Chem Soc Rev

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Journal:	*Chemical Society Reviews*
Manuscript ID	CS-SYN-08-2015-000657.R2
Article Type:	Tutorial Review
Date Submitted by the Author:	09-Dec-2015
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TUTORIAL REVIEW

Tracking single viruses infecting their host cells using quantum dots

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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Single-virus tracking (SVT) technique, which uses the microscopy to monitor the behaviors of viruses, is a vital tool to study the real-time and *in-situ* infection dynamics and virus-related interactions in live cells. To make SVT a more versatile tool in biological research, the researchers have developed quantum dot (QD)-based SVT technique, which can be utilized for long-term and high-sensitivity tracking in live cells. In this review, we describe the development of QD-based SVT technique and their biological applications. We first discuss the advantage of QDs as tags in the SVT field by comparing with the conventional tags, and then focus on the implementation of QD-based SVT experiments, including the QD labeling strategy, instrumentation, and image analysis method. Next, we elaborate the recent advances of QD-based SVT in biological field, and mainly emphasize the representative examples to show how to use this technique to acquire more meaningful biological information.

Key learning points

- Advantages of quantum dots (QDs) in single-virus tracking (SVT) field
- Strategies of labeling viruses with QDs
- Instrumentation for QDs imaging
- Image processing and data analysis for QD-based SVT
- Applications, challenges and perspectives of QD-based SVT

1. Introduction

Virus is a kind of obligate parasites and can infect host cells, which utilizes the metabolic system of the cells to obtain the substance and energy for replication. The infection process starts from the interaction between the virus and the receptor on the cell surface. Some types of viruses can break the barrier of plasma membrane and release their genome into the host cell directly. Also, many viruses are trapped into endosomes by endocytosis and transported to specific organelles for genome release. Next, the viral genome is transported to appropriate regions in cytosol or nucleus for replication and expression. The newly-synthesized proteins and genomes assemble progeny viruses, which can be released into the extracellular environment by exocytosis or lysing the host cells.[1] Evidently, the virus infection is a very complicated process, involving multiple infection steps and interactions with cellular structures. Thus, an effective method is urgently required to study the spatio-temporal dynamics and mechanisms of virus infection in live cells.

Single-particle tracking (SPT) techniques utilize the microscope to monitor the movements of individual particles, and then analyze the dynamics of particles on the basis of video images, which are vigorously developing in the field of life science and have been a kind of indispensable tools for biological researches to solve many basic biological problems successfully. The main reason of this significant influence is that most of conventional techniques do the analysis by the ensemble average of a large number of particles, and the averaged properties cannot characterize the heterogeneous particles in complex biological environments accurately. SPT techniques can solve the conflict between traditional bulk measurements and heterogeneities of single particles. Thereinto, single-virus tracking (SVT) technique is essential to study the infection mechanisms of virus precisely, which offers the opportunity to probe real-time and *in-situ* dynamics of individual viruses in live cells and to explore the interactions between viruses and cellular structures.[2,3]

To visualize the infection behaviors, viruses should be labeled by fluorescent tags primarily. Quantum dots (QDs), colloidal semiconductor nanocrystals, have attracted the attention of many researchers over the past two decades in

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virtue of their excellent optical properties, such as high brightness, excellent photostability, moderate size and multicolor, *etc*.[4] QDs used for biological applications are mainly made by CdSe cores coated with a ZnS layer, which possess high quantum yields, and excellent harsher conditions resistance (for example, acid, alkali and oxidation in live cells). The high-quality QDs generally use the pyrolysis of organometallic precursors in high-temperature organic solvent/ligand mixtures. Since these types of QDs with hydrophobic surface have no aqueous solubility, the QDs solubilization is the essential step for further use in biology. Likewise, the biofunctionalization of QDs is critical to facilitate biological applications of QDs. The pioneering researches on the synthesis and biofunctionalization of QDs promote the development of biolabeling, bioimaging and biosensor of QDs.[5,6] Along with the improvements of the synthesis, surface modification and bioconjugation of QDs, more and more biomolecules can be labeled with QDs. QDs are widely employed in real-time, long-term and high-sensitive SPT field, owing to the remarkable optical properties. So far, QD-based SPT technique has been successfully performed in the researches on the dynamics of cell membrane receptors, intracellular/intercellular molecules, and molecular motors.[7,8] It is worth mentioning that QDs as a kind of emerging fluorescent tags have been developed for virus labeling and shown their unique advantages in SVT field in recent years.[9,10]

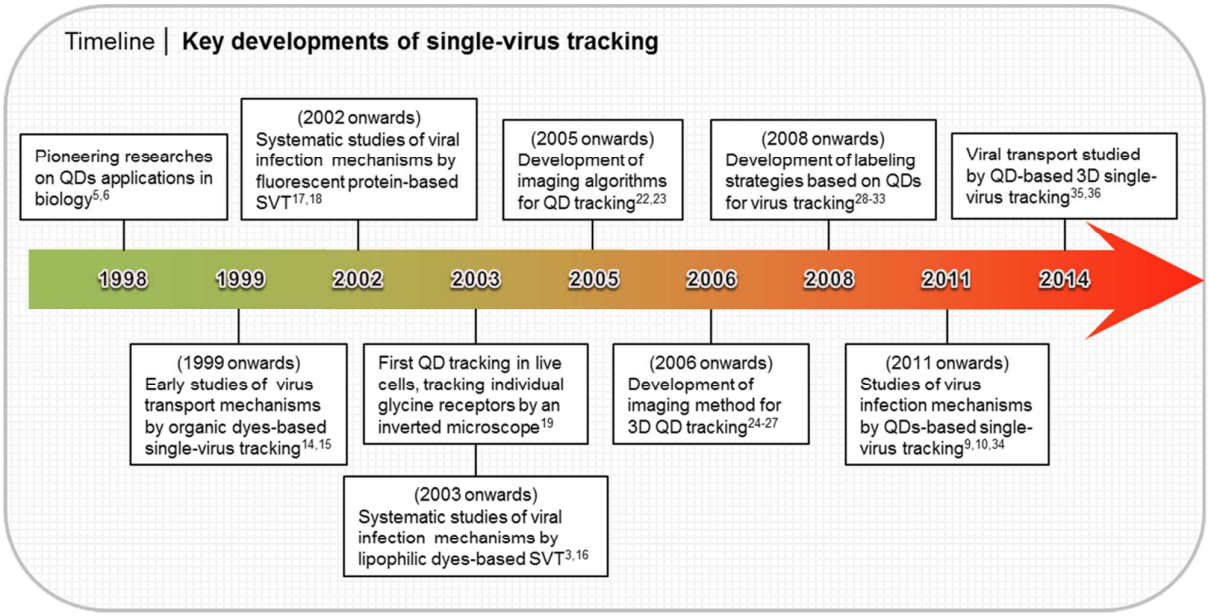
The synthesis, biofunctionalization, and other biological applications of QDs have been reviewed elsewhere.[11,12] In this review, we mainly summarize the current developments of QD-based SVT. We begin with a short overview about the emergence of QD-based SVT and give a brief discussion on the advantages of QDs for SVT technique. Next, we focus on the current state of QD-based SVT technique, including the labeling strategy, imaging instruments, image processing and trajectory analyzing methods. Subsequently, we describe the recent application of QD-based SVT in biological fields, which may shed a light on the coming researches.

2. The emergence of QD-based SVT

For tracking individual viruses in live cells, the primary task is to label the viruses with tags. Early SPT experiments widely utilized colloidal gold particles to track individual particles in live cells. Notably, Kusumi et al. established a high-temporal resolution tracking method using colloidal 40 nm gold particles, and provided a meaningful "picket fence" model for plasma membrane, which broke the traditional concept of "fluid mosaic" model for the plasma membrane proposed by Singer and Nicholson.[13] However, owing to the intrinsic properties, gold nanoparticles cannot be used for multicolor imaging and studying of the interactions between different particles. Hence, gold nanoparticles were rarely utilized to label single viruses in SVT field.

As a kind of conventional fluorescent tags, organic dyes are much smaller than the gold nanoparticles, which usually have functional groups for coupling to the ligands, and are easily obtained from commercial company. Organic dyes are utilized to track individual viruses in live cells in the early 2000s. Texas red-labeled adenovirus was tracked in live cells, and the results implied that the labeled virus engaged with two types of microtubules-dependent plus and minus end-directed motilities, which is an early research on studying the dynamic motilities of virus.[14] Subsequently, Cy5-labeled individual viruses were tracked and visualized the infection pathway of adeno-associated viruses.[15] Zhuang et al. used lipophilic dyes-labeled enveloped viruses to monitor infection behaviors, and detected the dequenching process of fluorescent signals to identify the occurrences of virus-endosome fusion events for genome release.[3,16]

Along with the development of genetic tags, fluorescent proteins (FPs) cover a broad range of colors at present, which are nearly distributed in the whole visible light region.



Especially, FPs can realize site-specific labeling of targets *in vivo* by genetic manipulation, and are also commonly used for labeling of viruses and intracellular structures. For example, by FPs labeling, human immunodeficiency virus (HIV) was tracked in host cells. The results indicated that HIV entered the cell by endocytosis and fused with endosome to remove the virus envelope. The virus genome was transported along microtubules to cell nucleus for replication.[17,18] However, organic dyes and FPs still have some drawbacks, such as poor photostability, relatively weak fluorescent signal, and broad absorption/emission profiles. For achieving decent signal-to-background (S/B) images, the excitation level should be carefully adjusted to minimize photo-bleaching. The broad absorption/emission profiles can cause the overlap of the fluorescent spectra, which leads to the difficulty of multicolor imaging. As a consequence, the drawbacks mentioned above require more strict imaging conditions, which limit their applications in long-term SVT field.

Compared with FPs and organic dyes, QDs possess unique optical properties such as high quantum yield and photostability, which have aroused great interest among biological researchers. In detail, the brightness of QDs is 10-100 times higher than FPs and organic dyes, which is benefit to improve the detection sensitivity and obtain high S/B images. The photostability of QDs is also 100-1000 times higher than FPs and organic dyes, so QDs are very suitable for long-term and high sensitive SPT. Moreover, QDs possess wide excitation spectrum and narrow emission spectrum, which can be used conveniently for multicolor tracking.[4] Dahan *et al.* first utilized QDs for tracking individual glycine receptors on the neuronal membrane, which opened a door to explore the dynamics of biomolecules in live cells by using QD-based SPT.[19] Inspired by this study, a series of researches were performed to systematically study the transport mechanisms of membrane receptors by QD-based SPT.[20,21] The emerging studies raised higher requirements of imaging algorithms in SPT field. Hence, the imaging algorithms for QDs tracking made headway on dealing with the blinking of QDs and constructing the trajectories by automatic reconstruction methods.[22,23] In addition, to achieve more accurate information in live cells, three-dimensional (3D) QDs tracking methods were developed from 2006.[24-27] Also worth noting is that the strategies for labeling viruses with QDs were developed from 2008,[28-33] and subsequently the researchers vigorously revealed the virus infection mechanisms by QD-based SVT technique.[9,10,34] Combined virus labeling strategies with 3D tracking techniques, several studies also successfully provided valuable information of virus infection behaviors in cells[35, 36] (Timeline).

3. Establishment of QD-based SVT method

Single-virus tracking uses fluorescent microscopy to monitor individual viruses in live cells, and extracts the dynamic information on virus infection. The virus must be labeled with fluorescent tags, the microscope should meet the requirements for tracking single viruses or viral components *in situ*, and the image analysis method must be powerful enough to acquire biological information about virus infection. In the following section, we will describe how to implement QD-based SVT method, including the strategies for labeling viruses with QDs, imaging instruments and image analysis.

3.1 Labeling viruses with QDs

Viruses display a diversity of shapes and structures. Nearly all viruses contain genome and capsid, and many animal viruses have lipid envelope. These are three main components of viruses. To visualize the different steps of virus infection, the virus components need to be labeled respectively. Although many researchers reported the strategies for labeling viruses with fluorescent tags, herein, we mainly expatiate on the QDs-labeling strategies used in SVT field (Fig. 1).

So far, QDs labeling strategies for biological imaging are mostly divided into four categories: (i) direct labeling protein with QDs using chemical reaction, such as click reaction and amino-carboxyl reaction; (ii) use of biotin-streptavidin reaction to bind QDs surface; (iii) labeling protein with QDs by antibody-antigen interaction; (iv) binding protein to QDs surface using peptides residues. Since antibody can block the viral functions in live cells, antibody-antigen interaction is less used for virus labeling to track QDs-labeled viruses.

3.1.1 Labeling external components

To label the external components of viruses, such as the envelopes of enveloped viruses or the capsids of non-enveloped viruses, direct chemical labeling methods can be readily used. Amino-carboxyl crosslinking reaction was utilized to label non-enveloped adeno-associated virus serotype 2 virus (AAV2) with quantum dots directly to study the infection behaviors of the virus in live cells.[34] Meanwhile, the click chemistry has been employed to label virus with QDs recently, which can directly generate productive labeled viruses.[30,31] These one-step labeling methods are readily obtained QD-virus conjugates and adapted to label other types of viruses. However, for one-step labeling, many tedious purification procedures are generally performed for biological application, such as size exclusion, ultrafiltration, and dialysis. These steps often lose a large amount of materials, waste a lot of time and energy, and influence the activity of the viruses. Moreover, conjugations obtained by using direct chemical reaction are often cross-linked and aggregated, because the functional sites of QDs are easy to bind/crosslink to the numerous protein binding sites. In addition, the QDs conjugations have very poor stability in neutral/acid buffers. The QDs complex cannot be long-term preservation, since the QDs can be unstable or precipitated below freezing and the protein can be degraded above freezing. Thus, this is inconvenient to perform a lot of parallel SVT experiments.

Biotin-streptavidin interaction is known one of the strongest non-covalent biological interaction (dissociation constant $\approx 10^{-14}$ M), which has now most often been used in diverse areas of biotechnology. The external components of viruses are easily biotinylated, which can then be interacted with

streptavidin-modified QDs (SA-QDs). Briefly, the biotin-modified viruses are exposed to the host cells and interact with receptors on the cell surface initially, and then are incubated with SA-QDs to complete the labeling process. The purification and labeling processes are finished simultaneously. In contrast to one-step labeling method, two-step labeling method has been developed to overcome the disadvantages of one-step labeling method for SVT tracking. This method can minimize the influence on the virus-cell interaction, since the viruses bind the cell surface before QDs labeling. The two-step biotin-streptavidin labeling is a very easy-implementation labeling strategy with high efficiency and commonality. The labeled viruses still possess high infectivity, which is very crucial for tracking infection behaviors *in situ*.[29]

Based on this, our group performed a series of single-virus tracking experiments for revealing various viral infection mechanisms.[9,10,36]

Peptide-based linkages use genetic cloning technique to put a small amino acids sequence into target protein as specific labeling sites. This approach possesses high binding specificity with targets in live cells. Hereinto, a 15-amino acid biotin acceptor peptide (AP) has been used to label the external components of the viruses.[28,32] The viruses can be biotinylated by the membrane incorporation of AP tags, and labeled with SA-QDs. Aptamers can recognize their ligands with high affinity and specificity, which can be constructed for labeling viruses. QDs-labeled influenza A virus was also successfully generated by the designed QDs-aptamer nanoprobe.[37]

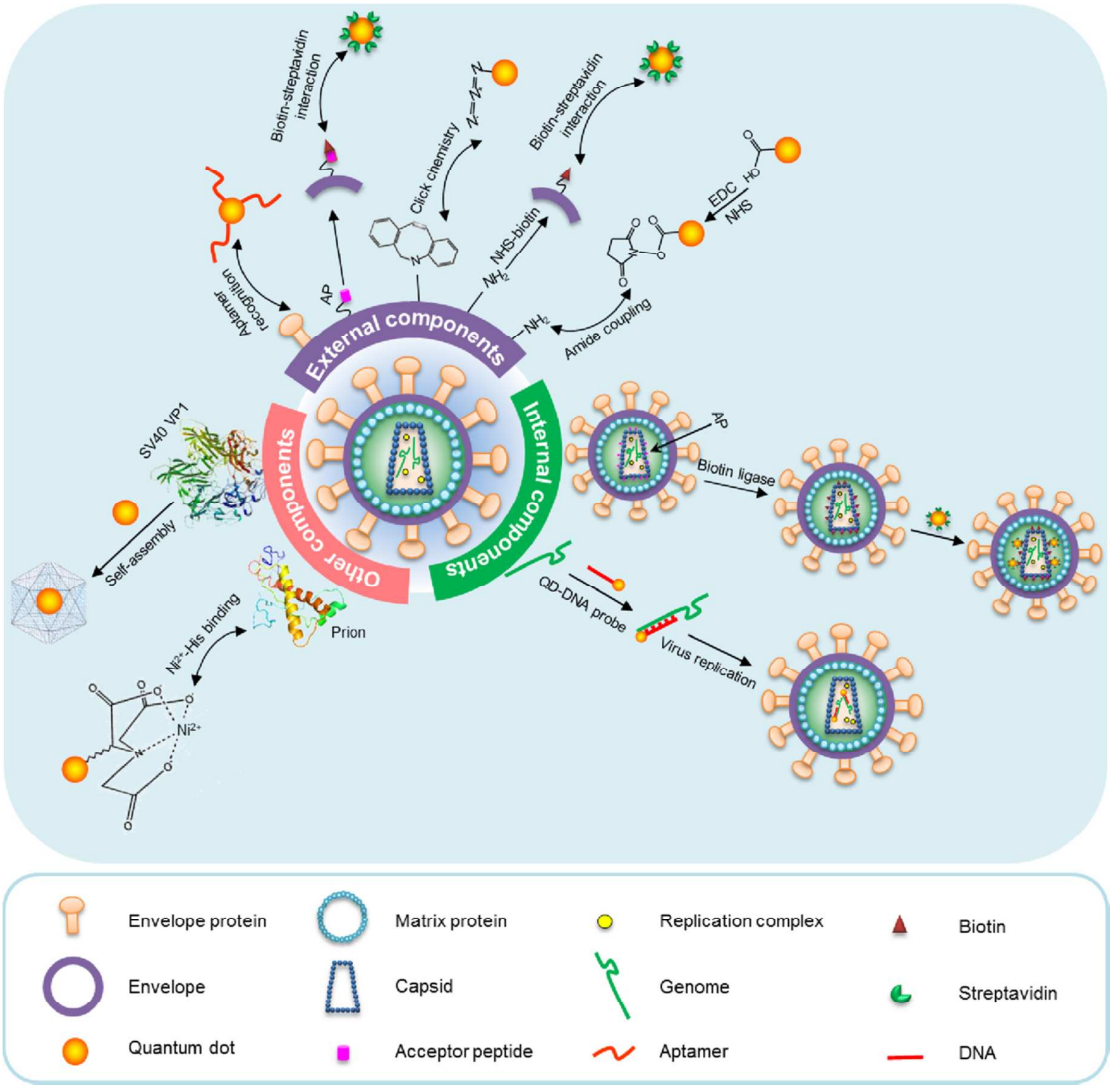


Fig. 1 The strategies for labeling various viral components with quantum dots (QDs).

3.1.2 Labeling internal components

Without modification of the viral surface, incorporation of QDs into internal components of virus showed minimal impact on the virus-cell interactions. Furthermore, it is important to label internal components for monitoring the late infection events after the loss of viral envelope. However, internal components, such as the capsid or genome of the enveloped virus, are difficult to label with QDs. So far, our group built a host-cell-assisted method to label nucleocapsid with QDs. We initially obtained the self-biotinylated nucleocapsid of enveloped baculoviruses during virus replication process in host cells, and then added SA-QDs to label the viruses.[33] By means of this labeling method, critical infection events before and after the envelope loss can be monitored in real time, which is helpful for revealing key steps of virus infection process. Zhang *et al.* utilized QDs to label modified genomic RNAs (gRNAs). During the virus assembly, QDs-conjugated modified gRNAs were successfully encapsulated into virions with gRNAs to realize the internal labeling. This packing method is very meaningful for studying the viral genome transport.[38]

3.1.3 Labeling other components

Prion is only composed of protein, and histidine (His) can provide specific binding site for divalent metal ions. Polyethyleneglycol-interspersed nitrilotriacetic acid modified QDs was employed to specifically label the His-rich prion protein, and the transport behaviors of prion were tracked in live cells.[39] There is an ingenious strategy to label the viruses with QDs through viral self-assembly, which can encapsulate QDs into the capsids of viruses. The major capsid protein VP1 of simian vacuolating virus 40 (SV40) can self-assemble into virus-like particles (VLPs), by which QDs can be encapsulated into SV40 VLPs through molecular self-assembly. The constructed QD-containing VLPs of SV40 were used to visualize the behaviors of VLPs in living cells.[40]

3.2 Instruments for QDs imaging

Usually, the most basic imaging device for SVT experiments is an epi-fluorescence microscope equipped with the detector, and the mercury lamp as excitation light source. Owing to the higher-power, narrow-band light beam, laser has been increasingly used as a light source for SVT instruments to reduce the background noise of images. To collect as much as possible excited fluorescent signals, the microscope always equips the objective with high numerical aperture. For real-time visualization, the imaging instrument also should have high-sensitivity and high-speed detector to acquire high S/B images. The low brightness and poor photostability of traditional FPs and organic dyes need researchers to use appropriate excitation intensity and short exposure time on the premise that the target signals can be acquired with high S/B images for long-term tracking. Owing to the excellent optical properties, QDs can lower the requirement of imaging equipment and are very convenient to obtain high S/B images. At present, QDs have been broadly exploited into various imaging methods, which can be mainly categorized into total internal reflection imaging (TIRF), confocal imaging, 3D imaging, and superresolution imaging (Fig. 2).

3.2.1 Total internal reflection imaging

As the basic imaging instrument, epi-fluorescence microscope has large excitation depth, and is easily interfered by non-focal signals. The acquired images often have very low S/B. Total internal reflection technique is still a wide-field imaging technique based on epi-fluorescent microscope, which uses evanescent wave generated by total internal reflection to excite fluorescent samples (Fig. 2A). This method can be used to observe the sample surface in only a few hundred nanometers. Therefore, the excitation depth and volume can be controlled to reduce the interference of the fluorescence from non-focal plane. This technique can obtain excellent S/B images, which is a powerful tool to visualize the membrane dynamics of biomolecules in the contact zone of the basement membrane.

3.2.2 Confocal imaging

Confocal microscope was developed in the 1980s as a new generation of imaging instrument. The basic principle of confocal microscopy is that lighting pinhole and detection pinhole are conjugated according to the focal plane, by which the out-of-focus fluorescent signals can be eliminated. Compared with epi-fluorescence microscope, confocal microscope possesses shallow depth of field and high resolution. Presently, there are two types of confocal microscopes: laser-scanning confocal microscope and spinning-disk confocal microscope (Fig. 2B). Laser scanning confocal microscope is using the scanning head to scan the sample in point-by-point format, which possesses the lateral resolution of 100-200 nm, and the vertical resolution of 500 nm. Thus, the images obtained by this microscope have very high S/B. However, the point-by-point scanning format limits the imaging speed, and cannot meet the requirement of studying the dynamic events. Spinning-disk confocal microscope is developed on the basis of laser-scanning confocal microscope. This type of confocal microscope is in surface scanning format, similar to that of the wide-field microscope. According to the basic principle of confocal microscopy, the microscope can realize simultaneous excitation of more points on the focal plane, and then utilize the charge coupled device (CCD) to collect the fluorescence signals, which greatly improves the imaging speed and increases the utilization of the excitation light. This instrument is very suitable for studying transport mechanisms of biomolecules in live cells.

3.2.3 Three-dimensional imaging

3D single-particle tracking technique can monitor the accurate spatio-temporal behaviors of the particles, which is playing an increasingly important role to explore the biological events in live cells. At present, due to the excellent photostability and brightness of QDs, many microscopies have been developed for 3D SPT based on QDs labeling, which can be mainly divided into two categories. One approach is to analyze the acquired 2D images for 3D tracking, and the other is a real-time feedback method.

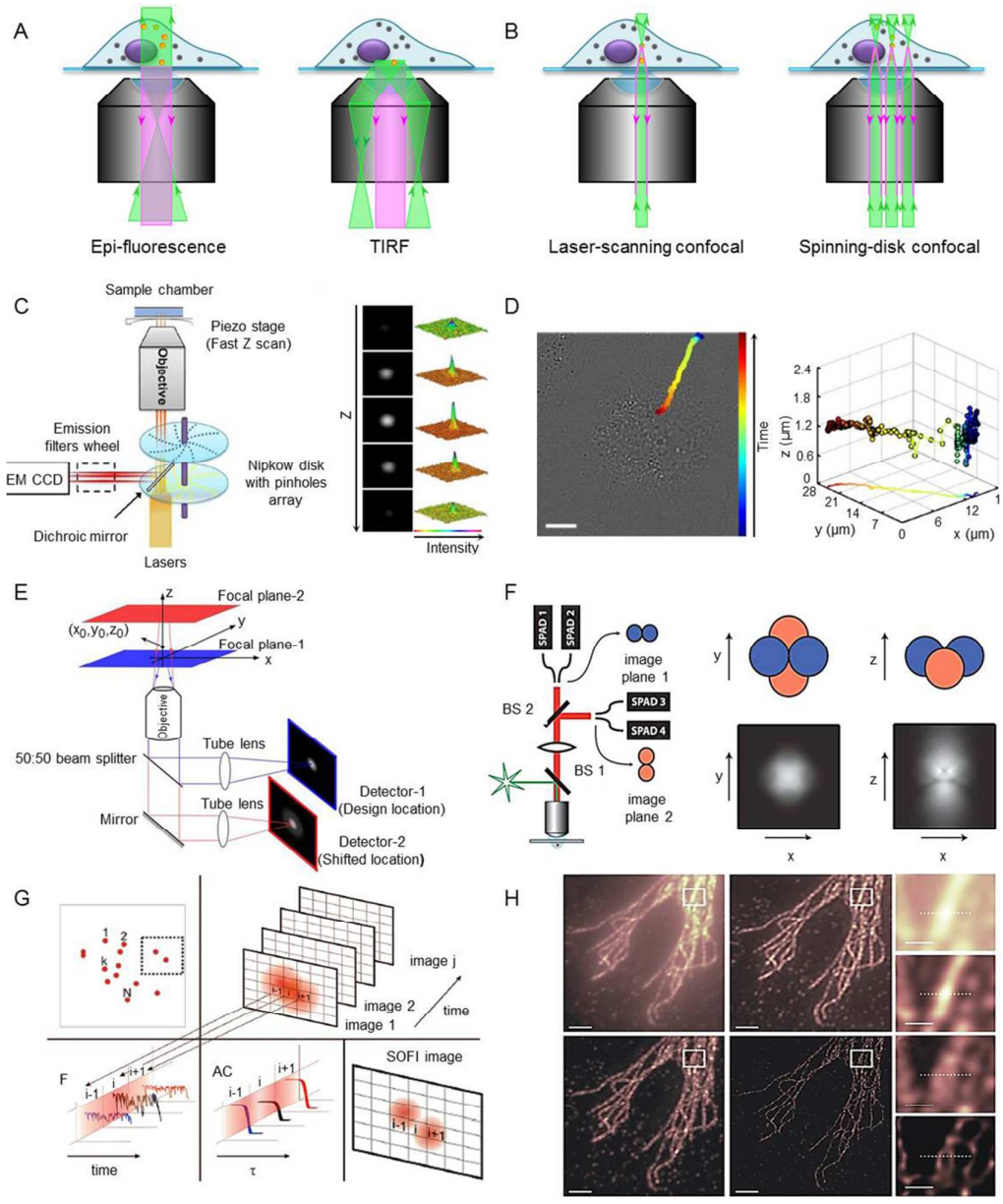


Fig. 2 Fluorescence microscopies for QDs imaging. (A) Epi-fluorescence microscopy (left) and total internal reflection fluorescence (TIRF) microscopy (right). (B) Laser-scanning confocal microscopy (left) and spinning-disk confocal microscopy (right). (C) Fast Z-scanning microscopy for three-dimensional tracking (left). The 2D images were acquired from the top to the bottom of the particle (right). (D) 2D trajectory of an influenza virus labeled with QDs in the host cell (left) and 3D trajectory of the virus (right). The colorbar indicates a time axis from 0 s to 250 s. Reproduced with permission from ref.35. Copyright 2014 WILEY-VCH Verlag GmbH & Co. KGaA. (E) Multifocal plane microscopy for 3D tracking. Reproduced with permission from ref. 26. Copyright 2008 Elsevier Ltd. (F) Feedback system for 3D tracking. Reproduced with permission from ref. 25. Copyright 2007 AIP Publishing LLC. (G) Superresolution optical fluctuation imaging (SOFI) method. (H) SOFI images of QDs-labeled 3T3 cells. Reproduced with permission from ref. 41. Copyright 2009 National Academy of Sciences.

The first-type techniques generally utilize optical modifications on standard microscope to acquire the 3D imaging. Hereinto, the spinning-disk confocal microscope combined with fast piezo z-scan device has been widely applied in 3D SPT field, which can get a series of z-stack 2D images of particles. Recently, we applied this method to tracking QD-labeled influenza virus in host cells (Fig. 2C and 2D).[35] Meanwhile, we developed a fast and high-efficiency single-particle localization algorithm for z-stack 3D tracking.[27] This method utilized the scaling of a 3D image in the axial direction and calculated the radial symmetry center of the scaled image to obtain the particle position, which is especially suited for 3D high-accuracy and high-speed SPT experiments and 3D single-particle imaging. To overcome the limitation of z-stack 3D tracking on time resolution, one or multiple 2D imaging methods have been developed to retrieve the z position based on the off-focal images or astigmatism images (Fig. 2E).[26,42] However, these methods also have the limitation on z spatial resolution.

The second type of 3D tracking methods determines the particle positions in real time by feedback system (Fig. 2F).[25] The focus is displaced to keep the particle in the center of the

focus. Therefore, as long as the particle is in the trackable area of the feedback system, the particle can be tracked with a very good temporal resolution. However, this method is unsuitable for tracking the particles within crowd environments.

3.2.4 Superresolution imaging

There are many subcellular structures with the size smaller than the diffraction limit, such as actin filaments, microtubules and transport vesicles. Thus, a new technique is urgently required to image the structures below the diffraction limit for biological research. Superresolution imaging is a new research field of microscopy developed in recent years, which can break the diffraction barrier and analyze the biological structure at the high-resolution level. There are two distinct conceptual strategies to realize superresolution imaging. One strategy is based on nonlinear optical approaches, such as stimulated emission depletion (STED) fluorescence microscopy and structured illumination microscopy (SSIM). The other strategy is as probe-based resolution, which generally uses photoswitchable, photoactivated or photoconverted molecules to resolve dense populations of molecules, and reconstructs a superresolution image by localizing individual points, such as photoactivated localization microscopy (PALM), stochastic optical reconstruction microscopy (STORM), and fluorescence photoactivated localization microscopy (FPALM). The principal criterion for this type is that fluorescent probes can be used to image individual signals of molecules. Owing to the blinking property, QDs have been used in superresolution imaging field. For instance, Lidke *et al.* exploited the blinking property of individual QDs to separate each emitter, and then acquired a high-resolution image reconstructed by individual localized points.[43] Dertinger *et al.* presented a 3D superresolution optical fluctuation imaging method (SOFI), which is based on higher-order statistical analysis of QDs blinking recorded in a sequence of images.[41] Meanwhile, Irvine et al. found that manganese (Mn)-doped ZnSe QDs can be switched on and off by using a continuous-wave modulation laser, indicating that this type of QDs may be useful for superresolution imaging, similar as organic photoswicher.[44]

3.3 Image analysis for QD-based SVT

The images recorded by microscope contain large amount of information. In detail, there are many methods of image processing and data analyzing to accurately track individual particles in live cells, including reducing the image noise, detecting the particle position, reconstructing the trajectory and analyzing the trajectory. Manual tracking is a general method used to extract image information. However, the eyes only can recognize the distinct movements of particles, such as mobility or immobility. A little information can be extracted from manual analyzing, and this method is time-consuming, low-flux, and strong-subjectivity. Thus, a set of corresponding analysis methods is required for high-accurate and high-flux extracting of the detail information. Automatic tracking method can get the movement information of nearly all particles from thousands of images. This is very convenient to statistically analyze the speed and diffusion coefficient of particle movement. There are mainly two steps for automatically tracking: 1) recognize the position of each particle in each image; 2) link the positions of each particle in series of images. The first step is particle detection and the second step is trajectory reconstruction. Next, we introduce how to perform the particle detection, trajectory reconstruction and trajectory analysis (Fig. 3).

3.3.1 Particle detection

The first step of getting the particle trajectory is to accurately localize the particle position. Under optical microscope, the resolution is limited by the diffraction limit, which is ~250 nm in lateral direction and ~500 nm in axial direction. Thus, the particle center should be localized by special method to meet the requirement of SPT experiment.

Centroid method is a simplest and effective method to estimate the particle position. The formula of this method is shown in Equation 1.

$$x_f = \frac{\sum_{ij} x_{ij} I_{ij}}{\sum_{ij} I_{ij}} \quad (1)$$

Where, x_{ij} is the position of the pixel (x_{ij}, y_{ij}) in x direction, I_{ij} is the intensity at the positon (x_{ij}, y_{ij}). The y positions are calculated analogously.

Compared with other localization method, this method has the advantage of simple formulation, easy programing and quick calculating without iterative, numerical fitting steps. It can be used to localize the big or asymmetric particles, without considering the distribution model of the light spot. However, centroid method has low localization accuracy due to the interference of background noise.

Since the intensity distribution of single particle is similar to a two-dimensional Gaussian distribution, researchers have developed Gaussian fitting method for single-particle localization (Equation 2). This method can localize the particle position with very high localization accuracy (in several nanometers), which can overcome the diffraction limit of the microscope on the low spatial resolution. Meanwhile, this method also has the several disadvantages, such as low calculating speed, tedious steps, and shape limitation of target particles.

$$I(\mathrm{x},\mathrm{y}) = B + A\ exp\left[-\frac{(x-x_f)^2}{2S_x^2} - \frac{(y-y_f)^2}{2S_y^2}\right] \quad (2)$$

Where (x_f, y_f) is the particle position by fitting, A and B are the amplitude and offset, S_x and S_y is the width of point spread function in x and y directions.

Parthasarathy developed a non-iterative fast single-particle localization method based on radial symmetry, which has very high accuracy, similar to Gaussian fitting. This method can be used for super-resolution imaging.[45]

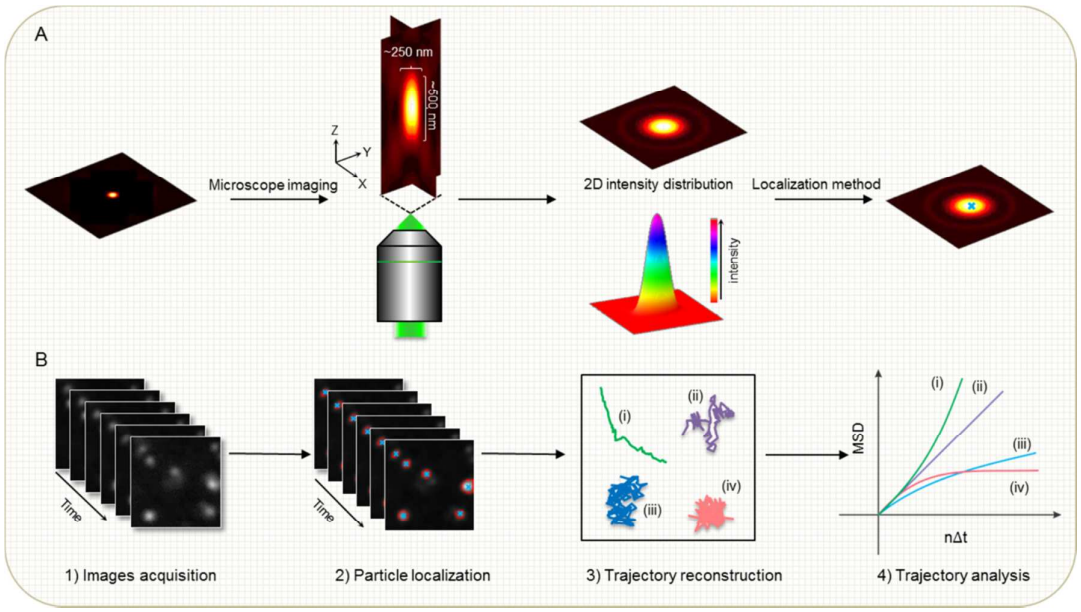


Fig. 3 Image processing for single-virus tracking. (A) Schematic diagram of particles detection. For one bright particle, the image can be acquired by fluorescence microscope, which has an ellipsoid shape with ~250 nm in lateral direction, and ~500 nm in axial direction. By 2D imaging, the intensity of particle is much like 2D Gaussian function. Localization methods are utilized to get the accurate position of the particle. (B) Four steps of image processing for single-virus tracking. 1) Recording the particle movements by microscope in a series of images. 2) Detecting the particle positions in each frame. According to localization algorithms, the accurate particle positions can be acquired. 3) Reconstructing the particle trajectories in the images. 4) Analyzing the trajectories of the particles. According the relationship between MSD and nΔt, the particle movements can be divided into four types. (i) Directed motion with diffusion. (ii) Normal diffusion. (iii) Anomalous diffusion. (iv) Corralled diffusion.

3.3.2 Trajectory reconstruction

After particle positions are localized accurately in each frame, the particle trajectory should be reconstructed by linking the positions of the same particle in different frames. However, the movements of labeled particles are very complicated in live cells, such as particle motion heterogeneity, particle merging, temporary particle disappearance (for example, QDs blinking), and particle splitting. Even though many these movements can be decreased under low particle concentration, the researchers always need to use the tracking methods to reconstruct the trajectories of particles. For huge amount of images, it is very important to find an automatic tracking method for reconstructing trajectories. Automatic reconstructing methods commonly use the size, shape, and intensity of particles to detect and link the positions of the same particle in different frames. Bonneau *et al.* and Bachir *et al.* developed the algorithms to deal with QDs blinking.[22,23] Thereinto, Jaqaman *et al.* built a robust single-particle tracking algorithm to solve the above problem, which is a highly applicable and versatile to study the dynamics of biomolecules.[46]

3.3.3 Trajectory analysis

For trajectories analysis, the main goal is to obtain a series of motion parameters quantitatively, which can provide adequate information on particle transport mechanism. The relationship between mean square displacement (MSD) and time interval (nΔt) is usually defined to specify the types of the particle motions, and further obtain the diffusion coefficient and fitting speed of the particle movement. In this part, we mainly introduce how to calculate MSD, and analyze the relationship between MSD and nΔt.

The MSD of the two-dimensional particle trajectory can be calculated by Equation 3.

$$MSD(n\Delta t) = \frac{1}{N-n}\sum_{i=1}^{N-n}\{[x(i\Delta t + n\Delta t) - x(i\Delta t)]^2 + [y(i\Delta t + n\Delta t) - y(i\Delta t)]^2\} \quad (3)$$

Where ($x(i\Delta t+n\Delta t)$, $y(i\Delta t+n\Delta t)$) is the particle position at the time point of nΔt. N is the total frames of the particle trajectory. Δt is the acquisition time of each frame. n and i are integers.

The relationship of MSD and nΔt can be analyzed according the following analytical forms for different motional modes (Equation 4-7).

(i) Directed motion with diffusion (Cytoskeleton-related motion)

$$MSD(n\Delta t) = 4Dn\Delta t + (Vn\Delta t)^2 \quad (4)$$

(ii) Normal diffusion (Brownian motion)

$$MSD(n\Delta t) = 4Dn\Delta t \quad (5)$$

(iii) Anomalous diffusion (Motion blocked with potential energy traps)

$$MSD(n\Delta t) = 4Dn\Delta t^{\alpha} \quad (6)$$

(iv) Corralled diffusion (Motion confined in limited area)

$$MSD(n\Delta t) = \frac{L^2}{3}\left(1 - exp\left(\frac{12Dn\Delta t}{L^2}\right)\right) \quad (7)$$

Where D is the diffusion coefficient, α is anomalous diffusion exponent (α<1), and V is the average speed obtained by fitting. L^2 is the confined area of the motion.[47]

4. Tracking virus infection using QDs

Virus infection includes many steps and has different pathways for virus replication (Fig. 4).[1] Virus entry is the first step for virus infection, and many viruses exploit endocytic pathways for entry. The initial interaction between viruses and host cells is the viruses binding to attachment factors or receptors, by which actives the downstream signaling pathway, and leads to the internalization of viruses by endocytosis. Next, the viruses are transported to the specific regions for genome release, which is essential for the replication of viral components. After viral replication, the virus assemblies occur during the virus egress process. At last, the new viruses exit the cell and release into the extracellular environments.

Early researchers mainly used transmission electron microscope (TEM) and biological experiments to investigate the virus infection mechanisms in host cells. The virus infection process cannot be visualized *in situ* in live cells. Thus, there are many remaining questions about virus infection, such as by which way the virus is transported dynamically into cells, and how the virus interacts with cellular components.

QD-labeled virus can be used to visualize the infection process of viral infection. To investigate the interactions between viruses and cellular structures, the researchers need to image fluorescent-labeled cellular structures and QDs-labeled viruses simultaneously. FPs can realize the specific

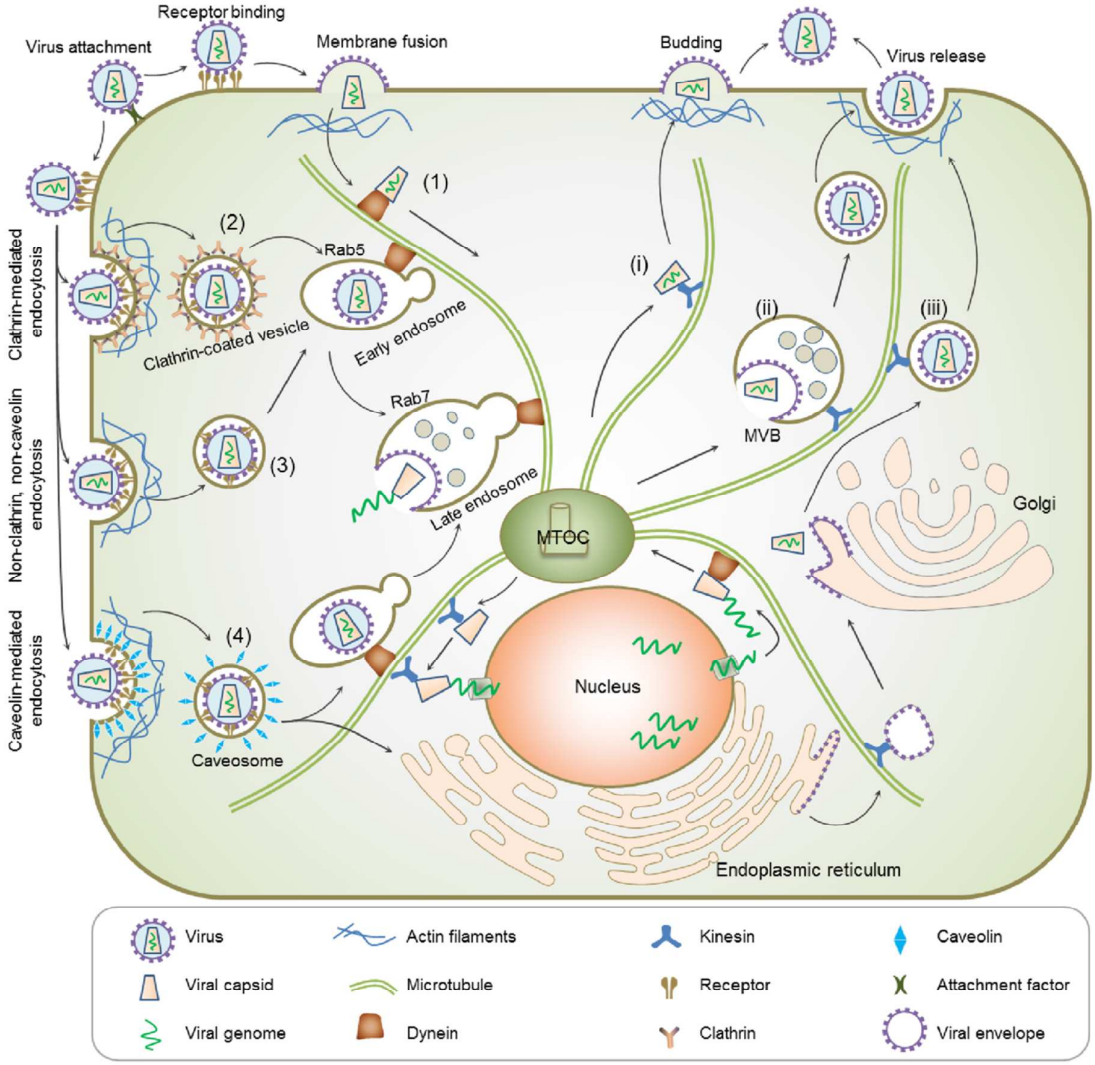


Fig. 4 Virus infection process in live cells, including virus entry, virus transport, virus assembly and virus egress. Viruses attach the cell surface and bind to the receptors. Viruses can fuse with the plasma membrane directly to release viral internal components into the cell (1). Viruses also can hijack the clathrin-mediated endocytosis (2), caveolin-mediated endocytosis (4) and non-clathrin, non-caveolin endocytosis (3) to enter the cell. Next, the viruses are transported long the microtubules by dynein to the microtubule organizing center (MTOC). The virus can fuse with the late endosomes to release the capsid or genome, and the capsids can also be transported by kinesin to release the genome into the replication site of nucleus. When virus components have been replicated, the viral genome is transported to the MTOC and packaged into the capsid. The capsid can be transported along microtubules to the cell periphery, then assemble and bud at plasma membrane (i). The capsid also can be enveloped in multi-vesicular body (MVB) (ii) or Golgi (iii) to form the complete virus. The virus can be transported to the plasma membrane to exit the cell.

labeling of the cellular structures (*i.e.* clathrin-coated vesicle, caveosome, endosome, cytoskeletons, *etc.*) by genetic engineering, which is a kind of excellent tags to label the specific cellular structures. Colocalization analyzing and multicolor tracking of the virus and cellular structures are two general approaches to investigate the interactions between viruses and host cells. For exploring the dynamics of genome release events, multicolor labeling and tracking of virus components is essential to monitor the viral disassembly process. So far, although QDs have already been used to label the viral internal components, QDs often need combine with organic dyes or FPs to realize the multicolor labeling of virus for tracking. For virus assembly and egress, it is still a challenge to monitor by QD-based SVT. Along with the development of labeling strategy of QDs, we expect this technique to become a valuable tool to study the dynamic process of the viral assembly and egress *in situ* for long term. Below we elaborate the representative studies in detail to show how to use the QD-based SVT experimentally and what information can be obtained by the SVT measurements.

4.1 Virus entry

Making clear of virus entry pathway is not only meaningful to augment our understanding of the pathogenic mechanism of virus in host cell, but also helpful to administer antiviral treatment and prevention of viral diseases.

By colocalization analysis of the endocytic structures and QDs-labeled viruses, it is revealed that QD-containing simian virus 40 (SV40) entered the host cell by caveolin-dependent endocytosis, and aggregated in the endoplasmic reticulum *via* microtubules. This was the first study using encapsulated QDs to investigate the virus pathways in live cells during the infection process.[40] QD-labeled vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped lentiviruses infected the

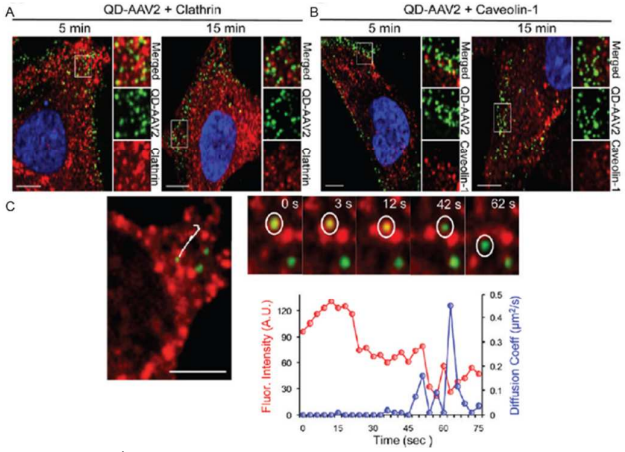


Fig. 5 Clathrin/caveolin-dependent entry of adeno-associated virus serotype 2 (AAV2). (A). Colocalization of clathrin and QD-labeled AAV2. (B) Colocalization of caveolin and QD-labeled AAV2. (C) Real-time monitoring of QD-labeled AAV2 internalization through the clathrin-coated vesicle. Reproduced with permission from ref. 34. Copyright 2011 American Chemical Society.

host cell *via* non-clathrin- and non-caveolin pathway, while QDs-labeled VSVG-pseudotyped retrovirus utilized the clathrin-dependent endocytic pathway to enter the cells.[28] The results indicated that the entry pathway of viruses is not only determined by viral envelope.

Real-time simultaneous monitoring of QD-labeled viruses and fluorescent endocytic structures revealed that most of QDs-labeled infectious hematopoietic necrosis viruses (IHNV) were internalized into the cytosol through *de novo* formation of clathrin-coated vesicles (CCVs). Time trajectories of viral velocity and clathrin intensity suggested that IHNV initially did not colocalize with clathrin, and CCVs formed roughly in 2 min after virus binding, and then the virus entered into the CCVs.[10] Joo *et al*. studied the entry mechanisms of adeno-associated virus serotype 2 (AAV2) by QDs labeling. They found that the virus may enter by clathrin and caveolin-dependent endocytosis by colocalization analysis. Next, QD-labeled AAV2 was tracked in the cells transfected with mRFP-clathrin. The virus signals colocalized with clathrin signals for 45 s and the clathrin signals disappeared rapidly, indicating that the virus was dissociated from the uncoated clathrin vesicles (Fig. 5).[34] These are the pioneering works using QD-based SVT to visualize the dynamics of virus entry, which provide the valuable information on studying the dynamics of virus infection.

4.2 Virus transport

After virus entry, the viruses need to release their genomes to specific sites for replication. Actin filaments and microtubules, as the major cytoskeletons of cells, are actively responsible for the cargos transport in the cytosol. Many viruses hijack the cellular transport systems to arrive the replication sites. Monitoring the dynamic behaviors of viruses and analyzing the motion parameters provide more accurate information on virus transport, and further elucidate the virus transport mechanisms.

Many viruses generally infect one host cell simultaneously, how to guarantee that the tracked virus can be infectious, and whether the virus behavior can reflect the whole features of this kind of viruses are the primary questions. Therefore, it is essential to track the maximum possible number of viruses simultaneously in one cell. In our lab, we used QD-labeled influenza viruses to monitor the global infection behaviors, and found that the viruses initially moved slowly around the plasma membrane, followed by a rapid movement towards nucleus, and finally converged to the perinuclear region. The entire infection process of individual viruses in live cells can be divided into five stages, which related to actin filaments, microtubules, and late endosomes (Fig. 6).[9] This is the first study on global infection behaviors of virus in single cells, which provided dynamic and mechanistic insights about population infection behaviors of influenza virus.

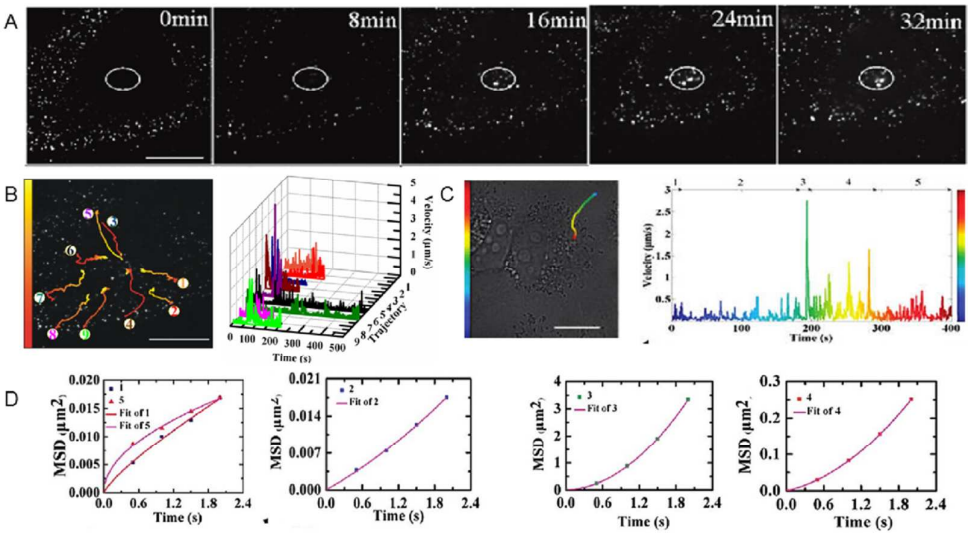


Fig. 6 Tracking infection process of influenza virus by QD-based single-particle tracking technique. (A) Snapshots of virus infection process in a single cell. The circles indicate the converging area of the virus in the cell. (B) Nine trajectories of the virus in the cell and the velocity and time plots of the nine viruses. (C) Typical trajectory of the virus in live cells, and the velocity and time plot of the virus. The colored bar indicates a time axis from 0 s (blue) to 400 s (red). Numbers 1, 2, 3, 4, 5 indicates the duration of different stages (Scale bar, 20 μm). (D) Mean square displacement-time plots in different stages. Reproduced with permission from ref. 9. Copyright 2012 American Chemical Society.

Further, the detailed transport behaviors of QD-labeled influenza viruses along microtubules were visualized dynamically and globally. This research revealed that the intersection configuration of microtubules can interfere with the transport of virus in live cells, which led to the changing and long-time pausing of the virus movements.[48] Meanwhile, we utilized QD-based 3D SPT to explore the Rab5 and Rab7-associated infection behaviors of influenza virus. The results indicated that the transition process of the virus-containing vesicles from early to late endosomes occurs during the intermittent movement in the perinuclear region. This revealed the distinct dynamic behaviors of Rab5 and Rab7-positive endosomes in the course of the intracellular transport of virus.[35] The host specificity of the influenza virus was extremely concerned for studying the cross-species transmission of influenza virus. The difference in sialic acid receptor-binding specificity for human and avian influenza viruses can cause the host specificity of the influenza virus partly. By using SVT, we investigated the infection behaviors of avian influenza virus in human bronchial epithelial cells, and found that this infection behavior exhibited a high degree of consistency with the sialic acid receptors, which can provide meaningful information on the cross-species transmission of avian influenza virus.[36] Additionally, for other types of viruses, QD-based SVT also revealed that the transport process of IHNV was cytoskeleton-dependent and low-pH-dependent forinfection in the cytosol.[10] QD-labeled AAV2 was trafficked through the early, late, and recycling endosomes through cytoskeletons.[34] QD-containing pseudotyped lentivirus (PTLV) transported to the microtubule organizing center through. Rab5-positive endosome *via* microtubules.[38] All the studies mentioned above clearly highlight the importance of QD-based SVT in the field of virus researches.

5. Conclusions and perspectives

In this tutorial review, we have discussed the implementation of QD-based SVT technique, including QD labeling strategy, instrumentation, and image analysis method related to SVT. Meanwhile, we have summarized the biological applications of QD-based SVT in biological field.

As mentioned above, SVT technique is a favorable tool to investigate the real-time, *in-situ*, and long-term dynamics of viruses in live cells. Although SVT technique has been widely used for biological researches, there is still a question that how to choose optimal fluorescent tags for SVT experiments. Organic dyes and FPs with small size are well-studied so far. Owing to easy operation, organic dyes used to label the external and internal components of viruses for tracking. Particularly, the lipophilic dyes-labeled enveloped viruses were monitored to detect the occurrences of virus-endosome fusion events for genome release by dequenching fluorescent signals of viruses. Meanwhile, FPs can realize the site-specific labeling of viral and cellular structures, which are essential to study the mechanisms of virus infection and the interactions between viruses and cellular structures. Due to low brightness and poor photostability, organic dyes and FPs require a sufficient number of fluorescent molecules to label individual viruses for single-virus detection, which may influence virus infectivity in live cells, and are not suitable for long-term SVT. QDs can overcome the limitation to easily realize the long-term, 3D tracking. To date, QDs-based SVT has commonly employed in studying virus entry, virus transport pathway and, by extension, the general viral infection mechanisms.

However, this emerging technique also has several issues that need to be ironed out. For instance, the size of viruses are in the range of about 10~300 nm. For labeling the smaller

viruses, the size of QDs (~10 nm) is a bit large. The large size may influence the infection route and dynamics of viruses in crowed environments. Also, the blinking property leads to the difficulty of single-QD tracking in live cells. Thus, QDs with nonblinking and small size prosperities is the major need for QDs engineering in recent years, which can facilitate effective and efficient labeling of the viruses. Especially, this can provide new opportunities for QD-based SVT in live cells. Meanwhile, although growing evidences indicated that the QDs labeling strategies have minimal influence on the biological characteristics of the labeled viruses and do not affect the viral infectivity. Compared with unlabeled wild-type viruses, the 'real' behaviors of the labeled viruses still need to be cognized. Besides, since viral infection process involves the interactions between viral and cellular constituents, site-specific QDs labeling was considered with the minimal disturbance of virus-host interactions. However, it remains unclear to what extent QDs-labeled viruses affect the interaction of viral and cellular constituents. Additionally, it is optimal to track one virus labeled with one QD. In this case, the influence of QDs on virus transport can be minimized. One protein labeled with one QD is easy to achieve as reported.[7] However, the viruses have complex structures, which contain a variety of proteins. The specific-site labeling performed by genetic engineering can modify one type of proteins to link QDs, but still cannot label one virus with one QD, because many of the same modified proteins exist in one virus. Thus, one virus labeled with one QD is still a challenge so far.

To overcome these drawbacks, the new-type QDs should be synthesized with novel methods and materials. New types of non-blinking QDs nanoparticles were generated recently.[49] In our group, we have made great efforts on synthesizing a series of new-type near-infrared fluorescent nanoparticles with low toxicity[50] and developing new methods of biosynthesis.[51]

Additionally, the interactions between viruses and biomolecules always occur in the nanometer scale, which is unable to investigate by the conventional microscopy. Super-resolution microscopy has shown the powerful ability to image the ultra-structural information of biological structures. However, these methods often need the fixed cell samples or long time to do imaging. To track the dynamics of biological events in real time and in live cells, the techniques still need further development for super-resolution SVT.

Finally, the key superiority of this technique is that it can provide differentiated information of individual viruses, but it comes with cumbersome imaging process and massive data, especially for 3D and super-resolution imaging. Thus, besides the improvement of QDs (e.g. small size, low toxicity) and labeling strategies, the imaging methods and data analysis approach should be taken more attentions. The following question is how to optimize data processing approach for meeting high-throughput analysis of data. It could be of great importance to establish the operation procedure for standardized experiments and easy-to-use data analysis software for researchers.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (No. 21535005), the National Basic Research Program of China (973 Program, No. 2011CB933600), the 111 Project (111-2-10), and Collaborative Innovation Center for Chemistry and Molecular Medicine.

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Table of Contents

We describe the implementation of quantum dot-based single-virus tracking, and show how to use this technique to acquire meaningful information.