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Site-Specific Labeling of Enveloped Viruses with Quantum Dots for Single Virus Tracking

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Abstract

This study reports a general method of labeling enveloped viruses with semiconductor quantum dots (QDs) for use in single virus trafficking studies. Retroviruses, including human immunodeficiency virus (HIV), could be successfully tagged with QDs through the membrane incorporation of a short acceptor peptide (AP) that is susceptible to site-specific biotinylation and attachment of streptavidin-conjugated QDs. It was found that this AP tag-based QD labeling had little effect on the viral infectivity and allowed for the study of the kinetics of the internalization of the recombinant lentivirus enveloped with vesicular stomatitis virus glycoprotein (VSVG) into the early endosomes. It also allows for the live cell imaging of the trafficking of labeled virus to the $Rab5^{+}$ endosomal compartments. This study further demonstrated by direct visualization of QD-labeled virus that VSVG-pseudotyped lentivirus enters cells independent of clatherin- and caveolin-pathways, while the entry of VSVG-pseudotyped retrovirus occurs via the clathrin pathway. The studies monitoring HIV particles using QD-labeling showed that we could detect single virions on the surface of target cells expressing either CD4/CCR5 or DC-SIGN. Further internalization studies of QD-HIV evidently showed that the clathrin pathway is the major route for DC-SIGN-mediated uptake of viruses. Taken together, our data demonstrates the potential of this QD-labeling for visualizing the dynamic interactions between viruses and target cell structures.

Keywords

quantum dot; virus tracking; nanobiotechnology; retrovirus; human immunodeficiency virus

INTRODUCTION

The ability to track individual viruses is a powerful tool for investigating viral infection routes and characterizing the dynamic interactions between viruses and target cells.[1] It enables the possible elucidation of previously unknown but critical steps involved in the penetration of viruses into cells and dissemination of viruses, revealing novel therapeutic opportunities for controlling virus pandemics and pathogenesis.[1, 2] The first step towards the realization of single virus tracking in live cells is to label the external and/or internal constituents of viruses with fluorophores, allowing imaging using fluorescence microscopy. While fluorescent proteins (such as green fluorescent protein, GFP) can be genetically engineered to be

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incorporated into the interior of viruses during viral assembly to accomplish the internal labeling of viruses,[3,4] labeling the external components of viruses is usually achieved by using organic dyes through either chemical labeling of capsid proteins of non-enveloped viruses[5] or physical incorporation into the membrane of enveloped viruses.[1,6–8] Good photostability of the labeling fluorophores is desirable for the continuous tracking of individual viruses because a high magnification objective has to be used in order to detect these tiny viral particles (20–100 nm), generating high excitation light intensity in the focal plane of the objective.[9] In this context, the recently characterized inorganic nanoparticle semiconductor quantum dots (QDs) exhibit many promising features (such as remarkable photostability and brightness) for these types of viral imaging applications.[10–12] Successful examples of tagging viruses with QDs in the literature include: 1) the use of two or three antibody layers (a virus-specific primary antibody, followed by a secondary antibody and streptavidin-QD);[13,14] 2) encapsulation of QDs in viral capsids;[15] 3) covalent linkage of QDs to viral capsids.[16,17] However, these labeling schemes likely affect the normal properties of viruses including their host interactions.[1] We have yet to see a trafficking study of live viruses using these labeling methods to determine whether their perturbations can be tolerated during the process of viral infection. On the other hand, development of methods that can direct the QDs to selected positions of the viruses to ensure minimal disturbance of virus-host interactions can open up new opportunities for single virus tracking in live cells.

The goal of this study was to develop and characterize a general method to site-specifically label live and membrane-enveloped viruses using QDs. Our strategy was to first incorporate a 15-amino acid biotin acceptor peptide (AP) tag onto the surface of a virion (Figure 1).[18, 19] Subsequently, biotin ligase (BirA) was used to specifically modify the AP-tag to introduce the biotin moiety to the viral surface. Due to the tight interaction between biotin and streptavidin ($K_d = 10^{-13}$ M),[20] further addition of streptavidin-conjugated QDs allows the site-specific labeling of viral particles with photostable and fluorescent QDs. Such a labeling method has been successfully applied to targeting QDs to surface proteins in living cells.[19] Although the study here was focused on retroviruses to demonstrate the viability and utility of this method for virus labeling, this method could easily be applied to other types of membrane-enveloped viruses.

RESULTS AND DISCUSSION

A construct was first designed to allow the efficient incorporation of AP-tag to the surface of virus-producing cells and subsequently to the surface of virions. The construct contained a signal peptide derived from the human CD5 protein fused with an AP-tag sequence and the transmembrane (TM) domain of the human CD7 protein, designated AP-TM (Figure 2A). This construct was tested for its ability to display AP-tag by transfecting virus-producing 293T cells with a plasmid encoding AP-TM. After two days post-transfection, cells were washed with the buffer solution (PBS containing $MgCl_2$), biotinylated by adding BirA, biotin and ATP, and further labeled with streptavidin-conjugated dye (R-Phycoerythrin) for flow cytometry analysis, or QD (QD525) for confocal microscopy analysis. Fluorescent signals were detected for cells transfected with AP-TM and treated with biotinylation (Figure 2B and 2C); approximately 40% of cells displayed the AP-tag, as analyzed by flow cytometry. No signal was detected for control cells without AP-TM transfection. Some background signals were obtained for transfected cells without exogenous biotinylation (no BirA treatment, Figure 2B and 2C); this was likely caused by the expression of endogenous biotin ligase in 293T cells, which can endogenously biotinylate AP-tag before it is transported to the cell surface.[19] Nevertheless, we confirmed that the AP-TM construct could successfully display functional AP-tag on the cell surface for site-specific biotinylation and labeling. It is noteworthy that both BirA and streptavidin are membrane-impermeable,[19] thus no intracellular protein can be tagged under our labeling condition.

It was next determined whether AP-tag could be incorporated onto the surface of lentivirus for QD labeling. HIV-1-based lentivirus is one type of retrovirus that possesses the unique feature of transducing non-dividing cells and has been recognized as one of the most efficient and potent systems for the development of gene delivery vectors.[21, 22] QD labeling of lentivirus can potentially enable us to visualize the process of viral entry and transport, which in turn can facilitate our understanding of the virus lifecycle and the design of more efficient vectors for gene therapy. To produce AP-tag-bearing lentivirus, 293T cells were transiently transfected with a lentiviral backbone plasmid FUW,[23] a plasmid encoding envelope glycoprotein derived from vesicular stomatitis virus (VSVG), a plasmid encoding AP-tag (AP-TM), and other necessary packaging plasmids (gag, pol, rev). Concentrated viral particles were resuspended in $MgCl_2$-containing PBS and biotinylated in the presence of BirA, biotin and ATP, followed by incubation with QD525-streptavidin. A previously reported method of confocal imaging was used to detect lentiviral particles.[3] The particle solutions were overlaid onto the coverslips, and adhered viral particles were immunostained with an antibody specific for HIV capsid protein (p24). Clearly the AP-tag was incorporated onto the viral surface, as the fluorescence signal of QDs was readily detected on biotinylated virions, and most of the QD signals (> 70%) were co-localized with p24 (Figure 3A, upper). No QD signal was observed for viral particles lacking the AP-tag (Figure 3A, bottom). To test whether the labeled viruses remained infectious, we made lentiviruses carrying a GFP reporter gene (FUGW,23 instead of FUW, was used as the lentiviral backbone plasmid for preparation of the virus). Half of the viruses were labeled with QDs and used to infect 293T cells; the other half were used as the control for unlabeled viruses. It was found that a similar transduction efficiency was obtained for the labeled (FUGW/VSVG+AP+QD525) and unlabeled lentiviruses (FUGW/VSVG+AP) (Figure 3B), suggesting that this strategy of labeling lentiviruses with QDs could allow us to retain viral infectivity.

The photostability of streptavidin-conjugated QDs bound to viruses was examined. The AP-tag-bearing lentiviruses were individually labeled with streptavidin conjugated with either QD (QD525) or fluorescent dye (fluorescein isothiocyanate, FITC). The labeled viruses were illuminated continuously with the Argon laser (8 mW) to excite QD or FITC. In order to clearly see viral particles, we needed to use a 63× oil-immersion objective. Because of high light intensity generated at the focal plane, this type of objective usually requires good photostability of labeling reagents. After continuous exposure by the laser light, the dye-labeled viruses showed marked photobleaching, much more significant than that with QD-labeled particles (Figure 4A and 4B). Thus, photostable QDs therefore have a great advantage to be used as fluorescent probes for virus imaging, especially for trafficking study of live viruses in cells, in which long-term illumination under confocal light is likely required.

Having established the QD-labeling of lentivirus with retained infectivity (Figure 3B), we tested whether such labeling could facilitate single viral particle tracking within target cells, which would be of importance for understanding the mechanism of viral entry and transport. As a demonstration, the experiment was focused on monitoring the movement of lentivirus to the endosomal compartment, where the membrane of the VSVG-pseudotyped lentivirus and the endosomal membrane are believed to fuse together, an important step of successful infection.[1,2] To mark the endosomes in living cells, the target cells were transfected with a construct to express a small GTPase (Rab5) fused with a fluorescent protein (DsRed); Rab5 is well-known to be associated with early endosomes.[24] It has been shown that the expression of this kind of chimeric protein does not affect intracellular trafficking of virus and viral infectivity.[6] Lentiviral particles (QD-labeled, VSVG-pseudotyped) were initially added to 293T cells in the cold to synchronize the binding. Cells were then shifted to 37°C for different time periods (10, 30, and 60 min), fixed, and analyzed by confocal microscopy. At 10 min, no virus (green) was colocalized with the early endosome marker Rab5 (red). After 30 min, a few viruses were seen to be colocalized with Rab5, as evidenced by the appearance of the yellow

color after overlay of both QD-virus and Rab5 images. After 60 min, most of the viral particles were observed to be located in $Rab5^+$ endosomes. The quantification of colocalization suggested that at 10 min, < 5% of viruses were located in $Rab5^+$ organelles (n=50), at 30 min, 31% in $Rab5^+$ organelles (n=65), and at 60 min, 68% of viruses were observed in $Rab5^+$ organelles (n=58). To monitor the transport of QD-labeled virus to endosomes in real-time, viruses were incubated with 293T cells for 10 min at 37°C to allow the initial internalization, and then began live cell imaging using time-lapse confocal fluorescence microscopy. Selected images obtained from a time series were shown in Figure 5B. The green virus (indicated by arrow) was initially separated from red endosomes (0–458 sec), and then a fluorescent DsRed spot emerged that was centered on the virus at 472 sec; the colocalization of the virus with an endosome was maintained for an extended period of time (up to 708 sec). Thus, we demonstrated the use of the photophysical properties of QDs to monitor the intracellular movement of lentiviruses in live cells.

Many viruses such as vesicular stomatitis virus (VSV) enter cells through endocytosis.[25] It is generally believed that retroviruses, including lentiviruses and gamma-retroviruses, once enveloped with VSVG, are also internalized to low pH endosomes to infect target cells. However, many of the molecular details of entry mechanisms for these pseudotyped viruses remain poorly understood and their entry has not been directly visualized. QD-labeling was tested to see whether it could be a useful tool to study some endocytic pathways exploited by these viruses to enter cells. Clathrin-and caveolar-mediated pathways were investigated in this study as these two are well-characterized pathways of endocytosis.[2] To track the endocytic structures in cells, we made constructs capable of expressing the fluorescent protein-tagged clathrin (DsRed-clathrin) and caveolin (DsRed-caveolin) based on a previous report.[26] 293T cells transfected to express either DsRed-clathrin or DsRed-caveolin were incubated with QD-labeled lentiviruses (FUW/VSVG+AP+QD) and imaged the individual viral particles and endocytic structures after different incubation time periods (10 min and 30 min). It was found that many viruses had been internalized into cells after 10 min of incubation (Figure 6). However, no significant colocalization of lentivirus and clathrin (Figure 6A) or caveolin (Figure 6B) was detected during these time periods (up to 30 min), suggesting that clathrin and caveolin were not involved in the entry of VSVG-pseudotyped lentiviruses. Although this observation is somewhat unexpected, considering that native VSV enters cells by a clathrin-dependent route,[25] it is consistent with a previous biochemical study, in which the entry efficiency of lentiviruses enveloped with VSVG was not altered by the expression of a dominant-negative dynamin in host cells;[27] dynamin is the cellular GTPase that is essential for clathrin- and caveolin-associated endocytosis.[28] This result highlights a fact that there are many different endocytic routes, some of which have yet to be defined, that cells can use to uptake particles and ligands.[2, 29–32] Similarly, the viral entry of VSVG-enveloped gamma-retroviruses (MIG/VSVG+AP+QD) was investigated. QD-labeling had little effect on the infectivity of gamma-retrovirus (Figure 3C). Interestingly, significant colocalization (67%, n=60) of discrete clathrin structures and QD-labeled gamma-viruses was seen at 10 min of incubation (Figure 6C, left). After incubation for 30 min, a lesser degree of colocalization (21%, n=57) was detected (Figure 6C, right), suggesting that several viruses had already dissociated from uncoated clathrin structures and were likely transported to early endosomes. Thus, we obtained clear evidence that clathrin-mediated endocytosis was involved in the entry of VSVG-pseudotyped gamma-retroviruses, which is consistent with the results from many biochemical assays.[33–35]

To further validate the result from the confocal imaging of QD-labeled viruses, assays were performed to examine the inhibitory effect of viral entry by drug treatment. Chlorpromazine is a drug known to prevent clathrin polymerization and obstruct the internalization mediated by clathrin-coated vesicles (CCV).[36] It was found that chlorpromazine at concentrations of 10 and 25 μg/ml, could markedly inhibit VSVG-pseudotyped gamma-retroviruses to infect 293T

cells, whereas no inhibitory effect was observed for VSVG-pseudotyped lentiviruses (Figure 6D). The treatment with filipin, a drug capable of depleting cholesterol to inhibit caveolin-dependent internalization,[37] did not affect the entry of both viruses (Figure 6E), indicating that their entry is independent of caveolin. Thus, QD-labeling of viruses allows us to visualize the clathrin- and caveolin-independent entry of VSVG-pseudotyped lentiviruses and the involvement of clathrin for the entry of VSVG-pseudotyped gamma-retroviruses, which are consistent with the results from infection assays using corresponding inhibitors. Our study also suggests that envelope glycoprotein is not the sole determinant of the viral entry pathway. The exact means of cell entry for VSVG-pseudotyped lentiviruses remains to be established.

The potential utility of QD-labeling for the study of HIV was further explored. To test whether our QD-labeling method could be used to tag HIV particles, virus-producing cells were transiently transfected with the plasmids FUW and AP-TM, a plasmid encoding a codon-optimized and CCR5-tropic HIV-1 envelope protein,[38] and other necessary packaging plasmids (gag, pol, rev). Viral supernatants were harvested and concentrated to obtain HIV particles, which were further subjected to biotinylation and labeling with streptavidin-conjugated QDs. The resulting particles were incubated with HeLa cells expressing viral receptor CD4 and coreceptor CCR5 (HeLa/CD4/CCR5) and stained with an anti-CD4 antibody. Green fluorescence signals could be clearly detected on the surface of HeLa/CD4/CCR5 (Figure 7A, upper), indicating the successful labeling of HIV particles. When the same viral particles were applied to HeLa cells lacking the expression of viral receptors, no green signal was observed (Figure 7A, lower), confirming that the specific interaction between envelope glycoprotein and its cognate receptor accounted for the observed binding of HIV to HeLa/CD4/CCR5.

In addition to the well known interaction of HIV envelope glycoprotein with CD4 and appropriate chemokine receptors (CCR5 or CXCR4), HIV also binds to DC-SIGN, a C-type lectin predominately expressed on immature dendritic cells (DCs).[39] Instead of mediating infection, this HIV-DC binding induces the internalization of intact HIV into a nonlysosomal compartment, where its competence of infection could be retained for an extended period of time before transfer to target cells, resulting in the trans-enhancement of HIV infection to T cells.[39–41] Although the study of this internalization using QD-labeled envelope glycoprotein gp120 is insightful,[42,43] there has been no report on direct visualization of the binding, entry, and trafficking of HIV particle in DC-SIGN-expressing cells. The feasibility of using the QD-labeled HIV (QD-HIV) to track this internalization was tested. Upon incubation for 30 min at 4°C, QD signals could be readily detected on the surface of 293T cells expressing DC-SIGN (293T/DC-SIGN) (Figure 7B). Significant internalization of QD-HIV was seen after prolonged incubation with 293T/DC-SIGN at 37°C. To examine whether the DC-SIGN-mediated uptake of HIV was clathrin-dependent, 293T/DC-SIGN cells were transfected with DsRed-clathrin. The resulting cells and QD-HIV were incubated for 10 min at 37°C, followed by fixation and confocal imaging. Approximately 65% of the QDs (n=51) were colocalized with CCV, indicating that the entry is mediated by a clathrin-dependent pathway. The colocalization of QDs with the Rab5 protein was further studied. About 62% of internalized HIV particles labeled with QDs (n=47) were colocalized with DsRed-tagged Rab5, confirming that DC-SIGN-mediated endocytosis of HIV particles relies on a clathrin-dependent pathway to enter the early endosomes.

To summarize, our goal in this study was to design and evaluate a general method to site-specifically label viruses with QDs. Membrane-enveloped retroviruses were demonstrated to efficiently incorporate a biotinylation tag (AP tag), which could be biotinylated and labeled with streptavidin-conjugated QDs. It was also demonstrated that this method could be used for tagging recombinant lentivirus and gamma-retrovirus and for tagging HIV. The viruses labeled with QDs exhibited much better photostability than that of organic dyes. Coupled with the

nature of the extremely stable binding between biotin and streptavidin, this QD-labeling could provide a potentially practical means to track single virus particles for prolonged periods of time. The small size of the AP tag and site-specific attachment of QDs make this method less likely to affect the property of viral envelope glycoproteins. Compared to the multiple layers of antibody-based QD-labeling,[13,14] our method of direct attachment of QD-conjugates to the small biotin molecule could introduce less perturbation to the virus. It was observed that this AP tag-based QD labeling had little effect on the viral infectivity. The kinetics of the internalization of the recombinant lentivirus enveloped with VSVG into the early endosomes could be studied using QD labeling and live cell imaging could be used to monitor the trafficking of QD-tagged virus to the $Rab5^+$ endosomal compartments. To further demonstrate that this labeling method could be a good tool to study the molecular mechanisms of viral entry, a comparative study of the clathrin- and caveolin-dependent pathways for the internalization of two different types of retroviruses was done and for the first time, direct visualization was used to demonstrate that VSVG-pseudotyped lentivirus enters cells independent of clathrin- and caveolin pathways, while the entry of VSVG-pseudotyped retrovirus occurs via the clathrin pathway. Importantly, the results from this imaging study of QD-labeled virus are consistent with the drug inhibition study by us and others,[27,33–35] suggesting that this labeling scheme can be reliably used for single virus tracking. Our initial studies monitoring HIV particles using QD-labeling showed that single virions on the surface of target cells expressing either CD4/CCR5 or DC-SIGN could be detected. Further internalization studies of QD-HIV evidently showed that the clathrin pathway is the major route for DC-SIGN to uptake viruses, consistent to the previous study using HIV envelope protein.[42,43] Taken together, this study reported and demonstrated a general and efficient means based on QD-labeling for detecting and tracking live viruses during infection. This labeling can take advantage of the excellent fluorescence property of QDs and may represent an attractive tool for elucidating the molecular details of entry and intracellular transport of many kinds of enveloped viruses.

MATERIALS AND METHODS

Cell lines, antibodies, reagents

The 293T/DC-SIGN cell line was generated previously in our laboratory.[44] 293T, 293T/DC-SIGN, and HeLa cells were maintained in a 5% CO_2 environment in Dulbecco's modified Eagle medium (Mediatech, Inc.) with 10% FBS (Sigma), and 2 mM L-glutamine (Hyclone). HeLa/CD4/CCR5 cell line (TZM-bl) was obtained from the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). The cells were maintained in D10 media with gentamycin (50 μg/ml). QD525-streptavidin conjugate and TexasRed-labeled goat anti-mouse IgG antibody were obtained from Molecular Probes. PE- and FITC-conjugated streptavidin were purchased from BD Bioscience. Alexa647-conjugated anti-human CD4 antibody was obtained from Biolegend. Mouse monoclonal antibody to human DC-SIGN was obtained from Abcam. Monoclonal antibody against HIV-1 p24 (AG3.0) was obtained from the NIH AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, NIH). Chlorpromazine and filipin were purchased from Sigma.

Cloning and expression of biotin ligase (BirA)

The previously described protocol was followed to clone and produce the biotin ligase (BirA) from *E. coli*.[45] Briefly, the gene encoding BirA was PCR-amplified from *E. coli* genomic DNA and cloned into pET28 expression plasmid to yield pET-BirA; the BirA gene contained a C-terminal His Tag and was under the control of the T7 promoter. The plasmid (pET-BirA) was transformed into *E. coli* expression strain BL21 by a heat-shock method. The single colony was picked and cultured in 10 ml of LB media overnight. The resulting culture was expanded into a 1 L culture. When OD reached ~0.6, BirA expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentration of 0.42 mM. After

shaking at 30°C for 3 hours (h), cells were pelleted by centrifugation. The cell pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 5 mM phenylmethylsulfonyl fluoride (PMSF), pH=8.0). BirA enzyme was then purified using Ni-NTA agarose according to the manufacture's protocol on native protein purification (Qiagen). Eluted fractions were subjected to SDS-PAGE analysis and those containing BirA were pooled and further purified using an ion-exchange PD-10 column (Amersham Biosciences) to remove the imidazole.

Plasmids

For the construction of plasmid AP-TM, assembly PCR was employed to fuse the DNA sequence of AP tag (amino acid sequences: GLNDIFEAQKIEWHE) to C-terminus of CD5 signal peptide using a synthesized oligonucleoitde (5′-GGTCTGAACGATATCTTCGAAGCTCAGAAAATCGAATGGCACGAAAGATCTG CGGATCCACCA-3′) and this PCR product was amplified using the forward primer 5′-GAATTCTGCAGATGCCCATGGGGTCTCTGCAACCG-3′ and the backward primer 5′-TGGTGGATCCGCAGATCTTTCGTGC-3′. The PCR product was then cloned into the modified pcDNA3 (Invitrogen), downstream of the CMV promoter but upstream of the transmembrane domain of CD7 via restriction sites Pst1 and BamH1. The cDNAs for human clathrin light chain (Gene accession #: M20472) were PCR-amplified using the forward primer 5′-TCGAGCTCAAGCTTATGCTGAGCTGGATCCGTTCGGCG-3′ and the backward primer 5′-GGCCCGCGGTACCTCAGTGCACCAGCGGGGCCTG-3′. Rab5 (Rab5a, Gene accession #: AF498936) were PCR-amplified using the forward primer 5′-TCGAGCTCAAGCTTATGCTAGTCGAGGCGCAACAAGACCCAAC-3′ together with the backward primer 5′-GGGCCCGCGGTACCTTAGTTACTACAACACTGATTCCTGGTTGGTTGTGTGG-3′. The PCR product was then cloned into the pDsRed-monomer-C1 (Clontech) via restriction sties Hind3 and Kpn1 to form DsRed-clathrin[46] and DsRed-Rab5,[47] respectively. For the plasmid encoding DsRed-caveolin[48], the cDNA for caveolin-1 (Gene accession #: NM_001753) was PCR-amplified using the forward primer 5′-GAGCTCAAGCTTATGTCTGGGGGCAAATACGTAGACTCGGAG-3′ and the backward primer 5′-ACCGGTGGATCCATTTCTTTCTGCAAGTTGATGCGGACATTGC-3′ and then inserted into the plasmid pDsRed-monomer-N1 (Clontech) via restriction sites Hind3 and BamH1.

Production of AP-tagged virus

AP-tagged and pseudotyped lentiviruses were produced by transient transfection of 293T cells using a standard calcium phosphate precipitation method.[49] 293T cells at 80% confluence in 6-cm culture dishes were transfected with 5 μg of the lentiviral plasmid FUW, together with 2.5 μg each of AP-TM, the envelope plasmid (VSVG or HIV gp160) and the packaging plasmids (pMDLg/pRRE and pRSV-Rev). For production of AP-tagged retroviral viruses, 293T cells were transfected with 5 μg of the retroviral plasmid MIG,[49] along with 2.5 μg each of AP-TM, the envelope plasmid (VSVG) and the packaging plasmid (gag-pol). The viral supernatant was collected after 48-h posttransfection, filtered through a 0.45-μm pore size filter, and then concentrated by ultracentrifugation (Optima L-90 K ultracentrifuge, Beckman Coulter) either for 90 min at 82,700 × g for VSVG-pseudotyped viruses or for 60 min at 50,000 × g for HIV virus. The pellets were then resuspended in an appropriate volume of cold PBS containing 5 mM $MgCl_2$.

Biotinylation and QD-labeling of virus

The concentrated viruses in PBS-$MgCl_2$ were incubated with 2.5 μM BirA, 10 μM of biotin, and 1 mM ATP for 60 min at 4°C, followed by incubation with 30 nM of QD525-streptavidin

for 60 min at room temperature.[19] Viral aggregates were removed with 0.45 μm pore size filters before imaging.

Viral transduction

293T cells (0.2×10^6 per well) were plated in a 24-well culture dish and spin-infected with viral supernatants (1 ml per well) at 2,500 rpm and 30°C for 90 min by using a Sorval Legend centrifuge.[49] Then, the medium was removed and replaced with fresh medium and cultured for 3 days before FACS analysis of GFP^+ cells. For viral transduction with drug-treated cells, 293T cells were pre-incubated with drugs (chlorpromazine: 10 and 25 μg/ml; filipin: 1 and 5 μg/ml) for 30 min at 37°C and then the cells (0.2×10^6 per well) were spin-infected with 1 ml of viral supernatants in a 24-well culture dish. The drug concentration was maintained during the spin-infection. The cells were further incubated for 60 min at 37°C. The drugs were then removed and replaced with fresh D10 media.

Confocal imaging

Fluorescent images were acquired on a Zeiss LSM 510 META laser scanning confocal microscope equipped with Argon, red HeNe and green HeNe lasers as well as attached to a Coherent Chameleon Ti-Sapphire laser for multiphoton imaging. Images were acquired using a Plan-apochromat 63×/1.4 oil immersion objective. For the detection of individual viral particles, QD-labeled viruses were overlaid upon polylysine-coated coverslips for 60 min at 37°C. The coverslips were then rinsed, fixed with 4% formaldehyde, permeabilized, and immunostained with monoclonal antibody specific to p24 capsid protein.[3] The coverslips were mounted in Vectashield (Vector Laboratories), which is an antifade mounting medium. Images were analyzed with the use of the Zeiss LSM 510 software version 3.2 SP2.

For photostability comparisons of fluorescent dye and QD-labeled viral particles, viral particles were labeled with FITC-streptavidin (20 μg/ml) or QD525-streptavidin (30 nM). The labeled viruses were overlaid upon polylysine-coated coverslips for 60 min at 37°C. The viruses were then continuously exposed to the Argon laser over 3 min. Images were captured at ~10 seconds (s) intervals. Fluorescence intensity versus time within the regions of interest was measured by using the Zeiss LSM 510 software package.

For the viral trafficking studies using clathrin, caveolin-1, and Rab5 constructs, 293T or 293T/DC-SIGN cells were transfected with individual plasmids encoding either DsRed-clathrin,[8] Dsred-caveolin1,[48] or DsRed-Rab5.[50] At 48 h posttransfection, cells were seeded onto glass-bottom culture dishes and grown at 37°C overnight. QD-labeled viruses were incubated with cells for 30 min at 4°C to synchronize infection. The cells were shifted to 37°C for the different time periods, and then fixed with 4% formaldehyde.

To visualize the interaction between HIV virus and target cells, Hela/CD4/CCR5 or 293T/DC-SIGN cells were incubated with QD-labeled HIV virus for 30 min at 4°C, fixed, and then immunostained with anti-human CD4 antibodies or anti-human DC-SIGN and counterstained with DAPI.

Live cell imaging of QD-labeled virus

For the real-time observation of colocalization of the labeled virus with early endosomes, QD-labeled viruses (FUW/VSVG+AP+QD) were incubated with cells for 30 min at 4°C to allow virus binding. The cells were then warmed to 37°C for 10 min to induce viral internalization, and confocal time-lapse images were then recorded.

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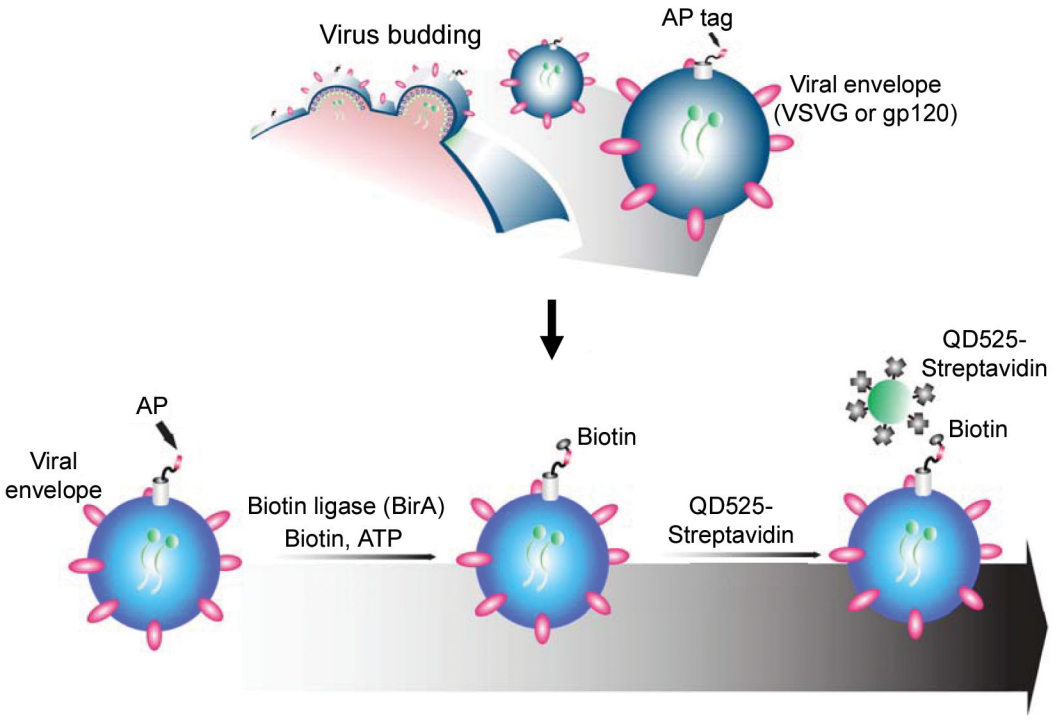

Figure 1.
General strategy for the site-specific labeling of enveloped viruses with QDs. Biotin acceptor peptide (AP) tag is incorporated onto the surface of viruses through the natural budding from cells expressing surface AP tag. The concentrated AP-tagged viruses resuspended in PBS-$MgCl_2$ buffer are biotinylated by adding biotin ligase (BirA), ATP, and biotin. Further incubation with streptavidin-conjugated QDs allows the site-specific labeling of QDs to the biotinylated surface of viruses.

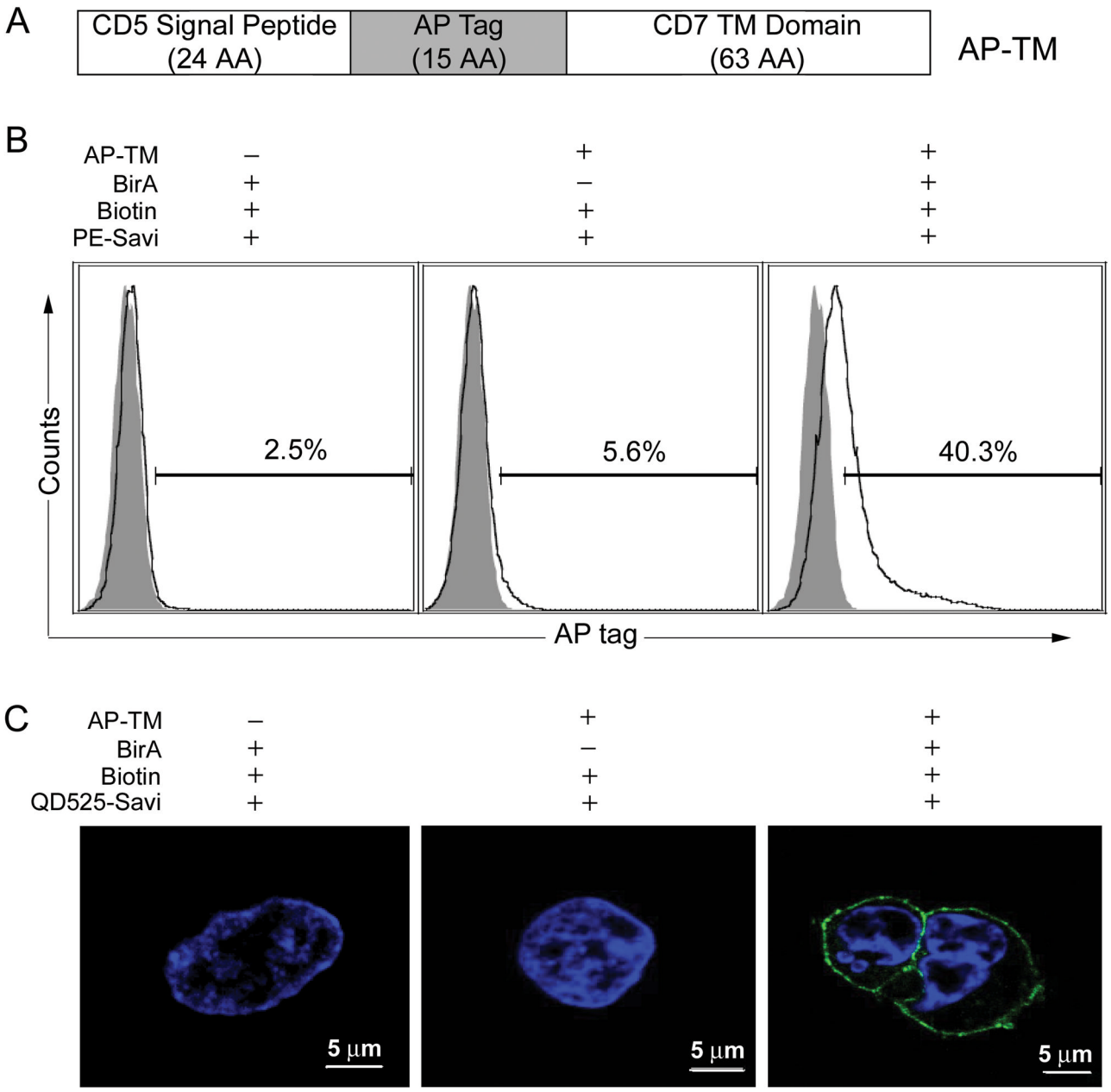

Figure 2.

Incorporation of AP tag onto the surface of 293T cells. (A) The schematic representation of the AP-TM construct. 293T cells were transiently transfected with a plasmid encoding AP-TM. After 48 h post-transfection, the cells were incubated with biotin and ATP in the presence (B and C, right) or absence of BirA (B and C, middle) and labeled with streptavidin-PE (PE-Savi) for analysis by flow cytometry (B) or streptavidin-QDs (QD525-Savi) for confocal microscopy analysis (C). Cells without AP-TM transfection were included as controls (B and C, left).

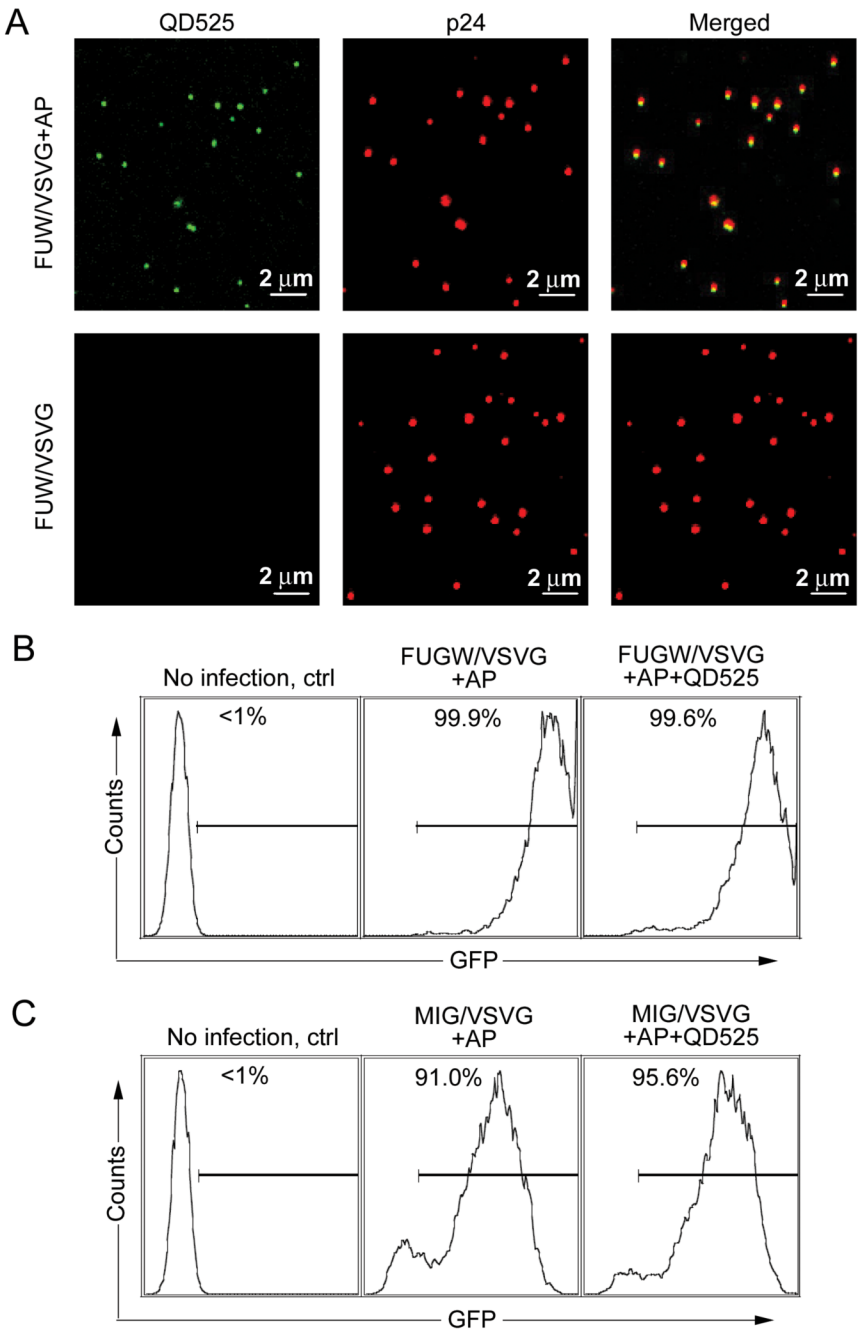

Figure 3.

Incorporation of AP tag onto the surface of lentiviruses for QD labeling. (A) Vesicular stomatitis virus glycoprotein (VSVG)-pseudotyped lentiviruses produced by cotransfection either with AP-TM (FUW/VSVG+AP) or without AP-TM (FUW/VSVG) were incubated with BirA, ATP, and biotin, followed by labeling with streptavidin-QD525. The viruses (green) were overlaid upon polylysine-coated coverslips for 60 min at 37°C. The coverslips were fixed, permeabilized, and immunostained with an antibody specific for HIV capsid protein p24 (red). Overlapping green and red signals appears as yellow in a merged image. (B) AP-tag bearing, VSVG-pseudotyped lentiviruses encoding a GFP reporter gene were produced and biotinylated. Half of the biotinylated viruses were further labeled with streptavidin-QD525

(FUGW/VSVG+AP+QD525), and the other half were not QD-labeled as the control (FUGW/VSVG+AP). 293T cells (2×10^5) were spin-infected with QD-labeled or unlabeled viruses. The resulting GFP expression was analyzed by flow cytometry. (C) 293T cells were spin-infected with QD-labeled gamma-retrovirus (MIG/VSVG+AP+QD) or unlabeled gamma-retovirus (MIG/VSVG+AP). The resulting GFP expression was analyzed by flow cytometry.

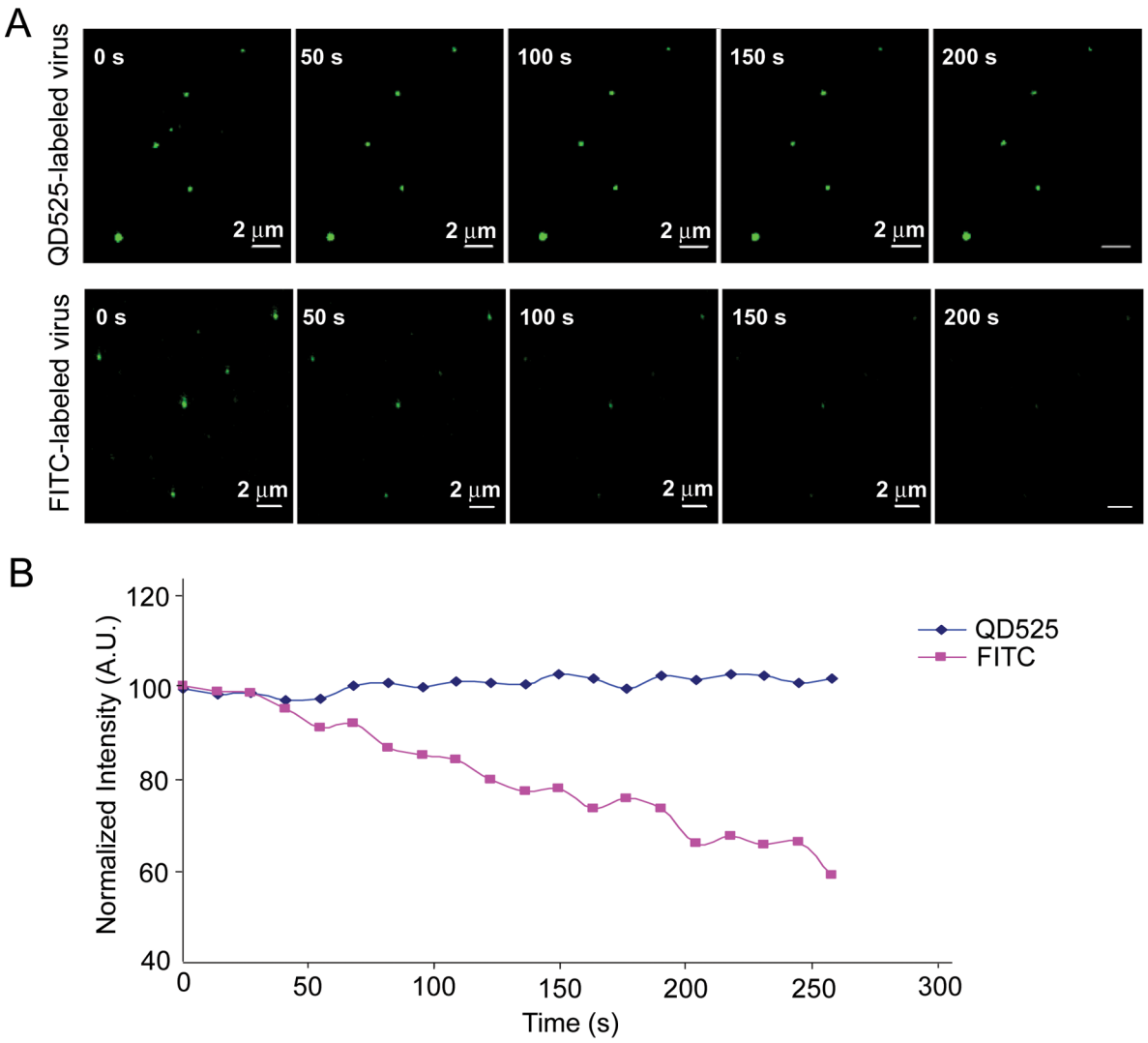

Figure 4.

Photostability comparison between QD-labeled or FITC-labeled viral particles. (A) Biotinylated VSVG-pseudotyped lentiviruses were labeled with streptavidin-QD525 or streptavidin-FITC and overlaid upon poly-lysine coated coverslips. The specimens were continuously illuminated by argon laser at 488 nm over 3 min. Images were captured at ~10 s intervals. (B) Kinetics of the fluorescence intensity of QD-labeled or FITC-labeled viral particles. The fluorescent intensity of viral particles was measured using the software package for the Zeiss LSM 510.

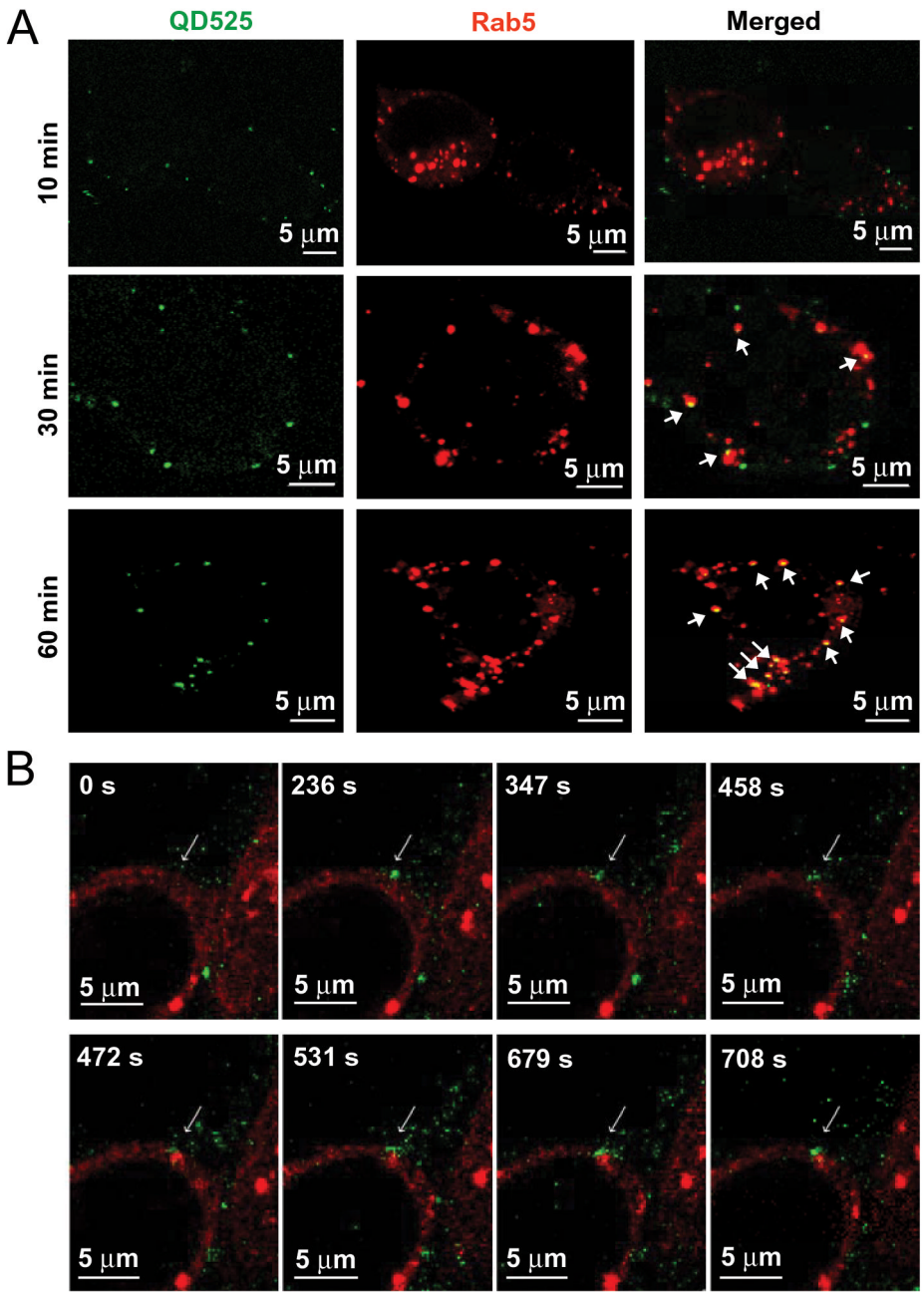

Figure 5.
The trafficking of QD-labeled viral particles through endosomes. (A) 293T cells transiently transfected with DsRed-Rab5 (red) were seeded on glass bottom dishes at 48 h posttransfection. QD-labeled VSVG-pseudotyped lentiviruses (green) were then incubated with the cells for 30 min at 4°C to synchronize infection. The cells were shifted to 37°C for various time periods (10, 30, 60 min) and then fixed. (B) Real-time monitoring of QD-labeled virus transport to endosomes. Rab5 (red) expressing 293T cells were incubated with QD-labeled VSVG-pseudotyped lentiviruses (green) for 30 min at 4°C and shifted to 37°C for 10 min to initiate virus internalization. Confocal time-lapse images were then recorded. The arrows indicate the internalized viral particle.

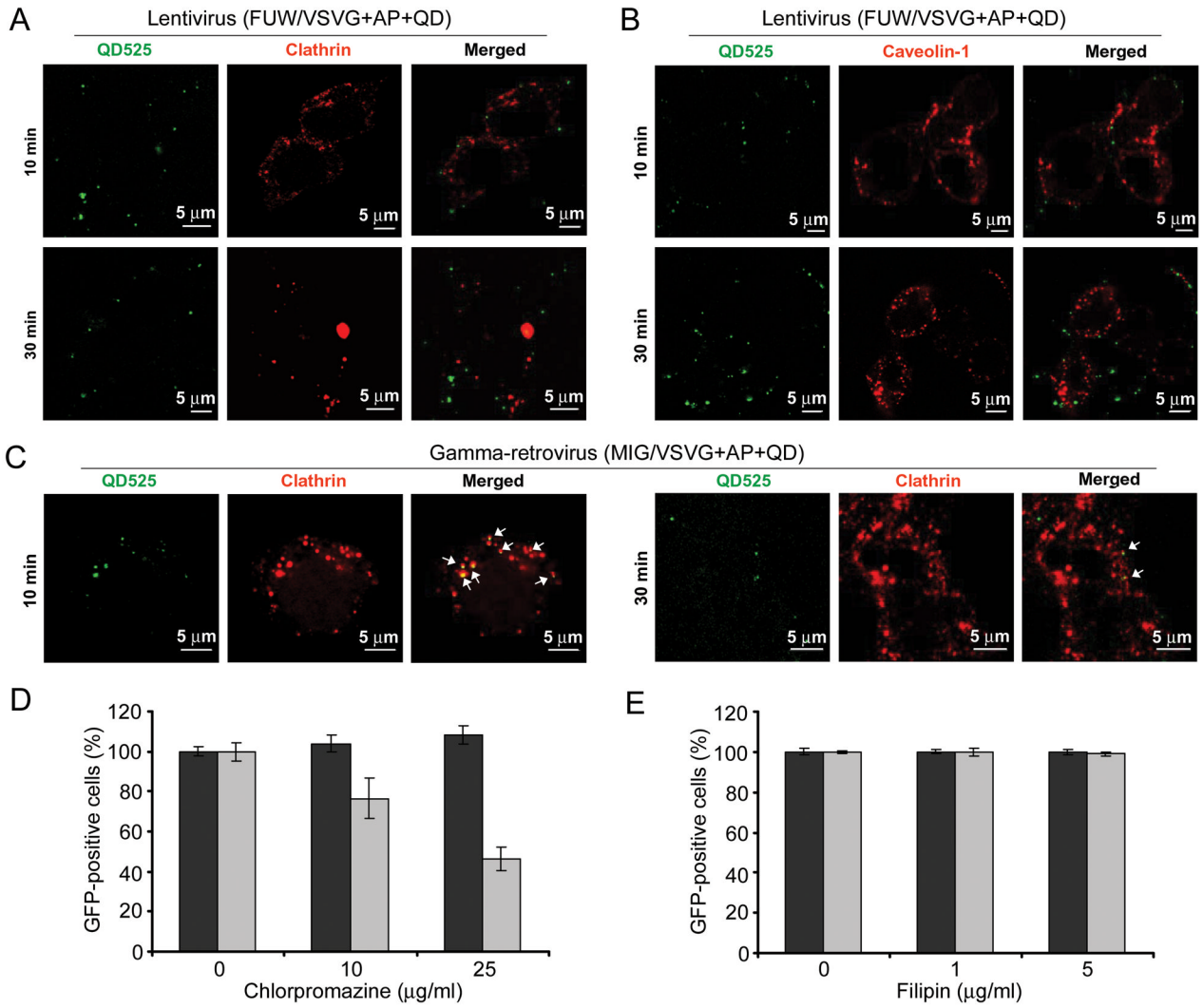

Figure 6.

Clathrin/caveolin-dependent entry of VSVG-pseudotyped retroviruses (green). 293T cells transiently transfected with DsRed-clathrin (red) or DsRed-caveolin (red) were seeded on glass bottom dishes at 48 h posttransfection. QD-labeled VSVG-pseudotyped lentiviruses were incubated with cells that express clathrin (A) or caveolin (B) for 30 min at 4°C to synchronize infection. The cells were shifted to 37°C for various time periods (10 and 30 min) and then fixed. The confocal images were acquired and colocalization was analyzed. Similarly, DsRed-clathrin-expressing 293T cells were incubated with QD-labeled, VSVG-pseudotyped gamma-retroviruses (MIG/VSVG+AP+QD) for 30 min at 4°C, shifted to 37°C, and fixed at different time points (10 and 30 min) (C). Arrows indicate the viral particles colocalized with clathrin. (D & E) Inhibition of clathrin-dependent internalization by chlorpromazine (D) or caveolin-dependent internalization by filipin (E). 293T cells were preincubated with chlorpromazine or filipin for 30 min at 37°C. The cells (2×10^5) were then spin-infected with supernatants of VSVG-pseudotyped lentivirus (FUGW/VSVG, black bar) or gamma-retrovirus (MIG/VSVG, gray bar). The percentage of GFP^+ cells was measured by flow cytometry. Both drug concentrations were maintained during the spin-infection. The drugs were then removed and replaced with fresh media.

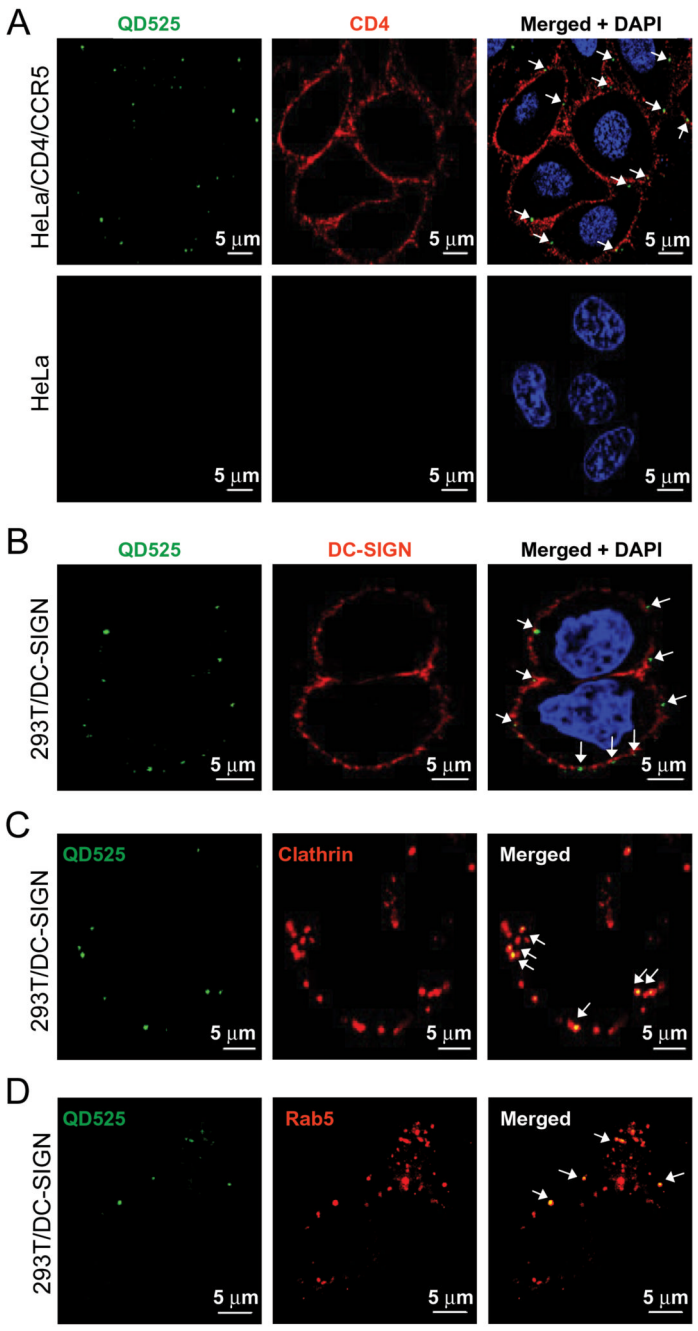

Figure 7.

Binding and intracellular trafficking of QD-labeled HIV. (A) Visualization of HIV viral particles on the surface of HeLa cells expressing viral receptor CD4 and coreceptor CCR5. HeLa cells or HeLa/CD4/CCR5 cells were seeded on glass bottom dishes overnight. The cells were incubated with QD-labeled HIV viral particles (green) for 30 min at 4°C, fixed, and then immunostained with anti-human CD4 antibodies (red) and counterstained with DAPI (blue). Arrows indicate QD-labeled HIV viral particles bound to the cell surface. (B) 293T/DC-SIGN cells were seeded and incubated with QD-labeled HIV viral particles (green) for 30 min at 4° C. The cells were fixed and immunostained with anti-human DC-SIGN antibodies (red) and counterstained with DAPI (blue). Arrows indicate HIV viral particles bound to DC-SIGN. (C)

Involvement of clathrin-dependent internalization of HIV in DC-SIGN-expressing cells. 293T/DC-SIGN cells were transiently transfected with DsRed-clathrin and seeded on glass bottom dishes at 48 h posttransfection. The cells were incubated with QD-labeled HIV viral particles for 30 min at 4°C to synchronize infection, shifted to 37°C for 10 min, and then fixed. Arrows indicate HIV viral particles colocalized with clathrin. (D) HIV viral transport to endosomes. 293T/DC-SIGN cells transiently transfected with DsRed-Rab5 were seeded on glass bottom dishes. The cells were incubated with QD-labeled HIV viral particles for 30 min at 4°C, shifted to 37°C for 30 min, and then fixed. Arrows indicate HIV viral particles colocalized with early endosomes.