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# Self-biotinylation and site-specific double labeling of baculovirus using quantum dots for single-virus in-situ tracking

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A B S T R A C T

Single-virus labeling and tracking represent a powerful tool to study virus–cell interactions. Using baculovirus as a model, here we developed a biochemical method for labeling both the viral envelope and the viral capsid of a virus. Viral envelope of the baculovirus AcMNPV was self-biotinylated and site-specifically conjugated with quantum dots (QDs) following one-step binding reaction, while the viral nucleocapsid was site-specifically labeled with green fluorescent protein (GFP) during viral replication. The established procedure of labeling did not affect viral infectivity, showing that the double-labeled virus retained functional structure and could be tracked for viral localization and movement in the host cells. The double-labeled virus also demonstrated the potential to be used for in-situ and real-time visualizing the internalization of a single viral particle into the host cells. Furthermore, the disassembly processes of the viral envelope and the viral nucleocapsid could be monitored for a long period of time (up to 2 h). Using the established method, several interaction details between the labeled baculoviruses and the host cells have been revealed. Given its advantages in high efficiency, high specificity, convenience and the maintenance of viral infectivity, the established approach provides a promising means for elucidating virus–cell interactions.

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## 1. Introduction

Single-virus tracking enables a better understanding of the interactions between viruses and the host cells through visualizing the process of infection, including attachment, entry, replication and egress. This powerful technology has been successfully used to elucidate the infections of many viruses [1–6]. To date, fluorophors for labeling viruses are mainly classified as organic dyes (e.g., Cy5 and DiD), fluorescent proteins (e.g., mCherry and GFP) and inorganic nanoparticles (e.g., QDs). Chemical dyes have a high quenching coefficiency with a very short excite-state lifetime and can be used as fluorescent probes for virus labeling. However, the fluorescence intensities of chemical dyes are not strong enough for in-situ and real-time monitoring [6–8]. Fluorescent proteins with longer wavelengths, which are genetically encoded, can be used to label specific viral components and endogenous cellular proteins for studying virus–cell interactions. QDs display unique optical and electrical properties as *in vitro* and *in vivo* fluorophors, and have been widely used for labeling nucleic acids, proteins and other biomolecules. QD-labeled virus particles have shown potential in clarifying the mechanisms of viral entry in several single-molecule fluorescence tracking and virus–cell interaction studies [1,2,9,10].

In addition to the selection of fluorophors, the methods for integrating fluorophors into virus particles are also critical. Chemical modification is usually laborious and would more or less affect the infectivity of the virus during the course of labeling [3,11,12], and in particular such non-specific labeling methods require a complex procedure including purification. However, recent studies suggested that biochemical methods could be used to specifically and efficiently label viral components when combined with genetic engineering and chemical coupling approaches [13], and

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the labeling could even be achieved during virus replication [14–16]. It is known that single-virus tracking in living cells requires the viral (internal and/or external) components being labeled with a sufficient number of fluorophors without compromising viral structures and infectivity [12]. In the current study, we attempted to develop a biochemical strategy to label enveloped viruses for visualizing viral entry into live cells.

Baculoviruses, a diverse group of DNA viruses most of which infect Lepidoptera, have been widely utilized as vectors for recombinant protein expressions and gene therapy as well as for biopesticides. The baculovirus display vector system (BDVS) is a eukaryotic display system that presents foreign polypeptides on the viral envelope or capsid. *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) is one of the most common isolates of the BDVS used in foreign gene display. To date, the entry mechanisms of baculoviruses have been addressed mainly using indirect methods to examine virus–cell interactions [17–19]. Although several strategies have been used to tag baculoviruses (envelope or capsid), which are applicable in studying the colocalization of the viral components as well as the specific sub-cellular structures, there is currently little information available concerning the dynamics of viral entry [20–22]. Furthermore, it has been proposed that productive baculovirus entry of the host cells can occur *via* clathrin-mediated endocytosis pathway [23–25], whereas other studies indicate that clathrin-independent pathway plays a role and viral entry is *via* phagocytosis or a direct fusion with plasma membrane triggered by low pH [18,26]. Therefore, further study which can visualize the entry events is crucial for clarifying the inconsistence.

We hypothesized that double-labeling strategy which labels both the viral internal and external components would be able to discriminate intact particles from subviral complexes during infection as well as to monitor the viral internalization. Owing to its large cloning capacity, high productivity and distinct biophysical property, we chose AcMNPV as a model. AcMNPV GP64 is the major envelope fusion glycoprotein which mediates low-pH triggered membrane fusion [27,28]. As a fusion partner, GP64 together with foreign proteins can be incorporated into cell membranes and budded virions [29,30]. The applications of the GP64-based surface display methodology have been well-characterized in the baculovirus display system [30–33]. In the presence of biotin ligase (BirA), biotin, and ATP, target protein fused to biotin acceptor peptide (AP) can be specifically biotinylated and therefore is able to bind to streptavidin-conjugated QDs (SA-QDs) [34]. Alternatively, protein can also be biotinylated by coexpression of the BirA and AP-tag [35]. QD labeling has been proven as an excellent tool for targeting and tracking viral particles [13,36]. VP39 is the major component of AcMNPV nucleocapsid. Previous studies showed that VP39–GFP fusion proteins were successfully incorporated into the viral capsid structure without affecting viral infectivity [19,37]. Similar capsid labeling strategy has been widely used for colocalization and tracking of different viruses [21,22,38].

In this study, using AcMNPV as a model, we developed a double-labeling strategy by labeling envelope with QDs and capsid with GFP. Genes encoding BirA, AP-tag and viral envelope protein GP64 were fused together, while GFP gene was fused to gene encoding viral capsid protein VP39. When cultured in host cells, the progeny recombinant viruses contained self-biotinylated envelope and GFP-labeled capsid. Subsequently, SA-QDs were specifically integrated to the biotinylated viral envelope following one-step reaction *in vitro*. The purified double-labeled viruses were used to monitor the dynamic interaction between viruses and the host cells *in situ*. We further investigated the internalization process into the host cells at the single viral particle level.

## 2. Materials and methods

### 2.1. Cells and viruses

*Spodoptera frugiperda* (Sf9) cells were cultured in Grace's medium (Invitrogen) supplemented with 10% (v/v) fetal bovine serum (Gibco) at 27 °C. Polymerase Chain Reaction (PCR) product was inserted into the downstream of an appropriate promoters of the transfer vector pFastBacDual (Invitrogen). The recombinant plasmid pFastBac-BirAAPGP64 encoding a second copy of the gp64 gene fused with BirA and AP-tag under the promoter PH was constructed. A second copy of the VP39 gene fused with GFP under the promoter p10 was inserted to generate recombinant plasmid pFastBac-BirAAP-GFP. The recombinant viruses (Ac-BirAAP-GFP, Ac-BirAAPGP64 and Ac-VP39GFP) and wild-type virus (WT virus expressing native GP64 and VP39 without labeling) were generated by transfecting indicated bacmids into Sf9 cells according to the manufacturer instructions (Bac-to-Bac baculovirus expression system, Invitrogen).

### 2.2. Virus infection, labeling and purification

Sf9 cells were infected with AcMNPV at a multiplicity of infection (MOI) of 1. The supernatants were collected 48 h after transfection, and cell debris was removed by centrifugation at 4000g for 20 min. The supernatants were filtered through 0.45 μm filter (Millipore) and subsequently centrifuged at 25,000 rpm at 4 °C for 90 min (Beckman SW28 rotor) on 5 mL of a 30% (w/v) sucrose cushion in Tris–EDTA (TE, 0.01 M Tris, pH 7.4, 1.0 mM EDTA) buffer [39]. The virion pellet was resuspended in TE buffer.

The concentrated virions and SA-QDs (625 nm, Invitrogen) were incubated for 3 h at room temperature. The mixture was carefully laid onto the top sucrose layer (there were two layers of sucrose cushion. The upper layer was 6 mL of 60% sucrose and lower layer was 6 mL of 25% sucrose in TE buffer). After centrifugation at 20,000 rpm, 4 °C for 3 h (Beckman SW40 rotor), the fraction containing the labeled viruses was collected and resuspended in TE buffer. Sucrose was removed by the ultrafiltration (Millipore). Purified viruses were resuspended in TE buffer and stored at 4 °C.

### 2.3. Fluorescence and western blot analysis

Sf9 cells were cultured in glass-bottomed petri dishes (NEST, China) overnight at 27 °C before infection. Cells were infected with the recombinant baculoviruses and WT virus at an MOI of 1, respectively. The expression of GFP was observed under the fluorescence microscope at 48 h post-infection. Confocal laser scanning microscopy was performed under Nikon Ti microscope with 20× objective lens. GFP was excited at a wavelength of 488 nm. Images of virus infection process were obtained by using the Velocity software (PerkinElmer, USA).

Virus-infected cells were treated with gel-loading buffer for SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) before western blots were performed [35]. 10 μL resuspended samples were subjected to western blot analysis using specific antibodies and HRP-streptavidin. Signals were detected using BeyoECL Plus kit (BeyoECL, China).

### 2.4. Transmission electron microscopy

The purified virus-QDs conjugates and viruses without labeling were loaded onto carbon-coated copper grids for a 5-min incubation and subsequently blocked with 5% bovine serum albumin for 10 min. Streptavidin–gold (1:25) was used for interaction analysis. The grids were washed three times with phosphate buffered saline (PBS) buffer and then negatively stained with 2% sodium phosphotungstate for 3 min at room temperature. All prepared copper grids were examined under transmission electron microscope (H-7000 FA, Hitachi) and used for analyzing the interaction between viruses and SA-nanoparticles.

### 2.5. Dynamic light scattering and spectrum analysis

Dynamic light scattering (DLS) measurement of double-labeled baculoviruses in PBS buffer was performed with Zetasizer instrument ZEN3600 (Malvern, UK). The UV–Vis absorption of double-labeled baculovirus Ac-BirAAP-GFP/QD and QDs were examined with UV-2550 spectrophotometer (Shimadzu, Tokyo, Japan) [5].

### 2.6. Viral infectivity assay

Sf9 cells were infected with recombinant baculoviruses Ac-BirAAP-GFP and Ac-BirAAP-GFP/QD at an MOI of 5, respectively. Culture medium was harvested at 12, 24, 36, 48, 60, 72, 84, and 96 h post-infection. All samples were stored at −80 °C before titer was determined by end-point dilution assay (EPDA) in Sf9 cells. One-step growth curve was performed to measure the infectivity.

### 2.7. Confocal microscopy

The purified double-labeled baculoviruses Ac-BirAAP-GFP/QD were incubated with the polylysine-coated coverslips for 60 min at 37 °C [13]. Ac-VP39GFP, Ac-BirAAPGP64/QD and QDs were treated under the same condition and served as controls. Hot-air dried coverslips were observed under confocal microscope.

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