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A Bright Light to Reveal Mobility: Single Quantum Dot Tracking Reveals Membrane Dynamics and Cellular Mechanisms

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Abstract

This perspective describes recent progress in single quantum dot techniques, with an emphasis on their applications in exploring membrane dynamics and cellular mechanisms. In these cases, conventional population measurements, such as fluorescence recovery after photobleaching, yield only a mean value on an ensemble or bulk collection of molecules, where the behavior of individual proteins and vehicles is missing. In recent years, the single quantum dot imaging approach has been introduced as a sub-category of single molecule fluorescent techniques to reveal single protein/vehicle dynamics in real-time. One of the major advantages of using single quantum dots is the high signal-to-noise ratio originating from their unique photophysical properties such as extraordinarily high molar extinction coefficients and large effective Stokes shifts. In addition to a brief overview on the principle of single quantum dot imaging techniques, we highlight recent discoveries and discuss future directions in the field.

Keywords

quantum dot; fluorescent microscopy; single molecule detection; membrane dynamics; trafficking

Plasma membranes, the physical barrier of the smallest living unit, a single cell, consist mainly of bilayer phospholipids with embedded proteins. The membrane not only allows for accomplishing vital biological functions but also governs the movement of materials and signals in and out of the cell. For the past four decades, the most well-known model of the plasma membrane is the fluid mosaic model,¹ in which the membrane proteins are viewed as freely floating boats in a phospholipid “sea.” However, recent investigations have found that the diffusivity of cellular membranes in eukaryotes have a significantly lower degrees of

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freedom than was predicted in the fluid mosaic model; this suggests the regulatory roles of the surrounding lipid environment in membrane signaling/interaction.² It has become increasingly clear that the cell membrane is far from homogeneous rather it contains various small microdomains for specialized functions.²⁻⁴ In many cases, the cholesterol-enriched membrane microdomains, also known as lipid rafts, were indicated to participate in cellular signaling by providing the microenvironment necessary for complex protein-protein interactions.^{4, 5} In addition, recent biochemical studies demonstrated a connection between membrane microdomains and the cytoskeleton. This connection was found to influence membrane compartmentalization,^{2, 5} further suggesting the role of membrane microdomains in the secretory and endo-exocytotic pathways. Together, these largely underdeveloped characters explain the recent emergence of the studies of membrane dynamics and cellular mechanisms.

To date, various techniques based on optical microscopy have been created to allow for observation and quantification of membrane dynamics. These approaches can be generally categorized into two groups according to the type of data collected: the first group, such as fluorescence recovery after photobleaching (FRAP) and conventional immunofluorescence microscopy, was developed to depict the ensemble average response of the movement of fluorescently labeled molecules; the second, known as single molecule microscopy, is designed to extract diffusion parameters from probing multiple single events.⁶ The ensemble-averaging approach, although convenient and widely used, presents an obvious disadvantage regarding the representativeness of the experimental data: loss of sub-population distribution in the ensemble average.⁶ To characterize the diffusion rates in different domains of the plasma membrane, the single molecule tracking (SMT) approach is clearly a better choice.² The basis of SMT is to follow the real-time movement of individual biological targets using high-speed fluorescent microscopy (typical time resolution is 10–100 milliseconds) with high spatial resolution (at the nanometer scale), resulting in a map of the real-time dynamics upon observing many individual events. Based on the above definition an ultimate sensitivity is required that allows individual single molecules to be monitored in a picoliter- to femtoliter-sized microscope sampling volume. A fundamental limit is, however, subject to Abbe's optical diffraction limit, which is ~ 250 nm in the visible spectrum. To achieve a sub-pixel localization in SMT, the FIONA (*Fluorescence Imaging with One-Nanometer Accuracy*) approach is typically performed (Figure 1).⁷ The diffraction pattern of a single emitter viewed through a microscope, known as the Airy disk, is thus analyzed in terms of the point spread function (PSF) where the localized centroid is determined by fitting the PSF to a two dimensional Gaussian profile with a least-squares estimator:

$$I_{xy}=A_0+A \cdot e^{-\frac{(x-x_0)^2+(y-y_0)^2}{w^2}} \quad [1]$$

, where I_{xy} is the intensity of the pixel, x_0 and y_0 is the designated local maximum coordinates of the Gaussian, A is the amplitude of the signal with local background A_0 , and w is the width of the Gaussian curve. It is important to note that the coordinate (x_0, y_0)

acquired by fitting function [1] is not a true position but only an estimate; the accuracy is strongly dependent upon the respective signal-to-noise ratio (SNR), which is defined as:

$$SNR = \frac{I_0}{\sqrt{\sigma_{bg}^2 + \sigma_{I_0}^2}} \quad [2]$$

, where I_0 is the maximum signal intensity above background, σ_{bg}^2 is the variance of the background intensity values, and $\sigma_{I_0}^2$ is the true variance of the maximum signal intensity above the background. However, common organic fluorophores used in single-molecule imaging have very limited photon yield and narrow Stokes shift (difference in excitation and emission wavelength), which makes them difficult to produce a sufficient SNR for single-molecule imaging. In addition, photobleaching associated with organic fluorophores prevents their usefulness in long-term monitoring. Hence, these shortcomings associated with organic fluorophores place a high demand for new fluorescent materials for single molecule tracking.

Fluorescent quantum dots (QDots) are semiconductor nanomaterials with a size in the range of 2–10 nanometers, smaller than their bulk Bohr exciton radius; this gives QDots discrete, quantized energy levels. Extraordinary optical properties of QDots include high quantum yield, broad adsorption with monochromatic, narrow emission, high molar extinction coefficients, and large effective Stokes shifts (for review, see Rosenthal *et al.*⁸). Over the past decade, QDots have been popularly used in biological labeling applications.^{9–14} As mentioned, the sub-pixel localization in single molecule imaging is dependent upon the SNR, which is highly correlated to the total photons emitted and the Stokes shift of a single emitter. Unlike conventional fluorescent dyes or fluorescent proteins, these two properties make QDots much more advantageous in single molecule imaging. Additionally the superior photostability makes QDots particularly useful for long-term tracking studies (see Table 1 for comparison of QDots, organic dyes, and fluorescent proteins for single molecule tracking). Since its emergence in the biological imaging field, single QDot imaging approach has played an essential role in investigating membrane dynamics and cellular signaling. In the following sections, we highlight recent biological applications using single QDot techniques, particularly the applications in exploring membrane dynamics and intracellular trafficking.

Mapping Receptor Membrane Diffusion

By utilizing the superior fluorescent properties of QDots and a high quantum-efficiency back-illuminated electron multiplying charge-coupled device (EMCCD) camera, Dahan *et al.* first reported the use of a single QDot imaging approach to investigating endogenous glycine receptors (GlyR) at the surface of cultured rat spinal cord neurons.¹⁶ More importantly, the authors were able to characterize multiple GlyR diffusion domains in relation to the synaptic, perisynaptic, or extrasynaptic GlyR localization, which is indeterminable by conventional ensemble methods. Since then, several studies inspired by

this work were directed toward addressing the specific biological functions of membrane protein sub-populations.

In line with the above study, noticeable efforts in the single QDot approach were made in recent years pertaining to the studies of the cholesterol and ganglioside GM1-enriched membrane microdomains.⁴ Pinaud *et al.* developed a raft-associated glycosyl-phosphatidyl-inositol anchored avidin test probe (Av-GPI) to study the correlation between the diffusion of individual Av-GPI and the location of GM1-rich microdomains and caveolae in the transfected HeLa cells.¹⁷ Single QDot-labeled Av-GPI complexes were found to exhibit dynamic partitioning in and out of GM1-rich microdomains in a cholesterol-dependent manner. In a native, non-transfected neuronal cell line, we used single QDot-labeled cholera toxin B subunit (CTxB) to investigate the diffusion dynamics and membrane compartmentalization of the GM1-rich microdomains.¹⁸ Diffusion measurements revealed that single QDot-labeled CTxB-GM1 complexes undergo slow, confined lateral diffusion with a diffusion coefficient of $\sim 7.87 \times 10^{-2} \mu\text{m}^2/\text{sec}$ and a confinement domain about 200 nm in size. Further analysis of the trajectories showed lateral confinement persisting on the order of tens of seconds, comparable to the time scales of the majority of cellular signaling and biological reactions.

The importance of lipid rafts in membrane protein signaling and regulation was also tested with the single QDot approach. In a recent publication, we reported the first use of a ligand-conjugated single QDot to track the membrane dynamics of the serotonin transporter (SERT).¹⁹ SERT involves the regulation of serotonergic signaling via clear neurotransmitter serotonin from the synaptic cleft back into the presynapse. Altered SERT activity and/or expression has been implicated in multiple neuropsychiatric disorders, including anxiety, depression and autism.²⁰ As such, selective serotonin reuptake inhibitors (SSRIs) that antagonize SERT at brain synapses are by far the most frequently prescribed drugs for the management of major depression.^{20, 21} We found two types of subpopulations of SERT in living neuronal cells; one exhibits relatively free diffusion and the other localizes to cholesterol and GM1 ganglioside-enriched microdomains, the latter of which displayed restricted mobility. Disruption of membrane rafts by gentle cholesterol depletion results in an increase of single SERT mobility and a loss of SERT confined diffusion (Figure 2). In addition, mobilization of transporters arises from a p38 MAPK-dependent untethering of the SERT C-terminus from the juxtamembrane actin cytoskeleton. This study demonstrates the potential of ligand-conjugated QDots for analysis of the behavior of single membrane proteins in relation to critical cellular signaling and regulation.

Endosomal Trafficking and Endocytosis

Endosomal trafficking and endocytosis are known to be highly heterogeneous in space and time. As one example, axonal transport of nerve growth factor (NGF), a small secreted protein that promotes the growth and differentiation of certain types of neurons, has been known to be critical to carry neural cell-survival. However, following endosomal transport/trafficking is a challenging field of research due to technical difficulties of visualizing the processes in real-time. Cui *et al.* presented a milestone study in which the real-time movement of NGF axonal transport was followed with single QDot imaging approach in live

rat dorsal root ganglion (DRG) neurons.²² To achieve a monovalent presentation of the NGF dimer on the QDot surface, a stoichiometric ratio of NGF to QDot of 1:1.2 was adapted in their conjugation procedure. In a clever setup, NGF–QDot conjugates were first added to the microfluidic chamber containing distal axons of DRG neurons and allowed to bind and form complexes with NGF receptors and then undergo subsequent internalization into early endosomes. Interestingly, endosomes containing NGF–QDots were found to exhibit “stop-and-go” retrograde transport toward the neuronal cell body with an average speed of $1.31 \pm 0.03 \mu\text{m}/\text{sec}$ (Figure 3A). This work also underscores the potential utility of single QDots for intracellular applications as the real-time observation of intracellular endosome movement is achieved in this study.

The same technique, with emphasis on monitoring internalization, was successfully extended by Fichter *et al.* to quantify the endocytosis of G-protein–coupled receptors (GPCRs), the biggest membrane protein superfamily that sense signaling molecules in a broad range of biological processes and are also the major drug targets.²³ In this study, a small group of 5-HT_{1A} receptors, a type of metabotropic GPCRs, were labeled with single QDots; moreover, the authors found that single QDot probes did not interfere with the internalization kinetics of receptors in live cells. In their analysis, sustained 5-HT exposure elicited receptor internalization that increased from 20 to ~ 60 – 70 % within 60 min, indicating sustained receptor internalization in response to continuous 5-HT stimulation (Figure 3B). This study provided new insights into the spatial and temporal control of the GPCRs in the endocytotic pathways. With further research, this could emerge as a powerful approach to elucidating the actions of GPCR-targeted therapeutics.

Dynamic Processes of Intracellular Targets

Recently, several groups presented brilliant ideas to take advantage of the nanoscale sized single QDots as a nanoscaled physical ruler in measuring the dynamics of intracellular processes. In an exciting example, Zhang *et al.* used single QDot fluorescence to investigate the molecular mechanism of vesicular secretion of neurotransmitters in living neurons.²⁴ Since a single-synaptic vesicle is so small (lumen diameter ~ 24 nm) that it can only load one QDot (~ 15 nm) at a time, the authors used single QDot imaging to see if a full-collapse fusion or a kiss-and-run fusion is taking place during neuron firing; the former refers to a preloaded single QDot being released, while the latter depicts the single QDot being trapped in the vesicle. In addition to the size of the QDots, the authors also cleverly took advantage of another important characteristic of single QDots that being the pH-dependent fluorescence fluctuation. The author found that the QDot fluorescence would increase by ~ 15% if the single QDots move from intravesicular space (pH ~ 5.48) to extracellular space (pH ~ 7.34). As such, in the kiss-and-run fusion, one can only capture the increase of QDot fluorescence but not the escape of the QDot, while in the full-collapse fusion a similar Qdot brightening would appear to be followed by a complete loss of signal as the QDot escaped from the synaptic vesicle. Using this sophisticated yet elegant approach, a stochastic vesicle dynamic is established in which the authors successfully correlate the increased neurotransmitter release probability with a higher prevalence of kiss-and-run fusion (Figure 4).

Another excellent example under this category is the use of peptide-conjugated single QDot as cargo to investigate the intracellular nuclear transport via nuclear pore complex.²⁵ It is known that nuclear pore complex acts as the gatekeeper for traffic into and out of the nucleus. Unlike small molecules, which can passively diffuse through nuclear pore complex, the transportation of larger protein-based cargo into the nucleus requires a dedicated transport mechanism. Generally the signal-dependent nuclear transport is facilitated by nucleoplasmic shuttling receptors termed importins, which are associated with Ran GTPase. In the study reported by Lowe *et al.*, the authors conjugated the importin- β binding domain to QDots (IBB-QDot) and used single IBB-QDots as pseudo cargo to visualize the nuclear translocation process (Figure 5).²⁵ Additionally, the conjugated IBB-QDot complexes have a mean hydrodynamic diameter of 18 ± 4 nm, similar to the size of native cargos that translocate into nuclei via receptor-driven transport through nuclear pore complex. Remarkably, with precise single QDot imaging analyses the authors were, for the first time, able to calculate the multi-step processes of Ran GTPase-dependent import through nuclear pore complex: cargo capture at a 25 % success rate; filtering and translocation, 80 % success rate; and cargo release into the nucleus, 50 % success rate with Ran and > 99 % abort without Ran.

Single Quantum Dot FRET

Fluorescence resonance energy transfer (FRET) is a photophysical process where the excited-state energy from a donor fluorophore can be non-radiatively transferred to a nearby acceptor fluorophore. The energy transfer is through a dipole-dipole coupling and the relative distance has to be in the range of 1–10 nm (for reviews of FRET principle, see Selvin²⁶ and Piston²⁷). Since the initial works of Willard *et al.*²⁸ and Medintz *et al.*²⁹ demonstrating the potential of a QDot as a FRET donor for biosensing, similar concepts have been applied to various applications such as explosive 2,4,6-trinitrotoluene (TNT) detection,³⁰ proteolytic activity assays,^{31–34} molecular sensing of microenvironmental changes in pH,^{35, 36} and calcium ion concentrations.³⁴

The bottleneck in traditional single-molecule FRET studies is the low SNR; however, with the assistance of high SNR QDot probes, Zhang *et al.* first developed a FRET-based single QDot nanosensor for specific nucleic acid detection (Figure 6).³⁷ A sandwich-type hybrid structure was designed to detect the target sequence by the presence of a biotin-labeled capture probe and a Cy5-labeled reporter probe. The hybridized sequence assembly then self-assembles on the streptavidin-conjugated QDot thus forming a FRET donor–acceptor ensemble. Extremely high femtomole sensitivity was achieved in this study. This is approximately 100 times more sensitive than the molecular beacon approach, the gold standard FRET method for nucleic acid detection. However, we wish to note that, despite various successes in molecular sensing, single QDot FRET probes are mostly limited in biochemical assays. To the best of our knowledge, single QDot FRET studies have not yet demonstrated in live- cell imaging applications.

3D Single Quantum Dot Tracking

While the application of live cell three-dimensional (3D) single QDot tracking is still in its infancy, recent advances in ultrasensitive detectors offer great promise in this field. Conventionally, 3D microscopic applications using confocal and two-photon excitation optical microscopy have already led to various advances in the biological detection. As such, approaches based on scanning confocal excitation, including the 3D single QDot tracking with confocal feedback^{38, 39} and two-photon 3D FIONA⁴⁰, have been readily adapted in 3D single QDot tracking. However, due to the simplicity in design and bigger imageable area with required time-resolution (> 10 fps), wide-field excitation using high-speed ultrasensitive EMCCD cameras are more often used for 3D single molecule imaging. In this section, only examples of 3D single QDot imaging based on wide-field excitation will be given in this section.

Two common approaches based on wide-field excitation using EMCCD cameras have been taken toward the construction of 3D single QDot trajectories in real-time. The first is to conduct multifocal plane imaging with a wide-field fluorescent microscope.^{41–43} Photons generated from a targeted single QDot are split into multiple paths. In each light-path, the signal is turned at a specific calibrated distance that allows each detector or certain portion of a detector to only reflect information from a specific focal plane (z). An obvious advantage of this technique is to simultaneously track multiple focal planes over a large z -range, which enables the observation of whole cell events. By configuring the microscope to image four distinct focal planes, a recent report from Ram *et al.* demonstrated an amazing ten micron z -range in 3D single QDot tracking of the transferrin receptor in a live epithelial cell monolayer.⁴¹

Another kind of 3D tracking approach based on wide-field excitation is aimed at 3D localization via altering the point spread function of a single emitter.^{44–47} An outstanding example established by the Moerner Lab is the engineering of a point spread function through a spatial light modulator.^{44, 45} This enables the display of additional z -directional information on a single detector, where the single emitter exhibits two lobes in the image plane that rotate continuously around the optical axis with propagation, termed the “double-helix point spread function” (DH-PSF) (Figure 7A). In a recent study with DH-PSF-based 3D fluorescent microscopy, Thompson *et al.* reported a nanometer localization accuracy along the x , y , and z directions, which allows one to follow the 3D movement of a single QDot-labeled structure inside a living cell.⁴⁵ Interestingly, the 3D single QDot trajectory showed a variety of diffusive and linear transport characteristics in live cells (Figure 7B), which would otherwise be missing if processed with 2D tracking.

Conclusion and Future Perspective

Since the first demonstrations in biological imaging, water-soluble QDots have become popular probes for various biological imaging and detection applications.^{48, 49} Their unique photophysical properties render them particularly valuable for revealing dynamic biological processes. However, owing to the size and surface chemistry, even single QDots can cause multivalent binding, which can be a major drawback in using QDots for single molecule

studies.⁵⁰ Recently, a monovalent QDot was introduced,⁵¹ which, if commercially available, would be an indispensable technology in the field of single QDot imaging.

Additionally, the pros and cons of QDot blinking in single QDot imaging have been previously discussed in great detail.⁵² On one hand, this property is popularly used as a criterion for identifying single QDots in live cells.^{16–19, 22–25} On the other hand, it raises concerns regarding the discontinuous trajectory of a single QDot tracking approach due to the temporary disappearance of the target position from the blinking events.⁵³ To address this deficiency, the synthesis of non-blinking QDots based on a multishell approach^{54, 55} and gradient alloyed core/shell structure⁵³ were recently reported. Researchers are currently taking advantage of the non-blinking QDots in single QDot tracking experiments.⁵⁶ We can expect the biological applications of non-blinking QDots to increase.

In summary, the biological applications of QDots have moved from a simple alternative of fluorescent dyes to more specialized topics for advanced biological research. This is particularly prominent in the cases of the recent development in single QDot applications. Although there are some limitations due to the physical nature of the QDots, advances in QDot synthesis and surface chemistry show significant potential to eliminate these pitfalls. Considering the multiple applications of single QDot approaches in the past few years, we are optimistic that the use of single QDots will largely advance our understanding in many areas of biological research. We specifically forecast that the additional dimensions of space and time that follow from the ability to dynamically track a single biological target will revolutionize our understanding of protein regulation, cell signaling, immune response, neuron function, and open horizons yet to be envisioned.

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Biographies

Jerry Chang received his Ph.D. in Chemistry in 2012 from Vanderbilt University under the direction of Professors Sandra J. Rosenthal and Randy D. Blakely. His graduate works, which involved the development of quantum dot-based drug screening platform and single quantum dot imaging techniques, led him to the Rockefeller University as a postdoctoral associate with Professor Paul Greengard. His research interests include development of quantum dot nanoconjugates for biological detection and optical imaging methods for drug discovery applications in psychological and neurological disorders.

Sandra Rosenthal is the Jack and Pamela Egan Professor of Chemistry, a Professor of Physics, Pharmacology and Chemical and Biomolecular Engineering at Vanderbilt University, and Joint Faculty with Oak Ridge National Laboratory. She serves as Director of the Vanderbilt Institute of Nanoscale Science and Engineering. She completed her PhD on femtosecond inertial solvation dynamics at the University of Chicago in 1993 and did an NSF Postdoctoral Fellowship at UC Berkeley and Lawrence Berkeley National Laboratory. Her current research concerns fundamental studies of nanocrystals and their implementation in biological imaging for neuroscience, solid state lighting, and photovoltaics. She has won the Popular Mechanics Breakthrough Award and is a Fellow of the American Association for the Advancement of Science.

Quotes to highlight in paper

Since its emergence in the biological imaging field, single QDot imaging approach has played an essential role in investigating membrane dynamics and cellular signaling.

Recently, several groups presented brilliant ideas to take advantage of the nanoscale sized single QDots as a nanoscaled physical ruler in measuring the dynamics of intracellular processes.

We specifically forecast that the additional dimensions of space and time that follow from the ability to dynamically track a single biological target will revolutionize our understanding of protein regulation, cell signaling, immune response, neuron function, and open horizons yet to be envisioned.

Figure 1.

(A) Schematic representation of the diffraction pattern from a point emitter passed through an optical device. Owing to diffraction, the smallest distance to which an imaging system can optically resolve separate light sources is limited by the size of the Airy disk. (B) Fitting a 2D Gaussian function to the PSF of a single emitter yields nanometer precision, termed *Fluorescence Imaging with One Nanometer Accuracy* (FIONA). Reprinted from ref. 7. Copyright © 2005 American Chemical Society.

Figure 2.

Single QDot tracking of SERT in living serotonergic RN46A cells under control and lipid raft-disrupted conditions. (A) Temporal profile of representative QDot-labeled, single SERTs with and without lipid raft disruption. Note the more mobile behavior of single QDot after lipid raft disruption. Scale bar is 10 μm . (B) Comparison of the mean squared displacement over time of the representative single QDots in (A). Single SERT under a lipid raft-disrupted condition shows the pattern expected of free diffusion whereas single SERT from untreated cells demonstrates a pattern consistent with confined lateral diffusion. Reproduced from ref. 19. Copyright © 2012 Society for Neuroscience.

Figure 3.

(A) Live imaging reveals that single NGF–QDot containing endosomes exhibit stop-and-go motion. Left: A typical axon showing four moving endosomes that contained NGF–QDot in pseudocolor. Center: Trajectory of a typical endosome, showing a switch between moving and pausing. Right: Trajectories of 120 endosomes showing that the moving speed and the duration of pauses vary greatly from one endosome to another. (Reprinted with permission from ref. 22. Copyright © 2007 National Academy of Sciences). (B) Cellular internalization of single QDot-labeled serotonin receptor complexes during continuous serotonin stimulation in transfected N2a cells. Red: QDot probe; green, plasma membrane, visualized with DiO. Scale bar is 10 μm . Reproduced with permission from ref. 23. Copyright © 2010 National Academy of Sciences.

Figure 4.

The dynamic control of kiss-and-run and vesicular reuse probed with single quantum dots (A) QDot-labeled single vesicles in presynaptic terminals outlined by subsequent FM4-64 staining. Scale bar, 3 μm . (B) Electron micrographs show QDots loaded into synaptic vesicles. Scale bars, 400 nm (right) and 10 nm (left). (C) Spectrophotometer measured QDot fluorescence change at different pHs and hypothesized QDot signals corresponding to K&R and FCF. (D) Sample records of field stimulus (arrowhead) evoked changes of QDot fluorescence. Figure kindly provided by Dr. Qi Zhang, ref. 24.

Figure 5.

Characterization of nucleocytoplasmic transport with single QDot approach. (A) Diagram of QDot-based cargo. The snurportin-1 IBB/Z-domain fusion protein is coupled via a bifunctional SMCC crosslinker to the amino-PEG polymer coat of a fluorescent QDot. The three helix Z-domain acts as a spacer to correctly present the IBB for biological function. (B) Dynamic light scattering size distributions of QDot-IBB cargos in the presence and absence of importin- β . (C) Dwell time distribution of all QDot interactions with the NPC. The time axis is truncated at 300 sec. (D) Bright-field image of a nucleus with a QDot fluorescence image (with background subtraction applied) overlaid in red. A single QDot cargo at the nuclear envelope is boxed. (E) Individual consecutive frames from a single-molecule experiment showing the arrival (first frame) and departure (final frame) of the cargo into the nucleus. The centroids determined from the fitting of the PSF are overlaid as red crosses. Frame numbers are at the bottom left hand corner of each frame. Movies were captured at 40 Hz. Reproduced with permission from ref. 25. Copyright © 2010 Nature Publishing Group.

Figure 6.

Single QDot FRET for DNA detection (A) Conceptual scheme showing the formation of a single QDot based DNA nanosensor in the presence of targets. The resulting assembly brings the fluorophore acceptors and the QDot donor into close proximity, leading to energy transfer from QDot to acceptors. (B) Representative traces of fluorescent bursts detected with QDot nanosensors. Fluorescent signals are acquired by both the donor and the acceptor detectors. In the presence of targets, fluorescent bursts were detectable in both donor and acceptor detectors. When targets were absent, fluorescent bursts were only detected by the donor detector, not by the acceptor detector. Reproduced with permission from ref. 37. Copyright © 2005 Nature Publishing Group.

Figure 7.

3D single QDot tracking with DH-PSF imaging approach. (A) Schematic of the DH-PSF imaging system. L1 and L2 are focal-length-matched achromatic lenses, and SLM is a liquid crystal spatial light modulator. Bottom images are a bead emitting on average 2000 photons/frame shown at five different axial positions (from left to right -960, -510, -20, 510, and 970 nm). Scale bar is 1 μm . (B) Images taken of the structure at different points along a single QDot trajectory showing different z positions, the bright aggregate at the top edge should be ignored. An average of 2400 photons/frame with a background noise of 8 photons/pixel/frame was measured for this trajectory. Scale bar is 1 μm . (C) 3D plot of a single QDot trajectory in live COLO205 cells, showing a variety of diffusive and linear transport

characteristics. Points on the curve that correspond to the images in (B) are shown by green shapes. Reproduced ref. 45. Copyright © 2010 American Chemical Society.

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Table 1

Comparison of QDots, organic dyes, and fluorescent proteins for single molecule tracking

Targeting Probe	Advantages	Disadvantages
Quantum Dots	<ul style="list-style-type: none"> Broad absorption (CdS: 350–470 nm; CdSe: 450–640 nm; CdTe: 500–700 nm) and narrow emission (FWHM^a: ~30nm). Tunable emission wavelength dependent upon the particle size, ideal for multi-color tracking. Extremely high extinction coefficients, often $>10^6 \text{ cm}^{-1}\text{M}^{-1}$ (e.g., QDot 605: $1,100,000 \text{ M}^{-1}\text{cm}^{-1}$ at 488 nm) ^b. Great photostability in SMT (> 10 min). Large effective Stokes shift (> 100 nm). 	<ul style="list-style-type: none"> Relatively large size (inorganic core: 2–10 nm). Significant increase in the hydrodiameter after bioconjugation (i.e.: QDot streptavidin conjugate: ~15–20 nm)^b. Additional conjugation, purification, and spectroscopic characterization may be required for QDot bioconjugate preparation. Potential issue in multivalency of the final conjugates. Concerned with non-specific binding, additional surface modification may be required.¹⁵ Irregular blinking is a minor drawback.
Organic Dyes	<ul style="list-style-type: none"> Very small (< 1 nm). Monovalent labeling achievable after conjugation. Rich, ready-to-use pre-labeled commercial products and resources. Comparable with fully automated solid-phase peptide synthesis and oligonucleotide synthesis. 	<ul style="list-style-type: none"> Small Stokes shift, often < 50 nm (e.g., Tetramethylrhodamine isothiocyanate: 24 nm; Tetramethylrhodamine: 25 nm). Relatively low extinction coefficients, often $< 10^5 \text{ cm}^{-1}\text{M}^{-1}$. Relatively poor photostability in SMT (< 10 sec).
Fluorescent Proteins	<ul style="list-style-type: none"> Small (< 5 nm). Monovalent labeling achievable after tagging. User-friendly protocol for protein tagging via transfections, advantageous in intracellular target detection. Good biocompatibility. 	<ul style="list-style-type: none"> Small Stokes shift, often < 50 nm. Relatively poor photostability in SMT (< 30 sec). Relatively low extinction coefficients, often $< 10^5 \text{ cm}^{-1}\text{M}^{-1}$. Not applicable for labeling endogenous targets. Concerned with altered subcellular localization and activity after tagging.

^aFull width at half maximum of the emission band;^bManufactured by Invitrogen.^cMonomeric fluorescent protein variants required.