such nanoparticles is local at the site of release and at the time of release (due to the relatively low diffusivity of QDs inside cells), this local concentration can facilitate efficient targeting of intended region and molecules and thus potentially allow spatially-resolved molecular experiments inside cells. For some cellular and molecular mechanics studies (e.g., mechanotransduction) inside living cells, spatially-resolved delivery of one or a traceable number of force probes (e.g., MNPs) would be desirable to pinpoint applied forces, which would then be uniquely achievable with this method.⁴¹ Similar to the QDs delivered through microinjection, the direct delivery of only a small number of nanoparticles with the nanoneedle would also minimize the effect of internalized nanoparticles on cell physiology.⁴² Furthermore, the delivery process can be done repeatedly at a desired time through the cell cycle and in conjunction with other cellular manipulations and measurements. An obvious limitation of this method is that one functionalized nanoneedle can only be used to deliver QDs into one cell (or at most, several cells if the nanoneedle is reused until the attached QDs are totally released). Beyond delivery, the nanoneedle-based approach can also be extended in many ways for single cell studies, for example, as an electrochemical probe²⁷ or an optical biosensor using QDs attached on the nanoneedle⁴³. Altogether, the nanoneedle-based delivery technique offers a powerful nanotechnology-based tool for studying biological processes and biophysical properties inside living cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Nanoscale mechanochemical delivery of QDs into living cells. (a) Schematic of the mechanochemical delivery of QDs into living cells. Inset, optical microscope image of a typical nanoneedle. (b) Procedure for functionalization of the nanoneedle and surface attachment of QDs. A NH_2 -terminated SAM was formed on the Au-coated nanoneedle by the chemisorption of thiols on gold. The nanoneedle was biotinylated by reacting the NH_2 surface group with *N*-Hydroxysulfosuccinimide (sulfo-NHS) esters of biotin (sulfo-NHS-SS-biotin), forming a stable amide bond. Streptavidin-coated QDs were attached onto the nanoneedle by the specific binding of streptavidin and biotin. Scale bar, 5 μm

Figure 2.

Delivery of QDs into the cytoplasm of living HeLa cells. (a-c) Optical microscope images of a nanoneedle functionalized with QDs during the QD delivery experiment, showing the nanoneedle penetrating through the cell membrane. The whole process was monitored in situ in the bright-field (a), the fluorescence (b), or the combined bright-field and fluorescence (c) image mode. The QDs attached on the nanoneedle are shown in red in (b) and bright white in (c). The gradually unfocused dark shade on the right side in (a) and (c) is the tip of the macroscopic needle on which the nanoneedle is attached. (d-f) Bright-field (d) and fluorescence (e) images of the cell after the QD delivery, and the overlay image (f) of (d) and (e) acquired

in one focal plane. (g) and (h) the fluorescence and overlay images acquired in two additional focal planes. The arrows indicate QDs (red). Scale bars, 10 μm

Figure 3.

Delivery of QDs into the nucleus of living HeLa cells. (a) Overlay of bright-field and fluorescence images of the cell after the nuclear delivery. (b) Enlarged fluorescence image of the region marked in (a). (c) Overlay of bright-field and fluorescence images of the region marked in (a). (d-e) Delivery of QDs into the nucleus of a living HeLa cell expressing GFP on the nuclear envelope. The nuclear envelope (d) of the cell (green) was identified with the GFP filter and the cell (e) was imaged at the same focus with the QD filter. (f) Overlay of (d) and (e). The dotted lines locate the boundary of the nucleus. The arrows indicate QDs (red). Scale bars, 10 μm in (a) and (d), and 5 μm in (b).

Figure 4.

Time trace of fluorescence intensity and tracking of QDs inside living HeLa cells. (a) Typical time trace of the fluorescence intensity of stationary QDs (red) in living cell plotted with the background signal of neighboring areas (black), showing the blinking pattern (see also Supplementary Videos 1 and 2). For comparison, the intensity variation of a single QD on a glass slide prepared by spreading a highly diluted QD dispersion (0.1 nM) on a glass slide is shown as labeled. (b) A fluorescence image sequence showing the QD in the living cell during the time period of 19-25 s in (a). (c) Tracking of a QD inside living cells (see also Supplementary Video 3). A series of fluorescence images shows the trajectory (the green line) of a tracked QD. (d) Mean-square displacement (MSD) versus time data show three types of

characteristic motion of QDs: free diffusive (red square), confined (blue square), and stationary (black square). The solid red line and blue line are the line fit on the data based on a free diffusion model and a confined diffusion model, respectively. (e) Diffusion of QDs in the cytoplasm. The diffusion coefficient D ($0.08\text{--}3.8\text{ }\mu\text{m}^2/\text{s}$, mean $0.8\text{ }\mu\text{m}^2/\text{s}$, $n = 20$) was determined by fitting a free diffusion model $\text{MSD}(t) = 4Dt$ to the initial few data points of each MSD versus time curve acquired from tracking of different QDs. The fitted lines are shown in solid red. The dashed blue line indicates the MSD expected for freely diffusing QDs in aqueous solution ($D_0 = 17\text{ }\mu\text{m}^2/\text{s}$). The dashed green lines are reference lines for D of 1.0 and $4.0\text{ }\mu\text{m}^2/\text{s}$ as labeled. Scale bars, 500 nm in (b) and $1\text{ }\mu\text{m}$ in (c).

Figure 5.

Confined diffusion of a QD in the nucleus of a living HeLa cell. (a-b) Confined diffusion of a QD in the region marked in (a) inside the nucleus of a living HeLa cell and the trajectory of the QD (b) (see also Supplementary Video 4). (c) Corresponding MSD versus time curve (blue) for this QD (A). The data for other stationary QDs (B) (black) are also shown for comparison. The solid line (blue) in (c) is the fit based on a confined diffusion model. The fit estimates the diffusion coefficient of the QD (A) to be $0.01 \mu\text{m}^2/\text{s}$ and the size of the confined domain L_A to be $\sim 300 \text{ nm}$, consistent with the typical size of the nuclear microdomains.