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Quantum dot coating of baculoviral vectors enables visualization of transduced cells and tissues

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ABSTRACT

Imaging of transduced cells and tissues is valuable in developing gene transfer vectors and evaluating gene therapy efficacy. We report here a simple method to use bright and photostable quantum dots to label baculovirus, an emerging gene therapy vector. The labeling was achieved through the non-covalent interaction of glutathione-capped CdTe quantum dots with the virus envelope, without the use of chemical conjugation. The quantum dot labeling was nondestructive to viral transduction function and enabled the identification of baculoviral vector-transduced, living cells based on red fluorescence. When the labeled baculoviral vectors were injected intravenously or intraventricularly for *in vivo* delivery of a transgene into mice, quantum dot fluorescence signals allow us monitor whether or not the injected tissues were transduced. More importantly, using a dual-color whole-body imaging technology, we demonstrated that *in vivo* viral transduction could be evaluated in a real-time manner in living mice. Thus, our method of labeling a read-to-use gene delivery vector with quantum dots could be useful towards the improvement of vector design and will have the potential to assess baculovirus-based gene therapy protocols in future.

1. Introduction

Gene therapy has caught the interest of researchers and clinicians for more than two decades, but has had only limited success so far [1–3]. One of the major hurdles encountered in clinical gene therapy trials is the inability to monitor gene transfection/transduction and transgene expression in the target tissue [4–6]. Biopsies or autopsy tissue samples are currently used for laboratory analysis of the distribution and pharmacokinetics of gene therapy reagents. Although valuable, the method does not provide adequate information on temporal and spatial distribution of the applied gene therapy vectors. To overcome the problem, non-invasive, *in vivo* imaging technologies have been developed for assessing the localization, magnitude and duration of transgene expression in situ.

The three major technologies currently under development for *in vivo* gene therapy imaging are magnetic resonance imaging, radionuclide imaging, and optical imaging [4–6]. Among them, optical imaging is considered to be convenient and relatively cheap. There are two commonly used optical imaging modes: imaging using fluorescent protein reporters and imaging using luciferase reporters. Genes encoding these reporter proteins can be employed and expressed, either alone or together with a therapeutic gene, from a gene delivery vector for *in vivo* imaging of transgene expression. Without the use of a reporter gene, vectors can be labeled to identify transduced cells and tissues. Such a labeling is essential if the tested vector has limited cloning capability and already contains a large expression cassette or multiple expression cassettes. Vector labeling would also allow quick identification of transduction before transgene expression and/or monitoring cell or tissue response after transgene expression is shut down.

Quantum dots (QDs) have become an important biological tool for labeling, imaging and sensing [7]. For biolabeling applications, QDs offer attractive advantages, including bright fluorescence, photobleaching resistance and multiplexed staining [8]. Thus, QD bioconjugates have been widely tested for molecular and cellular imaging, mainly intracellular imaging in cultured cells [7,8]. Labeling viral gene delivery vectors with QDs appears challenging. It has been reported that covalent labeling of retroviruses with QDs leads to a very low transduction efficiency of the treated viruses [9]. A

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multiple-step, biotin–streptavidin interaction-based procedure has been developed for site-specific labeling of lentiviral vectors with QDs [10]. The method first uses genetic engineering approach to incorporate a biotinylation tag, a biotin acceptor peptide of 15-amino acid residues, into the virus envelope and then through biotin ligase-mediated reaction introduces biotin molecules to the virus surface, followed by mixing streptavidin-conjugated QDs with the biotinylated viruses. A simple method by mixing QDs and retroviruses in a polycationic solution to label the viral vector results in a relatively low QD delivery efficiency, with only several QDs being observed inside the cells transduced with the complexes [9]. Using copper-free click chemistry, Hao et al. recently reported a mild and less destructive method to label enveloped virus with quantum dots and demonstrated that the labeled virions were of intact infectivity in cultured cells [11]. Nevertheless, possibly due to complicated labeling processes that affect virus infectivity or low QD delivery efficiency, the use of QD-labeled gene transfer vectors for *in vivo* imaging is not well investigated by the existing studies.

To explore the feasibility of using QDs for non-invasive, *in vivo* imaging in gene therapy, we used glutathione-capped CdTe QDs [12] to label insect baculoviral vectors by a simple coating procedure. Baculovirus Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) is able to transduce a wide variety of human cell types and has in the recent years captured much interest as a gene delivery vector for *in vitro* and *in vivo* applications [13–15]. This insect virus has the ability to enter mammalian cells, but neither replicates inside the transduced cell and nor causes toxicity to the cell, a property that makes them easy for production and far less harmful to humans. Baculoviral vectors (BVs) hold large gene cloning capacity and can be used to transfer large (at least 100 kbp) and multiple DNA inserts [13]. An important advantage of using BVs as a gene therapy vector is the absence of pre-existing antivirus immunity in humans since this virus is not infectious to humans. We report here that glutathione-capped QDs can efficiently bind to BVs after mixing the QDs with the viruses. Notably, the virus bioactivity is reserved and the bound QDs can be delivered into the cell by viral transduction, thus facilitating the visualization of transduced living cells and tissues.

2. Materials and methods

2.1. Baculovirus preparation and QD coating

BVs with an eGFP or luciferase reporter gene were constructed in our previous studies [16,17]. Viruses were produced and propagated in *Sf*9 insect cells according to the manual of Bac-to-Bac Baculovirus Expression System (Invitrogen, Carlsbad, CA, USA). To label BVs with QDs, viruses (2.5×10^7 plaque forming units, pfu) were suspended in 50 μl of HBS buffer (10 mM Tris HCl, 150 mM NaCl, pH 7.4) and mixed with glutathione-capped CdTe QDs (40 μg/μl) [12]. In some preparations, Triton X-100 was added to HBS at a concentration of 0.01% or 0.07%. After incubation overnight at 4 °C, the BV–QD mixtures were centrifuged at 28,000*g* for 1 h at 4 °C. The supernatant was then removed and the virus-QD mixtures were re-suspended in 120 μl of HBS and stored at 4 °C until use.

2.2. Cell transduction and analysis

Human U87 cells, purchased from American Type Culture Collection (Manassas, VA, USA), were used to test QD delivery and transgene expression. Cells were seeded in 24-well plates at density of 40,000 per well one day before viral transduction. Cells were transduced with appropriate amounts of BV–QD sample in 400 μL of DMEM at 37 °C for 4 h. After transduction, the cells were washed twice with PBS and fresh DMEM with 10% FBS was added into the wells.

For flow cytometric analysis, cells transduced with BV–QD samples were washed with PBS, trypsinized, dispersed in suspension and subjected to analyses using the FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Untransduced cells served as negative controls. Confocal microscopy analysis was performed using Carl Zeiss LSM510 confocal laser scanning microscope.

To measure luciferase transgene expression, cells were washed with PBS and permeabilized with 80 μL of a reporter cell lysis buffer (Promega, Madison, WI, USA). The cells were then subjected to a single freeze–thaw to ensure complete lysis of cells. Cell extracts (10 μL) were used for luciferase activity assay using an assay regent from Promega. Measurements were performed in a single-tube luminometer (Berthold Lumat LB 9507, Bad Wildbad, Germany) for 10 s. Luciferase activity was normalized to total protein content using a protein assay (Bio-Rad, Richmond, CA, USA). Untransduced cells served as negative controls.

2.3. Characterization of QD-coated baculoviruses by transmission electron microscopy and Zeta potential measurement

To prepare samples for transmission electron microscopy (TEM) analysis, 5 μl of viruses or viruses coated with QDs were dropped onto carbon-coated 200 mesh copper grid and allowed to bind to the grid by incubating the samples at room temperature for 15 min. The samples were fixed with 4% paraformaldehyde for 20 min at 4 °C, followed by gentle rinse with deionized (DI) water three times. The samples were than stained with 2% phosphotungstic acid (pH 7.0) for 60 s and air dried. Samples were viewed under a 200 kV TEM (FEI, OR, USA). For Zeta potential measurements, viral particles and QDs were re-suspended in DI water and loaded into a capillary cell. Zeta potential measurements were carried out using a Zeta sizer (Nano ZS, Malvern).

2.4. Animal experiments

Adult female Balb/c athymic, immuno-incompetent nude mice (weight 20 g, aged 6–8 weeks) were used. In one experiment, BV–QD samples (5×10^8 virus pfu) were prepared with 0.01% Triton X-100 and re-suspended in 200 μL HBS buffer for tail vein injection and the liver was collected 24 h later for tissue sectioning. In the 2nd animal experiments, BV–QD samples (1×10^7 virus pfu in 1 μl HBS) were injected into the cerebral lateral ventricle of in the mouse brain at a speed of 0.5 μl/min using a 10-μl Hamilton syringe connected with a 30 G needle and the brain was collected 48 h later for tissue sectioning. In the 3rd animal experiment, a xenograft tumor model was established by inoculating 1×10^6 U87 human glioblastoma cells subcutaneously into the back of nude mice. At day 14 post-tumor inoculation, BV–QD samples (1×10^7 virus pfu) were prepared with 0.01% Triton X-100 and re-suspended in 50 μL HBS buffer for intra-tumor injection. BV-mediated luciferase reporter gene expression and QD loading were monitored using the IVIS imaging system (Xenogen Corp., Alameda, CA, USA) coupled with cool CCD camera and the ICG filter (Caliper Life Sciences). Firefly luciferase bioluminescence signals were acquired using the IVIS imaging system with an emission filter of 560 nm after intraperitoneal injection of D-luciferin (100 mg/kg, Promega, Madison, WI). Images and measurements of QD fluorescent signals were acquired and analyzed using Living Image® 3.2 (Caliper Life Sciences).

All handling and care of animals was carried out according to the Guidelines on the Care and Use of Animals for Scientific Pur-

poses issued by the National Advisory Committee for Laboratory Animal Research, Singapore.

3. Results and discussion

We coated BVs containing an eGFP reporter gene with glutathione-capped, red fluorescence CdTe QDs and then added the QD-coated BVs to cultured U87 human glioma cells. After washing the cells three times with PBS and culturing the treated cells for 24 h, we observed many eGFP-positive, BV-transduced cells contained the QDs (Fig 1A). Quantitative flow cytometric analysis revealed that approximately 27% of all cells were red fluorescence-positive (Fig. 1B). Confocal microscopy analysis demonstrated the cytoplasmic localization of the QDs (Fig. 1C). Since addition of the QDs alone did not stain the cells (Fig. 1B), we believe that QDs were bound to BVs during the coating and baculoviral transduction had brought them into the cells.

Transmission electron microscopy (TEM) analysis confirmed the interaction of QDs with baculovirus envelope (Fig. 2A and B). These bound QDs appeared to be located primarily at one end of the rod shaped viruses (Arrows in Fig. 2B). Both baculoviruses and glutathione-capped CdTe QDs carry a net negative surface charge at physiological pH of 7.4 (Fig. 2C). However, there is a polar and locally concentrated distribution of the gp64 envelope glycoprotein on the surface of baculovirus, which are clustered only at one end of the virus. Our previous study demonstrated that the gp64 protein can be purified using cation exchange membrane chromatography [18], suggesting that the protein can bind to a negatively charged molecule. We speculate that electrostatic interaction between our glutathione-capped QDs and the clustered gp64 proteins might be a driving force for the binding process that held the QDs only at one end of baculovirus.

To improve QD loading efficiency, we used 0.01% Triton X-100 to permeabilize baculovirus envelope during the coating and then centrifuge the mixtures to collect precipitated viruses and remove the detergent. We observed a significantly improved intracellular delivery of QDs using the Triton X-100-treated BVs, with 83% of cells being red fluorescence-positive (Fig. 1B). Although not all these red fluorescence-positive cells were eGFP-positive, all green eGFP positive cells were red fluorescence-positive (Fig. 1A). Flow cytometric analysis revealed 10% of red fluorescence-positive cells being eGFP-negative (Fig. 1B), possibly because bright QDs have a lower concentration threshold for detection than eGFP. After increasing Triton concentration from 0.01% to 0.07%, we observed a significant decrease in eGFP expression, while the percentage of red fluorescence-positive cells remained 75%. This finding indicates that 0.07% Triton X-100 treatment did not significantly affect virus capability to enter the cell but affect the intracellular process of the virus that is necessary for transgene expression, for example intracellular transport of virus capsid.

To explore the feasibility of *in vivo* QD delivery by BVs, we injected QD-coated BVs containing a luciferase reporter gene through mouse tail vein. The liver was collected 24 h after injection. As assessed by ex vivo organ imaging, an improved tissue delivery of QDs with the use of baculoviral vector was observed

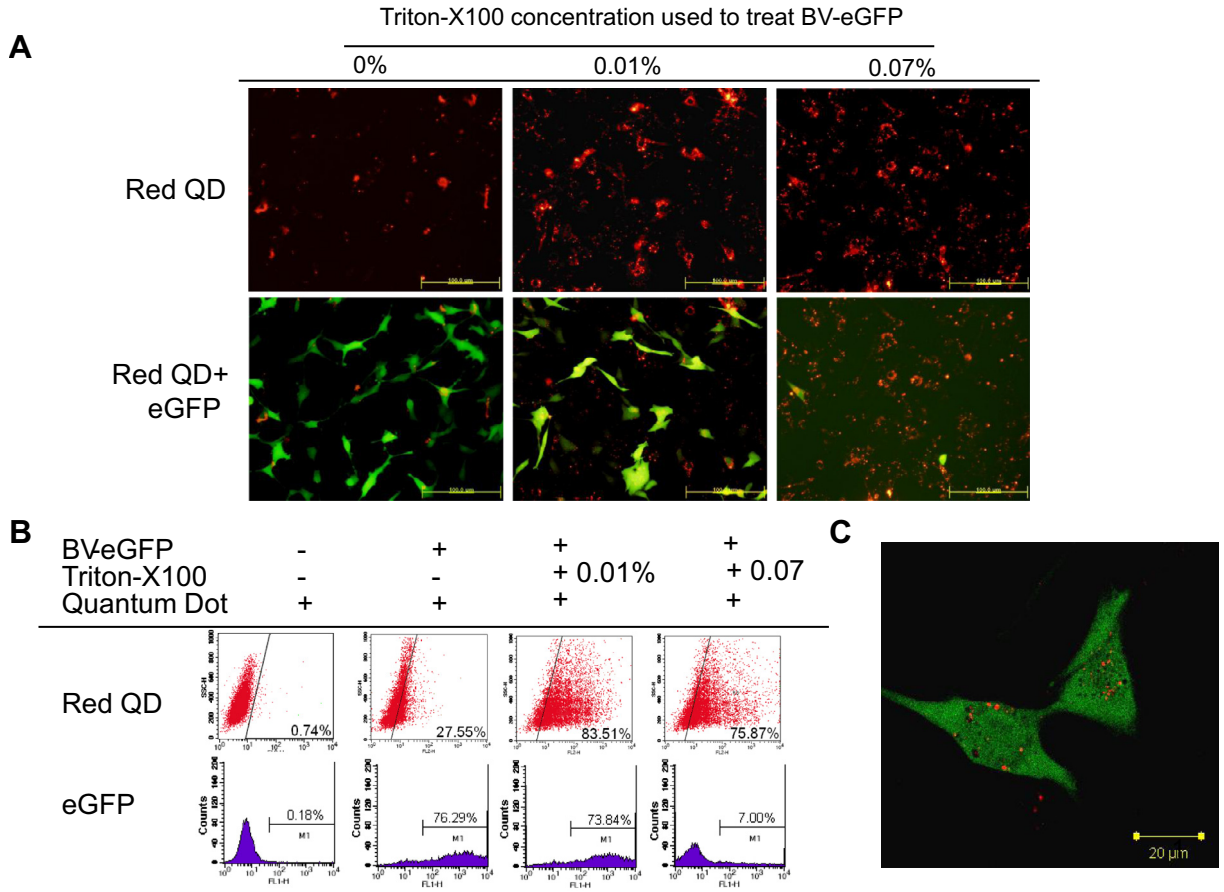

Fig. 1. Intracellular delivery of QDs by QD-coated BVs. QD coating was performed in a HBS buffer with or without Triton-X100. A BV containing the eGFP gene was used to monitor transduction efficiency in human U87 glioma cells. (A) Fluorescence imaging to show red QDs and green transgene products in transduced cells 24 h after viral transduction. (B) Quantitative flow cytometric analysis of red and green fluorescence-positive cells. (C) A confocal microscopic image demonstrating the cytoplasmic localization of QDs in eGFP-positive cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this book.)

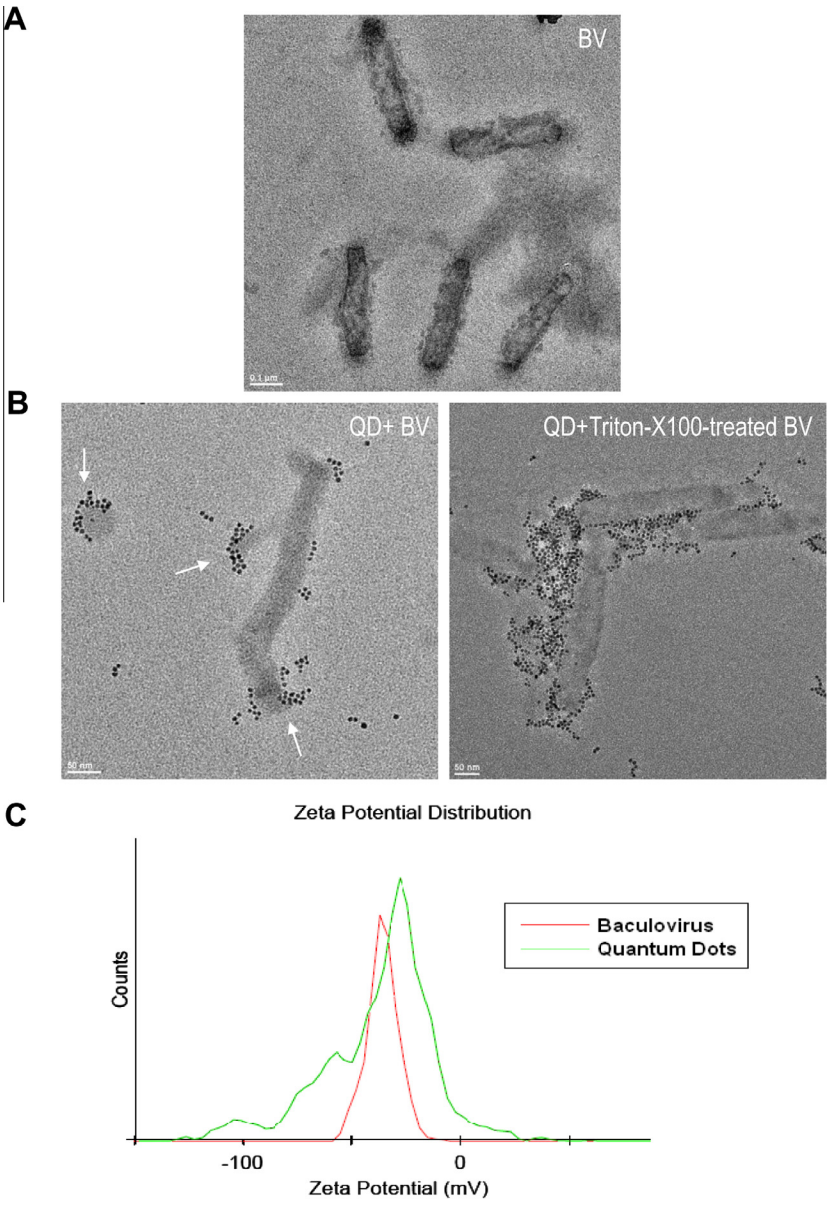

Fig. 2. Interaction of QDs with baculoviruses. (A) and (B) QDs on the surface of baculovirus (BV) envelope as visualized by transmission electron microscopy (TEM). Unmodified baculoviruses (A, bar = 1 μm), viruses coated with QDs in HBS (B, left, bar = 50 nm), and viruses coated with QDs in HBS with 0.01% Triton X-100 (B, right, bar = 50 nm) are shown. Clear rod-shaped particles with a length of 200–250 nm were observed. The bound QDs were located primarily at one end of the viruses (arrows in B, left). (C) Zeta potential of baculoviruses and glutathione-capped CdTe QDs.

over QD injection without a vector (Fig. 3A). The intensity of red fluorescence in the liver organs collected from the mice that were injected with QD-coated BVs were two times higher than that in the livers of mice injected with QDs only. When the liver tissue sections were examined, we noticed that more QDs were accumulated in the organ of mice injected with BV–QD samples, as compared to the liver of mice injected with QDs only (Fig. 3B). Especially, most of QDs were trapped in the blood capillaries surrounding the hepatocytes after tail vein injection of QDs only, while QDs were effectively taken up by the cells when QD-coated BVs were injected.

Luciferase transgene expression was not observed in the liver (data not shown). In view of the fact that baculovirus is highly sensitive to serum complement factors, which is a major barrier to baculovirus-mediated *in vivo* gene transfer [19], no luciferase transgene expression in the current study indicates that QD coating cannot protect tail vein-injected BVs against complement-mediated inactivation. However, as observed above when 0.07% Triton X-100 treatment was used for virus envelope permeabilization, such inactivation by complement factors probably did not affect cell uptake of QD-coated BVs, although the intracellular transport of viral genomes to the nucleus or other processes that are necessary for transgene expression from virions were obviously damaged.

Serum complement factors, like many other serum components, do not usually cross the blood–brain barrier. Thus, stereotaxic injection into the brain evades the complement-mediated inactivation and becomes one of the most effective *in vivo* gene delivery methods for baculoviral vectors [20,21]. We then tested injection of QD-coated BVs into the cerebral lateral ventricles of mice. BVs used were the one bearing the gene encoding green fluorescent protein (eGFP) placed under the control of the CMV promoter. The mice were killed 48 h later, and their brains were prepared for histology. We found the widespread eGFP expression along

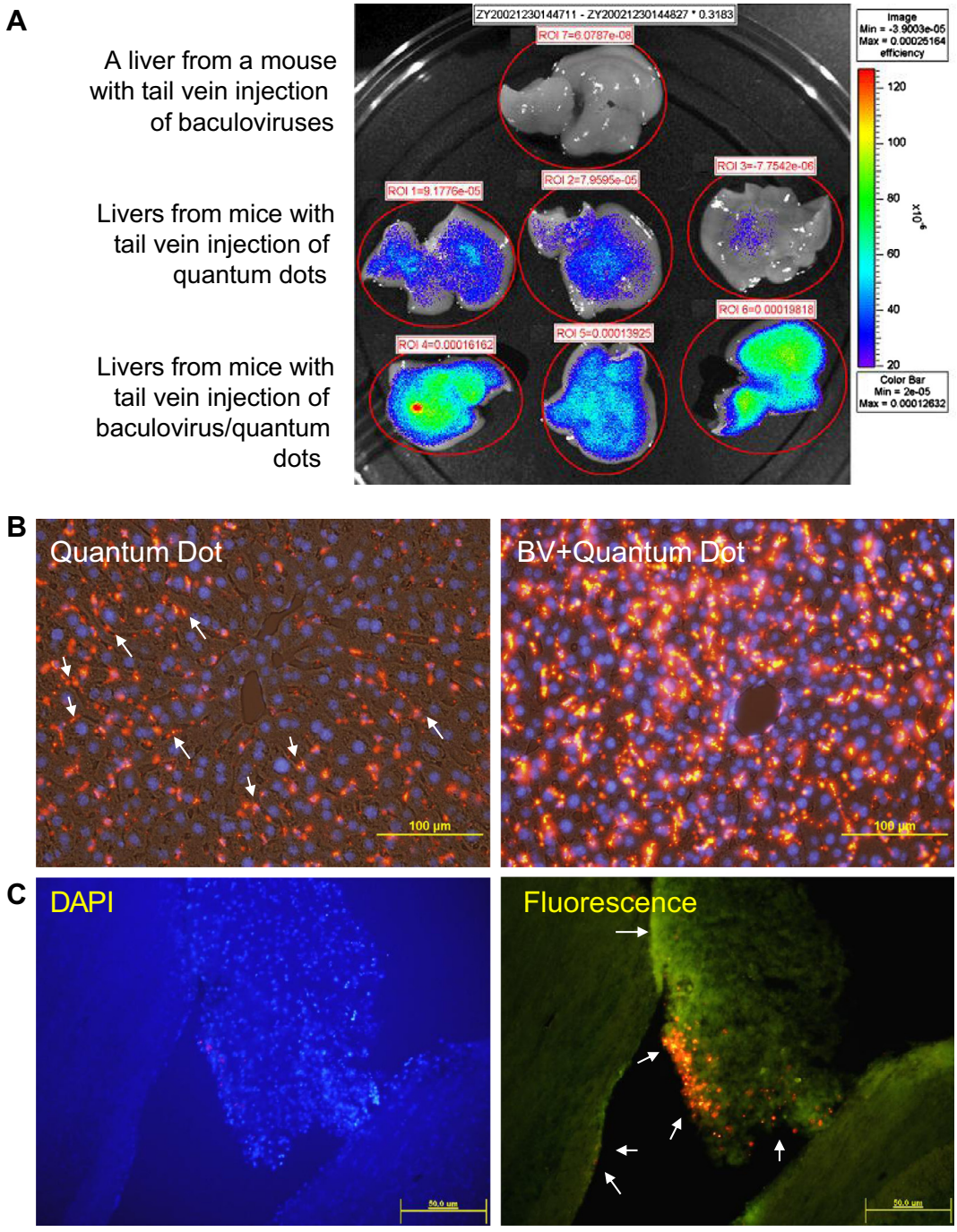

Fig. 3. Baculovirus-mediated in vivo delivery of QDs in normal tissues. QD coating of baculoviruses was performed in HBS with 0.01% Triton X-100. (A and B) Baculoviral transduction facilitates intracellular uptake of QDs in the liver. The coated viruses were injected into mice through the tail vein. The livers were harvested 24 h later. (A) Organ images to show fluorescence intensity of mouse livers. A liver collected from a mouse injected with BVs only (one at the top) is included to show background fluorescence. Fluorescence intensity of the livers from the mice that were injected with virus-QD samples (three livers on the bottom) were about two times higher than that in the livers from the mice injected with QDs only (three livers in the middle). (B) Histological sections of the livers from a mouse injected with QDs alone (left) and a mouse injected QD-coated baculoviruses (right). Arrows indicate QDs trapped in the blood capillaries surrounding the hepatocytes. (C) Visualization of transduced brain tissues by QD-coated BVs. A BV containing the eGFP gene was used to monitor transduction. The brain was collected 48 h after intraventricular injection of QD-coated BVs. Arrows indicate QDs in the eGFP-positive ependymal regions.

the ventricular ependyma, however the eGFP expression was restricted in the ependymal layer, with little parenchymal expression of GFP (Fig. 3C), indicating that viral penetration outside of the subependyma was minimal. Bright QD red fluorescence was easily observed in the regions of the ependyma with eGFP green fluorescence (Fig. 3C), suggesting that QD can be used to assess the location of transduced brain regions.

To investigate whether QD coating can be used to assess the localization and duration of baculoviral transduction in an animal tumor model, we established tumor xenografts in nude mice by subcutaneous inoculation of human glioma U87 cells. Tumors were allowed to form for 2 weeks. Then, QD-coated BVs containing a luciferase reporter gene were injected into the tumors. A noninvasive *in vivo* imaging technology was employed to longitudinally

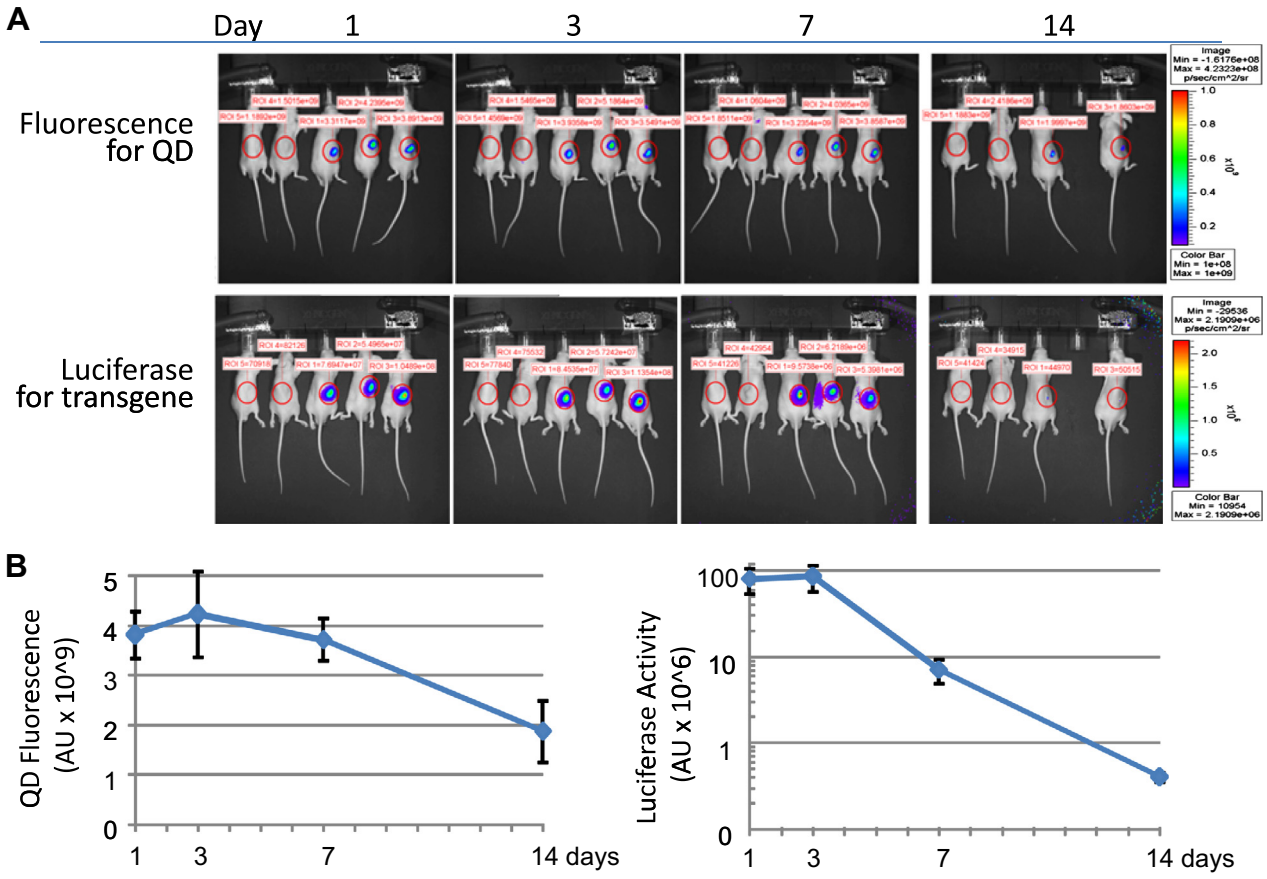

Fig. 4. Baculovirus-mediated co-delivery of quantum dots and the luciferase transgene into subcutaneous U87 tumors. A baculoviral vector containing the luciferase cDNA was used to express the transgene. QD coating of baculoviruses was performed in HBS with 0.01% Triton X-100 and the coated viruses were injected directly into the tumors. The same set of living animals ($n = 3$) were subjected to whole-animal imaging of fluorescence and bioluminescence with the IVIS imaging system on the day indicated. (A) Fluorescence imaging to show QD loading and bioluminescence imaging to show luciferase transgene gene expression. Two animals with tumor inoculation only were included to measure background fluorescence and bioluminescence signals. One animal bearing QD/BV injected tumor died after 12 days. (B) Quantitative analysis of fluorescence and bioluminescence signals over 2 weeks. For luciferase activity measurement, *Y*-axis values are shown in log scale.

monitor fluorescence intensities, indicative of QD loading, and bioluminescence intensities, indicative of transgene expression, at multiple time points in the same set of living animals ($n = 3$). Whole-body imaging showed that after intratumor-injection of QD-coated BVs, QD signals and the transgene signals were co-localized in the same region (Fig. 4A). In the images captured on days one through 14, their changing trends were similar: the intensities remained stable in the first 3 days, started decreasing on day 7, and became very low by 14 days (Fig. 4A and B). Thus, monitoring QD signals could be useful in evaluating the magnitude and the duration of transgene expression from BVs after the viruses are coated with QDs.

4. Conclusion

We demonstrated that BVs coated with glutathione-capped QDs acts as effective vectors for co-delivery of both transgenes and QD bioimaging probes not just *in vitro* but also in vivo. Our simple coating procedure avoids chemical reactions for covalent conjugation that might deteriorate virus activity. On the other hand, the function of gp64, the major envelope protein of baculovirus involved in host cell binding and cellular uptake of the virus, did not appear to be grossly affected by QD coating. Looking ahead, this technology will be valuable in assisting the design and implementation of BVs and in developing detailed understanding on transgene expression-mediated cellular functions in transduced cells, thus reinforcing the use of baculoviruses for *in vivo* transduction and human gene therapy.

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