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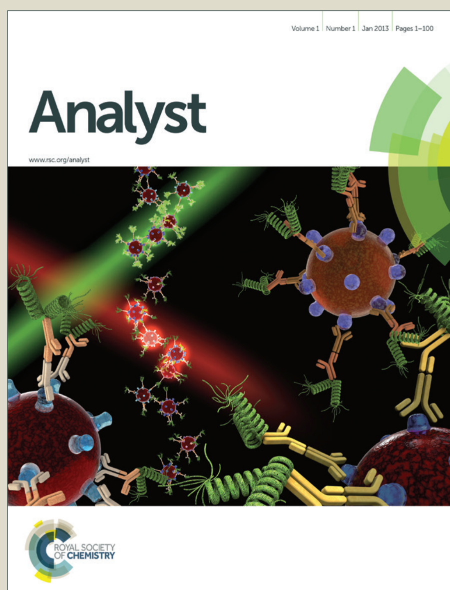

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CRITICAL REVIEW

Progress on the labeling and single particle tracking technologies of viruses

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Understanding and unravel the invasion mechanisms of virus infection is of high importance for preventing and treating viral diseases. Single virion tracking is a powerful way for exploring the mechanisms of viral infection. To successfully tracking, the virus and cellular structures of interest must be fluorescently labeled; the microscope imaging technology must be sufficiently powerful for real-time single virion or viral component tracking. All fields of scientists have made great efforts and improvements. Here we will review recent advances in virus labeling and the emerging fluorescence imaging technologies used in viruses imaging and tracking.

1. Introduction

Viruses are of great threats to human health. Viruses cause many serious diseases such as AIDS, avian influenza and SARS. A large number of infectious diseases are due to virus infection. Viruses vary tremendously in their shape, size and composition. But they are generally composed of three parts for enveloped viruses or two parts for non-enveloped viruses (except prion). No matter what kind of viruses, they all have a small genome, made up of single-stranded or double-stranded nucleic acid—either DNA or RNA. For nonenveloped viruses, the genome is wrapped in a protein coat, which is called capsid. For enveloped viruses, the capsid is further enclosed by an envelope, which is a lipid membrane acquired from the host cell membrane system in the process of budding. Viruses are generally considered to be fully parasitical and must enter host cells to replicate themselves. Viral infection is a complex and accurate process, in which, a virion interacts with various cellular structures and takes advantage of different cellular environments to replicate themselves. In general, the infection starts with the virus binding to the cell surface through specific receptors or attachment factors. Then some viruses inject their genomes directly into the cell by breaking the plasma membrane barrier,[1-2] and more viruses release their genomes after they have entered the cells.[3-10] After the replication of the viral genome and the synthesis of the viral proteins, the reassembly of these components produces progeny virions, which are finally released. Understanding the process and mechanisms of virus infection is crucial for preventing and curing viral diseases. But each kind of virus infects and replicates in a different way, which makes it a challenge to draw definite conclusions about the mechanisms of virus infection. Moreover, conventional *in vitro* static methods commonly used for virus infection study, such as transmission electron microscope (TEM), fluorescence imaging at discontinuous time and other cell or molecular biology technologies, are difficult in achieving detail and successive information about virus infection.[7] Much critical information is missed, and even some results are not accurate because both the interaction and cellular structures are complex and dynamic. For example, the pathway by which PV (poliovirus) enters cells settles a long-lasting debate. Early studies suggested that PV enters cells via clathrin-mediated endocytosis,[11-12] but more-recent studies have demonstrated that PV enters the cell by a clathrin-, caveolin-, flotillin-, and microtubule-independent, but tyrosine kinase- and actin-dependent, endocytic mechanism.[7] In the past 10 years, the development of new fluorescence labeling methods combined with the emerging dynamic or super-high resolution imaging technologies have greatly promoted the study for accurately elucidating the mechanisms of virus infection. By following the fate of individual virions or monitor the dynamic assembly process of viral and subviral particles, a great deal of detailed information which is previously unobservable can be revealed. In this paper, we review recent efforts and progress for virus labeling and the emerging fluorescence imaging technologies used in viruses imaging and tracking.

2. Labeling technologies for viruses

Fluorescent labeling of virions is critical for virus imaging and tracking. In general, an ideal virus labeling method, first of all, must be mild and reliable enough to ensure viral infectivity. Secondly, it should provide strong and photostable enough signal for single-particle and long-term tracking. In addition, to track different steps of infection process, it is necessary to respectively label different parts of the virus, such as the capsid, envelope and genome. For these purposes, both ideal fluorescence reagents and feasible labeling methods are needed. There are three kinds of fluorescence reagents are used so far in virus labeling: organic fluorescence small molecules, fluorescent proteins and quantum dots. There are also mainly three strategies for virus labeling:

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genetic engineering labeling, chemical and physicochemical labeling, and virus assembly labeling.

2.1 Progress of genetic engineering labeling methods

In theory, the genetic engineering labeling is thought to be the most efficient and useful labeling strategy for living-cell applications. It can label virus at a well-defined position with good reproducibility by fusing fluorescence proteins (FPs) to certain domain of virus proteins, no matter they are inside or outside the virion.[13-22] Now, it is feasible to respectively fuse several FPs with different colours to different viral proteins in the same virion,[18-21] which is useful in tracking the infection and assembly processes. Besides, viruses can be labeled with FPs by decorating modified green fluorescence protein (GFP) forms to their surfaces, which is realized by fusing GFP to either glycosylphosphatidylinositol (GPI)-anchor acceptor sequences[23-26] or viral membrane targeting sequences.[13, 16, 19] FPs can also be used to label viral nucleic acid if it includes sequence motifs specifically recognized by RNA binding proteins.[27-28] The signal is strong enough for single-virion tracking if sufficient fusion-protein is expressed. However, it is well known, this method has great challenge in the complex processes, technical barriers and even the inactivation of labeled virus.[28] Moreover, a significant fraction of FPs may be non-fluorescent due to misfolding of the protein, immature chromophores, or self-quench, which depresses the fluorescence of labeled virions, especially for those viruses with small size.[29] In addition, the big size of FPs (~27 kDa) results in reduced replication efficiency of the labeled virus under most conditions. An alternative of genetically fusion proteins is the small tetracysteine-tag (TC-tag) which comprises only 6–12 amino acids and can be labeled by biarsenical dyes,[30-32] or the SNAP-Tag, a 20 kDa variant of the human O(6)-alkylguanosyl-transferase which undergoes covalent self-labeling with various benzylguanine derivatives.[33] Both of them have been employed to generate labeled viruses. But these derivatives have not been commonly used in live-cell labeling because of the technology limit.

2.2 Progress of chemical and physicochemical labeling methods.

Organic fluorescence small molecules are traditional reagents for fluorescence labeling. Two labeling strategies are usually used: physical incorporation and bioconjugate chemistry. The former incorporates lipophilic fluorescence reagents, such as DiD, DiI, DiO, DIR, DIA, Rhodamine-18 and analogues, into lipid membrane structured envelope.[6, 10, 16, 34-39] The later links the fluorescence reagents with reactive groups, such as amino-reactive cynine or Alexa dyes, with viruses through covalent reactions,[7, 35, 39-43] both the specificity and efficiency of the reactions are not high.[5, 7] With the development of bioorthogonal chemistry in recent years, Wang's group modified the cowpea mosaic virus with azadibenzylcyclooctyne, which was subsequently used to label the virus with azidocoumarin dye through strain-promoted azide-alkyne cycloaddition reaction. The reaction is highly specific and biocompatible. Moreover, the fluorescent signal appears only upon the linkage between the virus and the dye, so unconjugated dye contributes little to the fluorescence and no false positive signal is observed .[44]

Organic fluorescence small molecules are also used in labeling the internal components of virions. But for intact virions, it is difficult to label internal viral components, typically nucleic acid, for they are inaccessible to dye attachment once incorporated into the virion. Moreover, bioconjugate chemistry strategies hardly can be used for nucleic acid labeling in living viruses because chemical modification usually influence the template function of nucleic acid. It is found that nucleic acid intercalating dyes can attach and release in turn during viral assembly and replication processes.[7, 45-46] Exploiting this property; Rosenke et al. reported a metabolic labeling strategy to incorporate bromodeoxyuridine into the DNA of human cytomegalovirus during the virus replication process which is 24-48 hrs in general.[47] Brandenburg et al. developed this strategy for RNA virus labeling and found only Syto82 works well although more than 20 RNA-binding dyes were screened.[7] Then, Liu et al. optimized this method to label the RNA of purified influenza virus at room temperature in 2 hrs (Fig 1). The viral envelope and genome were labeled with QDs and Syto 82, respectively. Using two-channel confocal microscopy, the viral envelope and RNA were imaged simultaneously (Fig. 1A). It was observed that nearly all the viruses with Syto 82 signals exhibited QDs signals. The line profile of the cell showed that the overlapped intensity peaks of QDs signals and Syto 82 signals, indicating that the genome was high-efficiently labeled with Syto 82 (Fig. 1B). The tMr (the percentage of red signals colocalized with green signals in the thresholded images), tMg (the percentage of green signals colocalized with red signals in the thresholded images) and intensity correlation quotient (ICQ) values further suggested that nearly all the viruses could be dual labeled with QDs and Syto 82 (Fig. 1C). In addition, the labeling process had less influence on the virus infectivity (Fig. 1D).[48]

Fig. 1 Dual labeling of viruses with QDs and Syto 82. (A) DIC and confocal fluorescence images of 540 nm QDs-labeled viruses, Syto 82-labeled viruses and the merge. (Scale bar: 20 mm). (B) Line profile indicating the distribution of QDs (green) and Syto 82 (red) signals on the line shown in the merge image. (C) Histograms of tMr, tMg and ICQ values. (D) Titers of H9N2, biotinylated H9N2 (Biotin-H9N2), QDs-labeled H9N2 (QDs-H9N2) and QDs and Syto 82-labeled H9N2 (Dual-labeled H9N2) replication in MDCK cells. The titers were determined by immunofluorescence focus assay. Asterisks indicate a statistically significant change in infectivity compared to H9N2 (*$P < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Reproduced with permission from ref. 48; Copyright 2012, Elsevier.

But the dyes used in these studies are easy to photo-bleach. To overcome this shortage, we succeed in using a molecular "light switches" of nucleic acids, $[Ru(phen)_2(dppz)]^{2+}$, to label the DNA of vaccinia virus through the metabolic labeling strategy. The fluorescence of labeled virions was stable and strong enough for long-term single-virion tracking.[46]

All the methods based on organic fluorescence small molecules require hundreds to thousands of dye molecules to attach to one virion in order to provide strong signal for tracking single-particle and distinguishing from the background fluorescence. However, the diameters of small viruses limit the numbers of fluorescent molecules that can attach to a virion; and for relatively large viruses, so many dye molecules are probable to weaken viral

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infectivity. The semiconductor nanocrystals, quantum dots (QDs) are of single-particle visible fluorescence strength and super long-time fluorescence stability.[49-53] The successful application of QDs in biology labeling and imaging provides another alternative for virus labeling and single virion tracking.

Labeling viruses with QDs is usually realized by bioconjugate chemistry[54-55] or bio-affinity interaction,[46, 56-61] despite electro-static attraction is also used occasionally.[62-63] The initial work uses the carboxylates on the QDs to react with amino on viral surface by using the 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) as cross-linkers.[54] The bio-affinity interaction labeling is generally based on the interaction between biotin modified virus and streptavidin modified QDs. The virus can be modified with biotin by either directly interacting with carboxyl activated biotin molecules[46, 59-60] or incorporating a short acceptor peptide (AP) tag which is susceptible to site-specific biotinylate the envelope or capsid of virus.[57-58] The former has no site specificity because all amino reactive molecules on the viral surface can react with carboxyl activated biotin. The later can label virus at specific site by expressing AP-tag on certain molecules. Though the methods based on bio-affinity interaction labeling are highly efficient, conjugating the QDs with streptavidin will remarkably increase the size of QDs, which might block the recognition site and change the movement behaviours of viruses to some extent, especially for small-sized viruses. We reported a new method for labeling enveloped viruses with QDs through copper-free click chemistry.[55] The QDs are modified with azide-polyethylene glycol or dibenzocyclooctynes-polyethylene glycol, which hardly increases their sizes. The viruses can be specifically and rapidly labeled under mild conditions. The labeling efficiency is high and the infectivity of viruses is scarcely affected.

For virus labeling, the most preferable methods should be those that can modify and label fully duplicative viruses through natural biosynthesis and metabolic processes. Taking advantage of the host cells deriving formation mechanism of virus envelope, Huang et al. reported a labeling strategy for enveloped virus by first metabolically incorporating biotin-functionalized phosphatidyl-ethanolamine (Biotin-Cap-PE) into cells and then naturally incorporating the Biotin-Cap-PE into enveloped viruses during virus *in-situ* assembling process. The obtained virus can be labeled with streptavidin modified QDs. This strategy is simple in technique and reliable.[61] Then we developed an alternative method for labeling fully duplicative enveloped viruses, in which both the biosynthesis and metabolic incorporation of phospholipids in host cells were simultaneously utilized to introduce an azide group to the envelope of the vaccinia virus, which could be subsequently used to label the envelope of the virus *via* the copper-free click chemistry. Furthermore, simultaneous dual labeling of the virus through the virus replication was realized by coupling this envelope labeling strategy with metabolic labeling strategy of viral nucleic acid. It is the first time that the virus was simultaneously dual-labeled in natural propagation process (Fig 2).[64]

Fig. 2 (1) Schematic illustration. (A) The chemical formulas of choline (Cho) and azide-Cho (AECho and APCho). (B) Biosynthesis of azide-Cho-containing phospholipids and its labeling by strain-promoted azide-alkyne cycloaddition using DBCO-Fluor 525. (C) The vaccinia virus (VACV) propagation in the presence of both azide-Cho and $[Ru(phen)_2(dppz)]^{2+}$ in the host cell. The biosynthesis and incorporation of azide-Cho-containing phospholipids in host cells were carried out at first (□). Then the cells were infected with VACV, and the $[Ru(phen)_2(dppz)]^{2+}$ was added to the medium at 2 h postinfection, which could enter the cells through the permeable cytomembrane resulting from cytopathogenic effects of the virus to label the nucleic acid of the virions (□). At 24 h postinfection, DBCO-Fluor 525 was added to the medium to label the envelope of the VACV (□). After another 24 h infection, the dual-labeled virions were finally assembled and released. Reproduced with permission from ref. 64; Copyright 2013, American Chemical Society.

(2) Fluorescence imaging of the dual-labeled VACV. The virions captured onto the glass slides were treated with Tris buffer solution (TBS) (a−c), TBS added with PLC and Ca^{2+} (d−f) or TBS added with PLC and EDTA (g−i). The DBCO-Fluor 525 was excited using a 514 nm laser, emitting 550−570 nm fluorescence. $[Ru(phen)_2(dppz)]^{2+}$ was excited with UV and the fluorescence was collectedin the wavelength range of 600−650 nm. Reproduced with permission from ref. 64; Copyright 2013, American Chemical Society.

2.3 Progress of virus labeling based on assembly

It is well known, the viral capsid proteins of nonenveloped viruses can self-assemble to form the capsid-like structure. By taking advantage of this, the virus assembly labeling methods are developed. In which, the fluorescence reagents are incorporated into the capsids of virus by partial or total replacement of viral nucleic acid. Different from normal labeling methods; virus-like particles (VPs) instead of intact virions are produced, which can be used to track the interaction between virus and host cells despite they cannot replicate. The incorporation is realized by either genetic engineering fusion or physical package strategy.[65-69] Early in 2004 year, Gilbert et al. proved the possibility of assembling fluorescent VPs from EGFP fused viral protein VP2 of canine parvovirus (CPV). The corresponding capsid-like complexes were very similar in size with authentic CPV. They could be utilized as a visualization tool to elucidate events related to CPV infection.[65] Minten et al. mixed the EGFP-capsid protein complex with wild-type capsid protein of cowpea chlorotic cottle cirus (CCMV) to produce EGFP-loaded capsids. The EGFPs are located inside the 28 nm diameter capsids with the incorporated number depending on the ratio of EGFP-capsid protein complex to pure capsid protein.[66] Yoo found that the VPs, formed by the self-assembly of hepatitis B virus (HBV) capsid protein fused with DsRed or EGFP, showed significant fluorescence amplification and much higher photostability, which is helpful for long-time optical imaging.[67] Organic fluorescence dyes are also used in virus assembly labeling. For example, the poly(styrene sulfonate) polymers with Rhodamine B were packaged into CCMV to form VPs.[68] Loo et al. directly packaged positive and neutral charged dyes within the red clover necrotic mosaic virus capsid and found 1000 dye molecules could be loaded into one virus capsid.[69] In general, the VPs' formation depends on the size of the virus, the structure and charge of the capsid interior, the ratio of the capsid proteins to the fluorescence reagents, and so on. QDs are of similar size with that of viral cavity. Besides, the structure and charge on their surfaces can be turned by modification. So it is possible to incorporate QDs into VPs and

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form QD-based VPs. This is a smart alternative to label viruses with QDs because labeling virus with QDs on the surface is faced with some problems, such as the influence on viral infectivity, the alteration of virus infection mechanism for the biological effects of QDs, etc.. The QDs with different surface structures and properties have been incorporated into viruses through different assembly mechanisms, and the viral infection process mimics that of wild viruses.[70-74] By coupling with genetic technologies, it is already feasible to assemble QDs in fixed point (Fig 3).[73]

Fig. 3 (1) Working principle of encapsulating SA-QDs into HIV-1-based lentivirus in living cells. In the HIV-1-based lentiviral system, the packaging plasmids express HIV-1 structure proteins Gag, Pol, and envelope protein VSV-G, and the transfer vectors generating gRNAs containing a packaging signal are co-transfected into packaging cells. gRNAs are encapsulated into the viral capsid through the interaction between Psi and Gag protein. Generated PTLVs acquire the envelope by budding from the infected cells (shown in the left part). When the transfer vectors are replaced with SA-QD-labeled gRNAs (shown in the right part), the QDs are encapsulated into the viral capsid together with the gRNAs to generate PTLV-QD. PTLV = pseudotyped lentivirus, LTR = long terminal repeat, Psi = packaging signal, gRNA = genomic RNA, SA-QD = QD625-streptavidin conjugate. Reproduced with permission from ref. 73; Copyright 2013, American Chemical Society.

(2) Purification and characterization of PTLV-QD. (a) Image of the SDGC tube after the separation of PTLV-QD under a UV lamp. (b) Image of fluorescent fractions F1, F2, and F6 under a UV lamp after further purification to remove sucrose. (c) Content of p24 in F1-F7 analyzed by Western bloting. (d) Fluorescence spectra of PTLV- QD compared with QDs. (e) DLS distributionversus D_h of PTLV-QD. (f-k) TEM images of negative staining reagent (f), SA-QD (g), PTLV encapsulating one QD particle (h, i), two QD particles (j), and wild-type PTLV (k). All samples were stained for 10 s (scale bar: 50 nm). Reproduced with permission from ref. 73; Copyright 2013, American Chemical Society.

3. Progress of virus imaging and tracking technologies

Wide-field microscopy is the most straightforward, sensitive and fastest imaging method for fluorescently labeled viruses. By combined with other imaging techniques, such as fluorescence recovery after photobleaching (FRAP), fluorescence loss in photobleaching (FLIP) or total-internal reflection fluorescence (TIRF), it can already be used to gain dynamic information of virus infection.[22, 75-80] But the strong photo-toxicity and the limited resolution restrict its general application in single-particle or long-term virion imaging. Fast developing imaging technologies offer increasing ways for real-time tracking of virus invasion and assembly.

3.1 Spinning Disc Confocal Microscopy used in single virion tracking

The confocal microscope is a powerful technology for biology imaging because it's high resolution and multi-dimension imaging ability. It uses point illumination and a pinhole to eliminate out-of-focus signal. As only light produced by fluorescence very close to the focal plane can be detected, the image's optical resolution is much better than that of wide-field microscopes. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity, so long exposure is required. As only one point in the sample is imaged at a time, two-dimension (2D) or three-dimension (3D) imaging requires scanning over a long time. Spinning-disk confocal microscopes use a series of moving pinholes on a disc to scan spot of light. It is capable of providing high acquisition speeds with acceptable contrast and minimal photo bleaching at the low light levels. To perform multicolour imaging with good signal-to-noise ratio, highly sensitive electron-multiplying charge-coupled device (EMCCD) was selected as the ideal detector, it is rapidly emerging as useful technique for dynamic investigation in living cells with high temporal and spatial resolution and sensitivity.[81-84] Over the last few years, spinning-disk confocal microscopes have increasingly been used to study the dynamic interactions between viruses and cells in all levers and are expected to become even more indispensable in the future. It has been used in studying the entry pathways of different kinds of viruses into living cells;[3, 5, 10, 43, 54, 60] in tracking the dynamics of virus assembly and release;[15, 20, 85-86] in tracking the dynamic behaviours of viral proteins and the interaction of viral proteins with host factors.[23, 41, 57] For example, the assembly of HIV-1 at the dorsal plasma membrane[84] and the assembly of murine leukemia virus in the direction of cell-cell contacts[87] were observed by using spinning-disk confocal microscopes in combination with very sensitive EMCCD cameras.

In the multidimensional temporal and spatial imaging, a great deal of trajectory information of particles can be obtained. How to exactly extract the information to elucidate the real movement and mechanism of virions or subviral particles is of great importance. This is a challenge because of the really complex movements of virions. The virions can assemble or appear, signals from two virions can merge or split, or virions can disappear from the observation plane. Hence, the verification of trajectories by visual inspection is imperative. The automated tracking softwares, such as Image-Pro Plus (IPP), Imaris Track, Andor IQ, etc., are needed to locate individual particles in each frame or channel and map the movement of each particle between consecutive frames. Based on the coordinates and intensities determined for each particle for all consecutive time points, its intracellular localization, co-localization with cellular organelles or structures as well as changes in fluorescence intensities indicative of dissociation or association processes can be analyzed. In addition, the 2D or 3D instantaneous velocity of the particle and the type of motional behaviour (diffusion, corralled diffusion or transport) can be determined by a mean-squared-displacement (MSD) analysis providing detailed insight into the mechanisms and dynamics of cell-virus interactions (Fig 4).[35, 43, 54, 59, 83]

Fig. 4 Analyzing the transport of individual influenza viruses in live cells. (a) Trajectory of a virus inside a cell. (b) Time trajectory of the instantaneous speed of the virus shown in (a). (c) Typical trajectory of a virus inside a nocodazole-treated cell. (d) Time trajectory of the instantaneous speed of the virus shown in (c). Numbers 1, 2, 3, 4, and 5 are the durations of stage 1, stage 2, stage 3, stage 4, and stage 5, respectively (scale bar, 20 μm). The color of the trajectory with the colored bar indicates a time axis from 0 s (blue) to 400 s (red). (e) Mean square displacement (MSD)-time plots in stage 1 (dark blue

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symbols) and stage 5 (plum symbols). The red and purple lines are the fits to MSD= $4D\tau^{\alpha}$+ constant (the constant term was due to noise and $\alpha < 1$) in stage 1 and stage 5, respectively. (f-h) MSD-time plots in stage 2 (blue symbols), stage 3 (green symbols), and stage 4 (red symbols). The purple lines are the fits to MSD = $4D\tau + (V\tau)^2$ + constant (the constant term was due to noise). Reproduced with permission from ref. 59; Copyright 2012, American Chemical Society.

3.2 Photoactivated localization microscopy and stochastic optical reconstruction microscopy used in virus imaging

In conventional wild-field fluorescence microscopy and confocal microscopy, the resolving power is limited to be approximately half the wavelength of the emitted light because of the diffraction of light. In general, the lateral (x–y) resolution is limited to 180-250 nm and the axial (z) resolution to 500-800 nm. This is a serious limitation for viruses and subviral structures imaging, as the dimension of most virions is smaller than the diffraction limit of light microscopy. During the last few years, advances in super-resolution fluorescence microscopy overcame the limits due to diffraction and increase the maximal resolution to a theoretical limit of 10 nm, which is approaching that of electron microscopy. It combines increased spatial resolution with the advantages of fluorescence microscopy and presents the opportunity to bridge the gap between conventional light microscopy and electron microscopy. The current available super-resolution fluorescence microscopic methods are stimulated emision depletion (STED),[87-91] reversible saturable optical fluorescence transitions (RESOLFT),[92-95] three-dimensional structured illumination microscopy (3D-SIM),[96-97] stochastic optical reconstruction microscopy (STORM)[98-100] and photo-activated localization microscopy (PALM),[101-102] etc..

STED microscopy is the most important hardware-based technique.[103] On the basis of confocal fluorescence microscopy, it employs two lasers that concurrently scan the sample: one pulse excites the fluorophores and the second beam de-excites the fluorophores, except for those in a zero-intensity point in the center of the doughnut, thereby decreasing the size of the excited area. No special probes are needed for STED; thus samples prepared for regular fluorescence microscopy are fully compatible with STED microscopy. Jakub Chojnacki et al. visualized the viral envelope (Env) glycoprotein distribution on the surface of individual HIV-1 particles with STED, and revealed maturation-induced clustering of Env proteins that depended on the Gag-interacting Env tail (Fig 5).[91] At present, the main defects of STED imaging are complex optical circuits, expensive equipment and high stability required system.

Fig. 5 HIV-1 Env distribution visualized by STED microscopy. Purified HIV-1 particles, containing EGFP. Vpr (green), were stained for Env (orange). Images were acquired using the STED setup with the EGFP. Vpr signal recorded in the standard confocal mode to define the localization of particles. Scale bars: 200 nm. (A) Env signal of mature particles acquired in the confocal microscope mode. (B) The same field of view as in (A), with the Env signal acquired in STED mode. (C) Control Env(-) particles with the Env signal acquired in STED mode. (D) Env signa l of immature particles acquired in STED mode. (E) Schematic illustration of the mature and immature HIV particles structures. Reproduced with permission from ref. 91; Copyright 2012, American Association for the Advancement of Science.

PALM and STORM microscopy belong to software-based methods. These techniques rely on the controlled activation and sampling of sparse subsets of photoconvertable fluorescent molecules. The xy position of individual fluorophores is determined with high precision by Gaussian fitting of the diffraction limited signals. The calculated localizations from all individual frames are computationally assembled into a final image.[103] Both these methods are based on the same principles, but were originally published using different photoswitchable probes. The development of PALM was largely prompted by the discovery of new species which displaying a controllable photochromism, such as Photo-activatable GFP. And the STORM originally made use of paired cyanine dyes. Although substantial improvement in imaging speed is needed before cellular processes can be imaged using these methods in real time, the virus imaging and tracking is sure to benefit from the advances in super-resolution microscopy since it provides the possibility to investigate viral assembly and movement in unprecedented detail. By using super-resolution microscopy imaging, Lelek et al. imaged the integrase enzyme (IN) of HIV in cells with 30 nm resolution and characterized the distribution of IN within virions and intracellular complexes to distinguish different HIV structural populations based on their morphology;[102] Pereira et al. visualized the changes in the molecular distribution of viral proteins with a lateral resolution of 15-20 nm and quantified the 2-D molecular distribution of the major structural proteins of infectious HIV-1 before and after infection of lymphoid cells;[104] Gabor revealed that caveolin-1 was required for spatial organization of CRFB1 and subsequent antiviral signalling in zebrafish.[105] Because the super-resolution fluorescence microscopy applies conventional fluorophores and fluorescence labeling technologies, they can also be used in multicorlor labeling imaging. Lehmann et al. labeled HIV-1 proteins and tetherin, respectively. Multicolor super-resolution microscopy revealed important structural features of individual HIV-1 virions, virus assembly sites and their interaction with tetherin at the plasma membrane.[106] By employing dual-color super-resolution microscopy, Muranyi et al. revealed specific recruitment of HIV-1 envelope proteins to viral assembly sites dependent on the envelope C-terminal tail.[107] Despite much work has already been done, relative progress is still suffering serious restrictions. Further efforts are needed to integrate the super-resolution microscopy with other technologies, such as multi-dimensioned imaging, long-time live cell imaging, quantitative analysis and statistic technology, so that it can be intensively used in virus imaging and tracking.

4. Conclusions and perspective

Single-particle tracking of virus has become one of the powerful ways for studying viral infection mechanisms and virus related diseases. It relies on the feasible fluorescence labeling methods for viruses and efficient fluorescence imaging technologies. It is already capable of simultaneously labeling both envelope and nucleic acid in natural propagation process now. In the future, developing those methods that can realize multiplex labeling in natural and mild conditions, and fully keep the viral infectivity is needed. At the same time, it is needed to develop super-resolution fluorescence microscopic methods to be applicable in living-cell

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and long-term imaging, so that it is feasible to track viruses in single-particle or subviral scale. And then, more detail information about the infection and pathogenic mechanisms of viruses are hopefully available, which will be of great help for viruses related diseases study and treatment.

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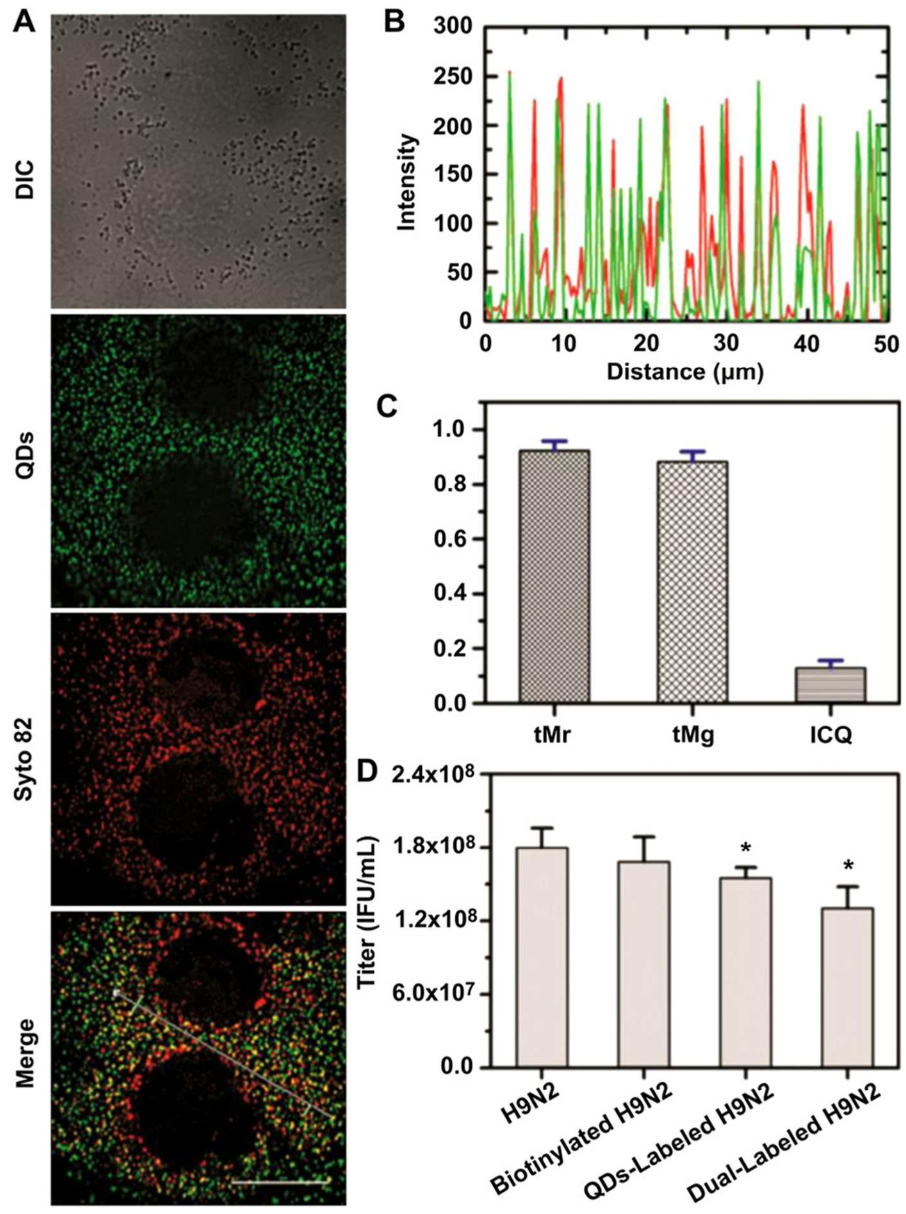

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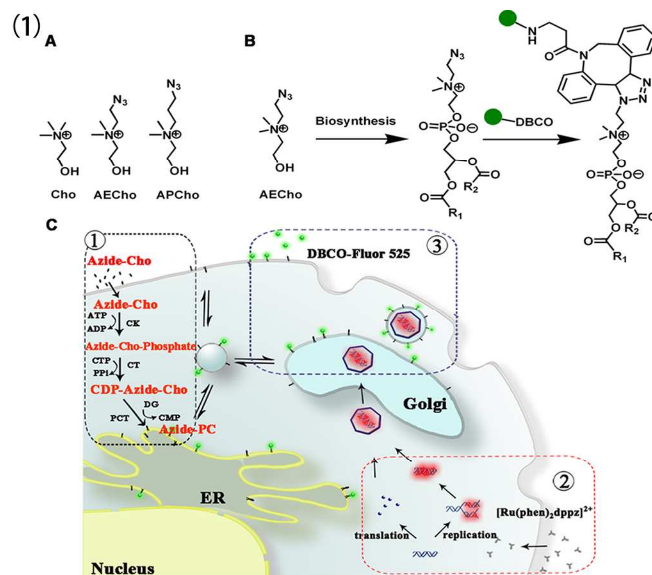

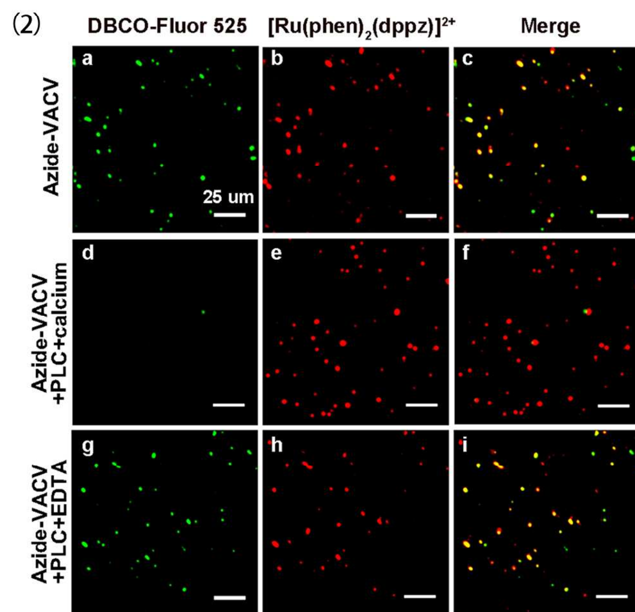

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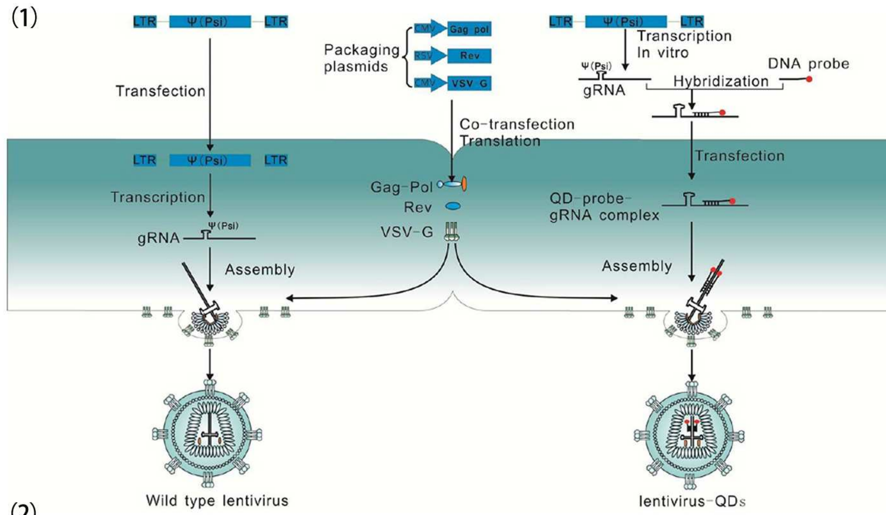

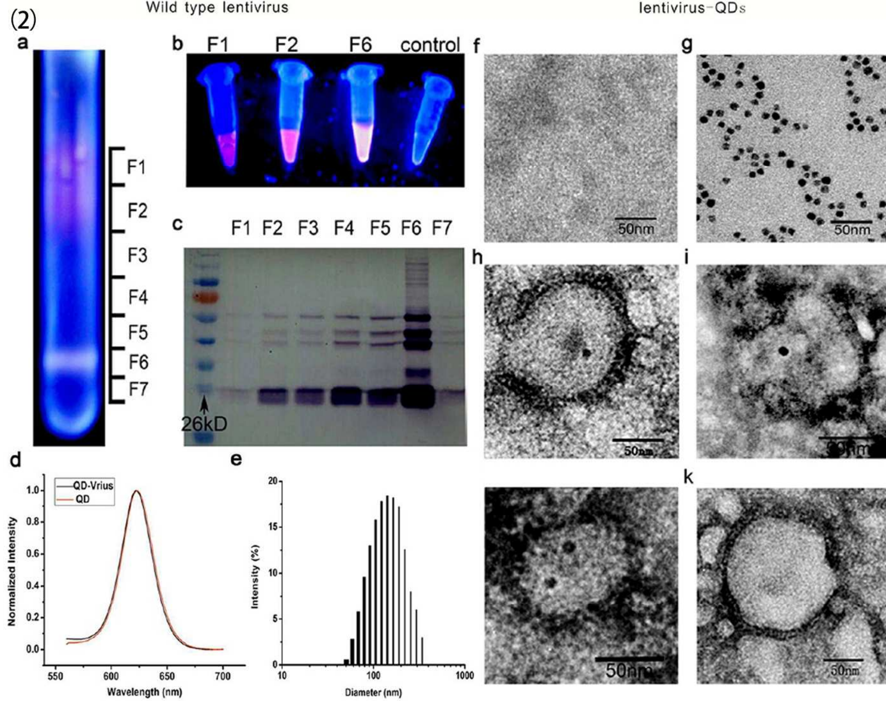

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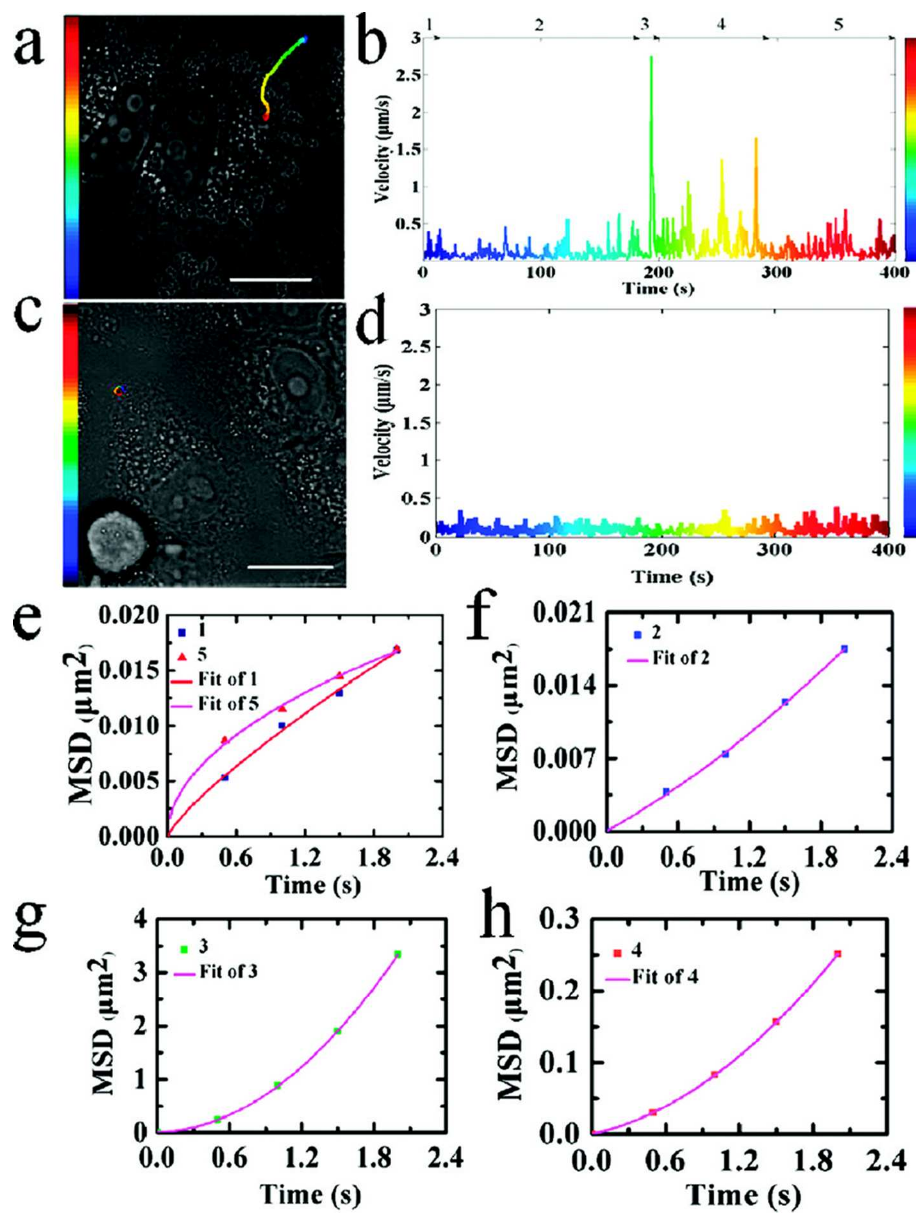

109x145mm (300 x 300 DPI)

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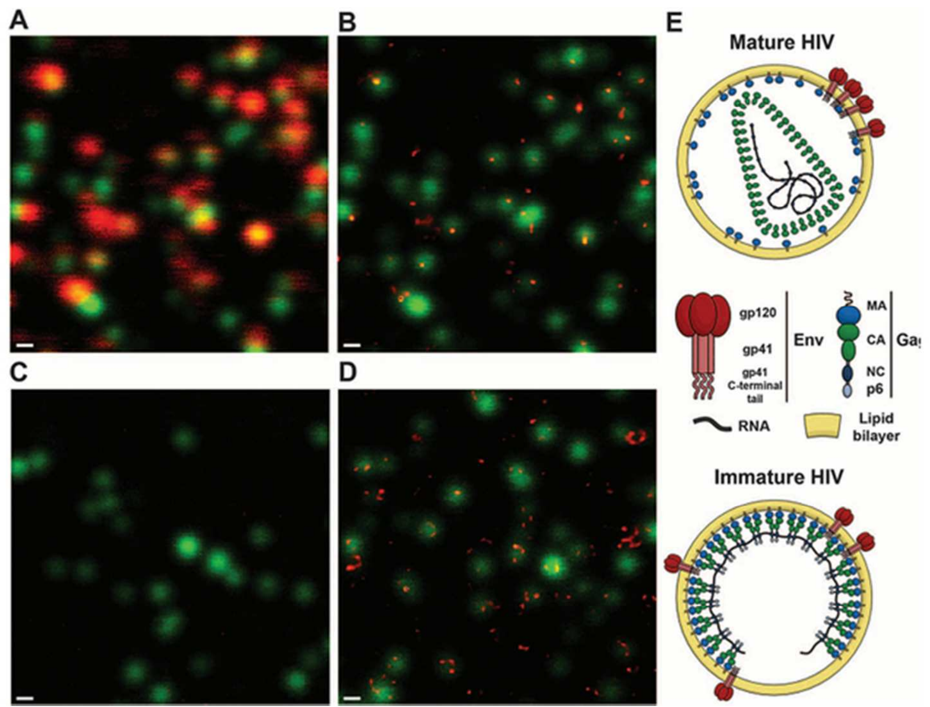

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