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Applications of T-lymphoma labeled with fluorescent quantum dots to cell tracing markers in mouse body

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Abstract

Photoluminescent semiconductor quantum dots (QDs) are novel nanometer-size probes that have found bioimaging. Here we imaged a cell line of mouse lymphocytes. QDs were actively taken into the target cells by endocytotic pathways. The fluorescence of QDs held in the endosomes could be studied for more than a week and remained stable luminescence against cell activation induced by concanavalin A, phytohemagglutinin, phorbol myristate acetate, and calcium ionophore A23187. These results suggested that QD-labeling was stable and did not affect either cell activation or cell function. When QD-labeled cells were intravenously injected into mouse, they remained in the peripheral blood in a concentration of approximately 10% up to 5 days after injection using both fluorescence microscopy and flow cytometry. In addition, approximately 20% of QDs were detected in the kidneys, liver, lung, and spleen and could still be observed 7 days after injection. These results suggested that fluorescent probes of QDs might be useful as bioimaging tools for tracing target cells over the period of a week in vivo.

Keywords: Bioimaging; Cell labeling marker; Cytotoxicity; In vivo; Living cell; Quantum dot; Semiconductor

Ultrafine nanocrystals are expected to be used widely in biotechnology and medical applications for separating biomaterials, immunoassay, diagnostics, and drug-carriers that are applied to the drug delivery systems [1–6]. Quantum dots (QDs) are novel inorganic fluorophores that consist of CdSe/ZnS-core/shell semiconductor nanocrystals. QDs have several advantages over organic fluorophores. QDs show high luminance, resistance to photobleaching, a range of excitement wavelengths from ultraviolet to red that depend on the size of the particles, and cover a range of fluorescent wavelengths from blue to red that can be excited using a mercury arc lamp [7,8]. At present, many organic fluorophores such as fluorescein isothiocyanate (FITC) and carboxyfluorescein diacetate succinimidyl ester (CFSE) have been used in various biological applications, such as fluorescent-labeled antibodies and molecules that are used to stain cells or cellar organs [8,9]. Experiments using organic dyes are limited to short-time assays such as flow cytometry due to the lifetime of fluorescence. Those dyes were not suitable for extended periods of bioimaging observations using fluorescent and confocal microscopy because organic fluorophores tend to quench rapidly [6,10]. Furthermore, it is sometimes difficult or impossible to record fine fluorescent images while the organic coloring probes fade in the course of adjusting the focus. In contrast, QDs are stabilized over a far longer exposure-time to light and can emit a fluorescence of high luminosity at an almost equivalent condition as the conventional organic fluorescence probes.

Initially, chemically synthesized QDs have not been applied to biochemical applications because they do not dissolve in water. Since hydrophilic surface treatment of QD was developed, the application range of QDs has been rapidly spreading to bioimaging [11–13]. However, QDs can easily aggregate at acidic- and even in isotonic-conditions because they are unstable in either acidic or saline conditions. Hence, QD-conjugated biomolecules are difficult to produce because most of the biomolecules

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exist in the isotonic condition in vivo [14]. The molecules that can conjugate with QDs are few: streptavidin, oligopeptides, and some antibodies [13,15,16]. We have considered methods to stabilize QDs in biological solutions such as culture media [11,17]. We previously examined albumin from 10 species to assess efficient stabilization since QDs could be conjugated to albumin non-specifically [17]. Time-lapse study of the intracellular distribution of QDs in culture cells has already been performed and the endosomal vesicles including QDs were observed [17]. In this study we assessed whether QDs could be applied to various applications using labeled cells for the purpose of long-time tracing. Labeled concentration of QDs did not appear to show any signs of cytotoxicity. Although there are many applications where QDs are used as dyes to stain mammalian cells and bacteria in vitro [17,18], no applications have been demonstrated where QDs can be used in cells that are transplanted into the living animals.

Materials and methods

Reagents and preparation of albumin-conjugated QDs. CdSe/ZnS-core/shell QDs (fluorescence wavelength: 520 nm) were conjugated with sheep serum albumin fraction V (Sigma) according to our study, as previously reported [17]. Briefly, albumin-conjugated QDs were prepared by mixing equal volumes of QD-sodium salt solution (10 mg/ml) and sheep albumin solution (10 mg/ml) in the presence of EDC (1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) coupling reagents purchased from Pierce Biochemical. The mixture was incubated for 30 min at room temperature and applied to a Sephadex G-25 column (Amersham Biosciences) to remove the excess fraction of reagents. The QD-working solution was prepared by diluting the QD-mixture with DMEM/F-12 culture media (Gibco), immediately filtered with a 0.1-μm centrifuge membrane filter (Millipore, Ultrafree-MC), and centrifuged at 10,000*g* for 10 min before use.

Assessment of QD-Uptake by cells. EL-4 cells, which were established from murine T-cell lymphoma, were cultured in DMEM/F12 supplemented with 5% heat-inactivated fetal bovine serum at 37 °C. The cells were plated at a volume of 1×10^6 cells/well on a 12-well culture plate (Iwaki) and were stimulated with the indicated concentration of QDs. After incubation, the cells were harvested and washed with phosphate-buffered saline (PBS) twice to remove the non-specific binding QDs. The cells were stained with 100 μg/ml propidium iodide solution. Then the cells were fixed with 10%-formaldehyde neutral buffer. As many as 1×10^6 cells were analyzed by flow cytometer (JASCO CytoAce300). Fluorescence was excited using argon laser (488 nm). Detection was triggered by forward-angle light-scattered signals at wavelength of 520 nm. Images were acquired with a digital camera D1X (Nikon) on a fluorescent microscope IX-81 (Olympus) using WIBA mirror unit to adjust the excitation wavelength to 470–490 nm and oil immersion objective lens.

Mice and separation of the cells and organ. BALB/c AnNCrj-nu/nu (nude) mice (5w, male) were purchased from Charles River Japan Inc. EL-4 cells used for administration were pre-stained with a PKH26 Red Fluorescent Cell Linker Kit purchased from Sigma before stimulation with albumin-conjugated QDs. The cells holding QDs were collected 2 h after stimulation and resuspended to the concentration of 5×10^7 cells/ml in serum-free DMEM/F12. Two hundred microliters of cell-suspended solution was intravenously injected to mice. The mice were sacrificed 2 h, 1 day, 3 days, 5 days, and 7 days after injection and the peripheral blood was collected. The blood was laid onto a Ficoll–Metrizoate density gradient (Histopaque-1083 solution, Sigma) and centrifuged at 700*g* for 30 min at room temperature to form a distinct layer at the plasma-media interface. The leukocyte fraction including EL-4 cells was separated from the interface of the two solutions. Then contaminated erythrocytes were removed by 2 washes in ACK-lysis buffer (150 mM NH_4Cl, 1 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.4). Each lysis was performed by incubating for 5 min at room temperature followed by washing the cells with PBS twice. Fluorescence intensity of QD-holding cells was detected by flow cytometric analysis and by fluorescent microscopy.

Collected kidney, liver, lung, and spleen organs were immediately washed three times by PBS to remove retained erythrocytes. Then each organ was incubated with 4% paraformaldehyde for 2 h at room temperature. After soaking, the organs were washed and incubated with 70% ethanol at 4 °C overnight. After embedding into paraffin, the section was sliced to 10 μm thickness. The section was affixed to slide glass (Matsunami).

Results and discussion

Many researchers have applied various techniques to recognize the introduced target cells specifically; labeled cells directly with organic fluorophores like CFSE [8,9], dyed X–Y chromosome by fluorescence in situ hybridization (FISH) using sex-mismatched target cells [19], transformed the gene coded specific molecule like green fluorescent proteins (GFPs), various kind of luciferases, or some cell surface expression molecules, etc. [20,21]. But unfortunately, it gets hanged up and it is costly to detect the target cells by these procedures. To remove those problems, we assessed the possibility of using QDs as the markers for the introduced target cells in vivo. First, we examined how long it took for the EL-4 cells to take up the albumin-conjugated QDs. EL-4 cells were plated at 1×10^6 cells/well and stimulated with 0.1 mg/ml QDs for 15, 30, and 60 min at 37 °C. Shown in Fig. 1A, the QDs were adherent to the cell surface 15 min after incubation. The vesicles including QDs started to form 30 min after incubation and QDs were recruited into the cellular granules after 60 min. We assumed that this uptake would be inhibited at chilled conditions if it were performed via endocytotic pathways. To investigate whether this uptake reaction was dependent on their temperature, EL-4 cells were cultured with QDs at chilled condition. As expected, QDs were also found adhering to the cell surface, but uptake into the cell vesicles was blocked even after 60 min incubation (Fig. 1A). In addition, re-incubating the cells at 37 °C, the QD uptake into the cells recovered again (Fig. 1B). This adhesion is not detached by washing the QD-holding cells and fluorescence of the cells was measured by flow cytometry, which was the same as at 37 °C conditions. These results suggested that uptake of QDs into the cells occurs via endocytotic pathways. This indicated that processing for 15 min to stain the cell surface is sufficient for the purpose of short-time cell tracing. Thus, QDs can become an effective cell marker for several days after only short-time treatment of the cell.

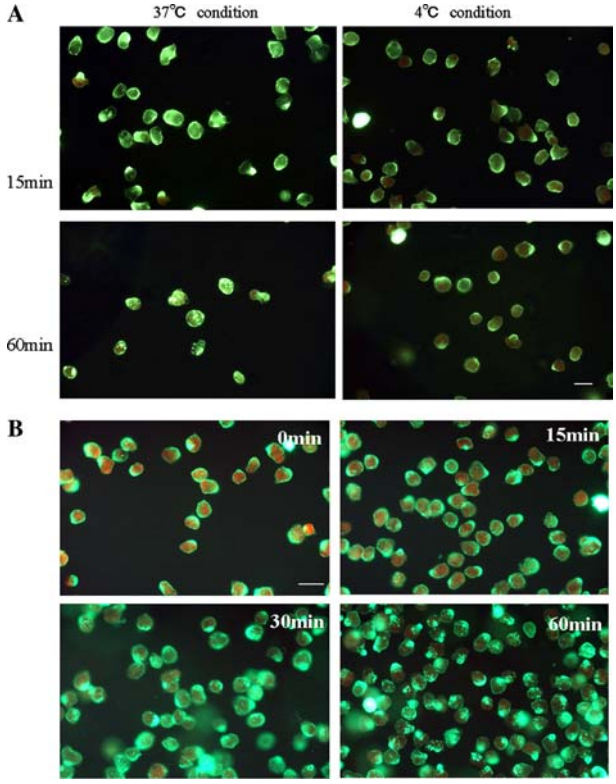

Fig. 1. Uptake of albumin-conjugated QD by EL-4 cells was dependent on temperature. (A) EL-4 murine lymphocytoma cells were plated and stimulated with albumin-conjugated QD at the concentration of 0.1 mg/ml and incubated for 15 and 60 min at 37 and 4 °C conditions. QD- labeled cells were observed by fluorescent microscopy. The bright green granules indicated fluorescence emitted from QDs. (B) Cells were pre-incubated for 20 min before stimulation. Then the cells were stimulated with QDs for 60 min at 4 °C condition and re-incubated at 37 °C for the indicated time. Bars indicate 10 μm. In all the figures, the observation data represent one out of three performed.

When QDs were used as long-term tracing markers, the acute cytotoxicity of QDs raises a problem. To investigate the relation between concentration and cytotoxicity of QDs, we assessed the cell viability after QD-stimulation. The cells were cultured at the concentration of 0.1, 0.2, and 0.4 mg/ml of QDs for 24 h and stained with propidium iodide to detect dead cells. As the fluorescence intensity of QD-holding cells increased by the concentration of QDs, the number of dead cells also increased (Fig. 2). The cytotoxicity was observed in proportion to the concentration of QDs and almost all of the cells were dead at 0.4 mg/ml more than 6 h after incubation. However, no remarkable cytotoxicity was observed at 0.1 mg/ml. Since using a high-concentration of QDs induced the cell death, we decided to use QDs at 0.1 mg/ml concentration.

Next we also examined how long QDs could be retained in the cells. To investigate this, EL-4 cells were further cultured after stimulation with QDs. Cell growth of EL-4 cells was choked by stimulation with QDs even

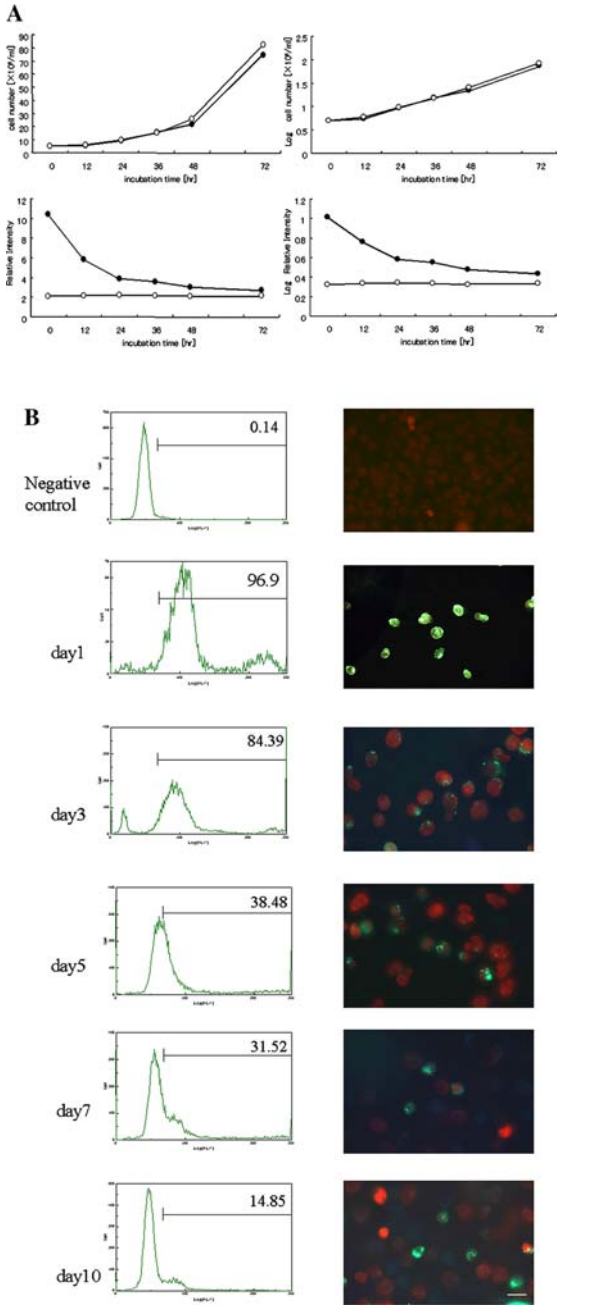

Fig. 3. QDs labeled on EL-4 cells were observed more than a week in vitro. (A) The *upper graphs* show a comparison between the growth curve of the unlabeled and QD-labeled EL-4 cells; open circles indicated the number of control cells and the closed circles are the QDs, respectively. The *lower graphs* show the relative fluorescence intensity of 10^5-collected cells measured by spectrofluorometer (JASCO, FP-6500); open circles indicated the fluorescence intensity of the control cells and the closed circles are the QDs, respectively. Each Graph in *right lanes* shows the logarithmic scales corresponding to the *left* ones. The data are presented as means ± standard deviation. (B) EL-4 cells were stimulated with QDs at 0.1 mg/ml concentration. The cells were incubated for 1, 3, 5, 7, and 10 days. The cells were harvested, stained with propidium iodide for detection of dead cells, and observed by flow cytometry and fluorescent microscopy, as in Fig. 1. The fluorescence intensity of the QD-holding cells in 10^6-collected cells was measured by flow cytometric analysis and the cell population in each region was calculated by CytoAce300 analyzer (JASCO).

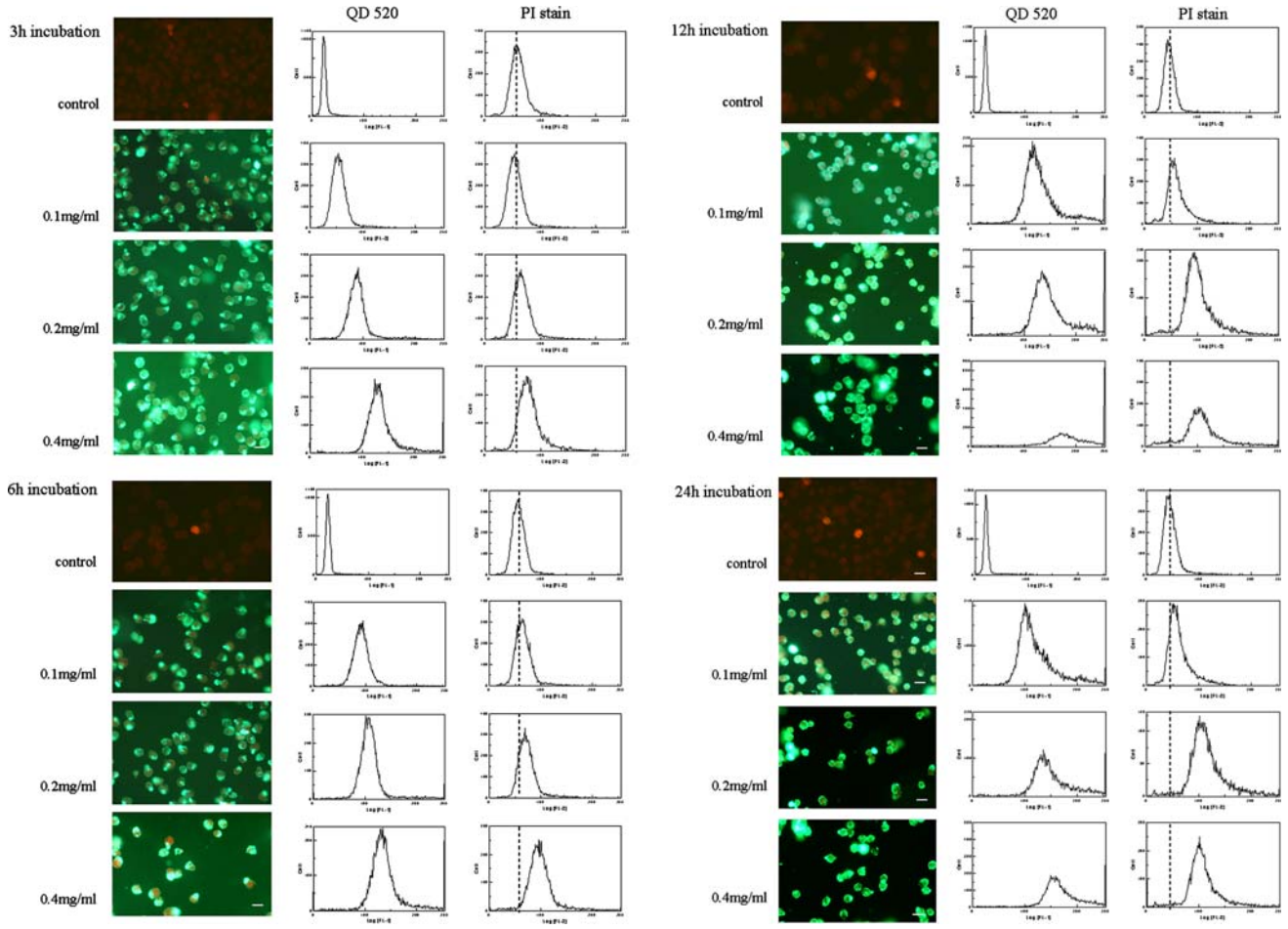

Fig. 2. The cytotoxicity of albumin-conjugated QDs depends on the QD-concentration. EL-4 cells were stimulated with culture media alone or with albumin-conjugated QD at the concentration of 0.1, 0.2, and 0.4 mg/ml. Cells were incubated for 3, 6, 12, and 24 h at 37 °C. The cells were harvested, stained for observation of dead cells, and measured by flow cytometric analysis. Fluorescence was measured by flow cytometry using an argon laser (488 nm). Detection was triggered by forward-angle light scattered signals. In the *graphs*, the relative cell number is given on the *y*-axis and the fluorescence intensity of QDs on the *x*-axis. Dotted lines in the *graphs* indicate the fluorescence medium of the unstimulated cells.

in 0.1 mg/ml concentration (Fig. 3A, upper graphs). Whereas the growth of both cells conformed to logarithmic growth phase, the doubling-time of QD-labeled cells was about 24 h and took 4 h longer than unlabeled cells (approximately 20 h). The fluorescence intensity of QD-labeled cells was logarithmically attenuated, but the attenuation ratio became lower after 24 h incubation (Fig. 3A, lower right). Moreover, the QDs that remained in the cells could be observed for more than a week (Fig. 3B). Approximately 10% of the cells still held QDs after 10 days culture, but fluorescent intensity of cells gradually decreased and highly concentrated in endosomes. This concentration of QDs was compatible with our previous study in Vero cells [17], implying that this labeling of cells by QDs could be applied not only adherent cell but also the cells that have the property of less endocytotic action and high-proliferation rate cells such as lymphocytes.

It is known that T cells were activated with various immune stimulants. Then, we investigated whether labeling of QDs held in the cells would be influenced by those stimulations. EL-4 cells were exposed to various stimulants after labeling of QDs. After stimulation, EL-4 cells were highly aggregated by stimulation with ConA and PHA. But no significant change was observed by stimulating for 24 h (Fig. 4). These results suggest that labeling of QDs were stable, and were not affected by either cell activation or cell function.

Then we compared the fluorescence of QD with those of other organic probes at the points of fluorescence intensity and photostability. Shown in Fig. 5A, fluorescence intensity of both QD and organic probes was approximately equivalent in the moment of excitation in the case of flow cytometric analysis. However after long-term exposure, e.g., long time observation using microscopes, fluorescence from organic probes was eliminated rapidly and almost disappeared within a minute (Fig. 5B). In contrast, the QDs kept emitting bright fluorescence for more than 30-min continuously (Fig. 5C).

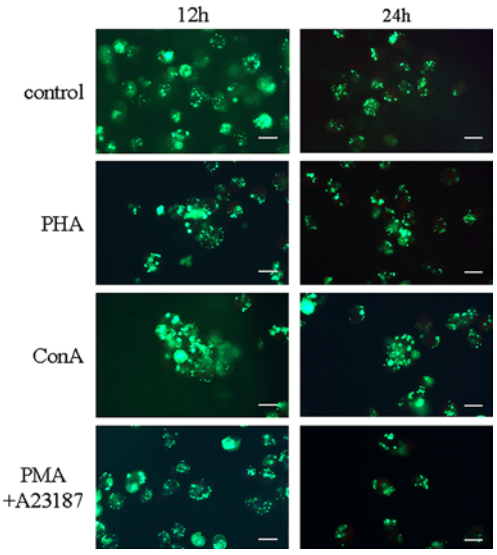

Fig. 4. Fluorescence of QDs was stable against activation of EL-4 cells. EL-4 cells were incubated with QD at 0.1 mg/ml concentration for 3 h. Then the cells were washed twice with DMEM and stimulated with 1 μg/ml ConA, 5 μg/ml PHA, and both 10 ng/ml PMA and 1 μM A23187. The cells were collected 12 and 24 h after stimulation. The cells were observed using a fluorescent microscopy, as in Fig. 1.

Next we considered the survival of QD-labeled cells in vivo. The cells labeled by QDs were intravenously injected into mice. The cells injected into mice were observed in the peripheral blood for 5 days after injection by microscopic analysis (Fig. 6). The fluorescence of QD-labeled cells was also observed until 5 days after injection, but the fluorescence intensity of cells was much smaller than expected. Unfortunately, fluorescence of PKH-dye was observed from cells by using neither microscopy nor flow cytometric analysis (data not shown). However, approximately 70% of QD-labeled cells were eliminated from blood circulation within 2 h after injection. We assumed that the excess number of QD-labeled cells would be homing on the other lymphatic tissue, as T-cell population in whole peripheral blood usually remained at approximately 20%. As expected, many cells including QDs were observed in spleen sections at 2 h after injection (Fig. 7A). In 2 h images, QD-labeled cells remained in the fringe area of white pulp and red pulp. After 5 days, fluorescence emitted from QDs was located in white pulp area. This result concurs with the fact that T-lymphocytes were first observed in red pulp and then moved and then

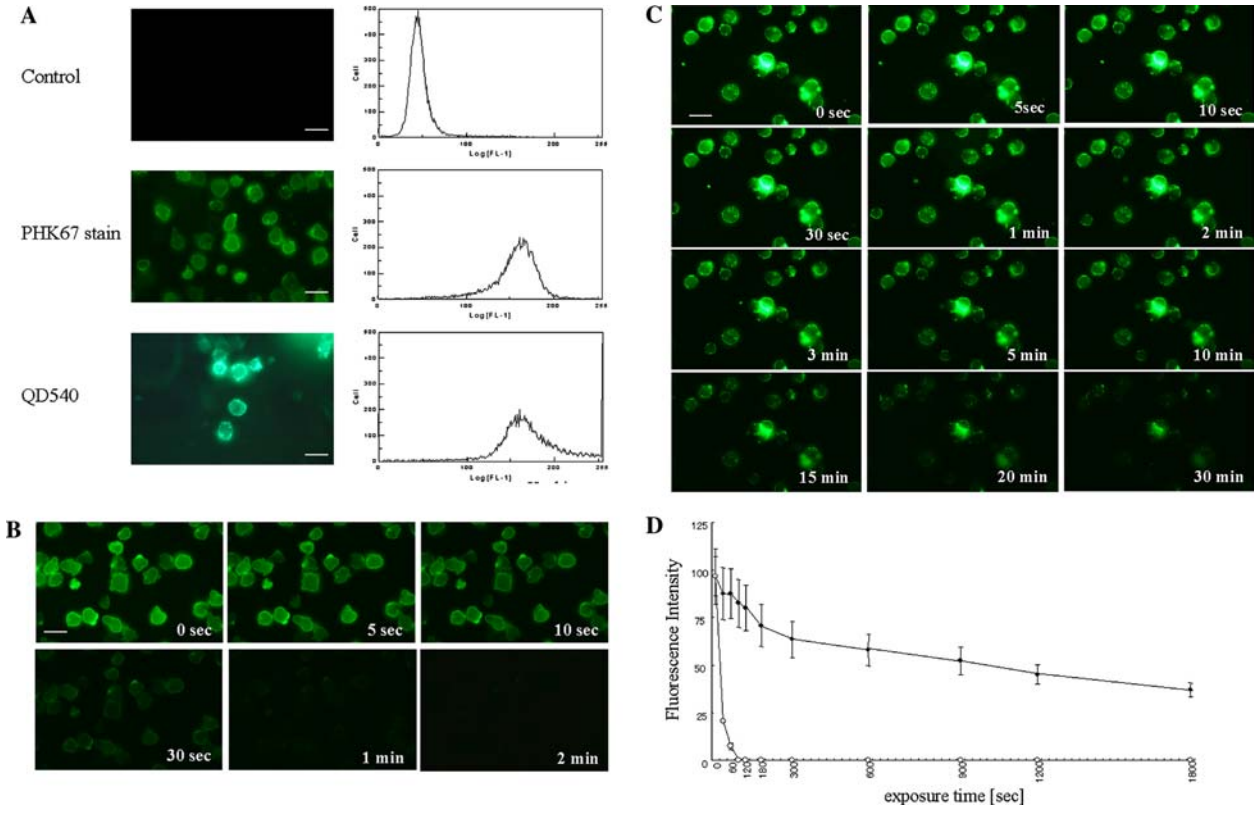

Fig. 5. Fluorescence of QDs was superior to that of organic flourophores with respect to the fluorescent lifetime. (A) Cells stained with QDs and organic dyes were observed by fluorescent microscopy and flow cytometric analysis. The cells were harvested, stained, and analyzed by flow cytometry and fluorescent microscopy, as in Fig. 1. (B) Observation of photostability of organic probe (PHK67). The cells were continuously excited and images were taken using D1X digital camera equipped with fluorescence microscope IX-81 at the indicated time by a 1.0 s exposure. Other conditions were the same as described in Fig. 1. (C) The photostability of QDs, as described in (B). (D) Comparison of the relative fluorescence intensity between QDs and the organic probes. The snapshots shown in Figs. 3B and C were calculated using the histogram analysis of Adobe Photoshop 7.0. Open circles indicate organic probes and closed circles are the QDs, respectively.

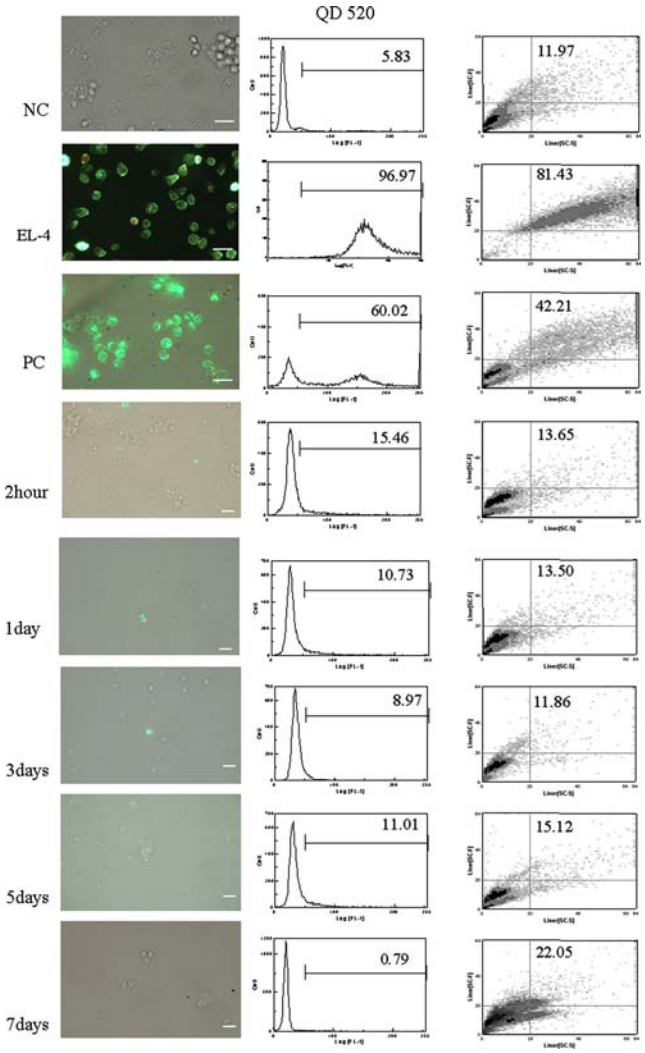

Fig. 6. Intravenously injected QD-labeled cells remained for several days in the peripheral blood. EL-4 cells used for administration were pre-stained with PKH26 Red Fluorescent Cell Linker Kit (Sigma) before incubated with 0.1 mg/ml QD. Then the cells were collected and re-suspended in serum-free DMEM and intravenously injected into the mice. The mice were sacrificed at indicated days ($n = 2$). The fluorescence intensity of the QD-holding cells was measured and calculated by a CytoAce300 analyzer, as shown in Fig. 3. In the *right graphs*, the forward scattered is given on the *y*-axis and side scattered on the *x*-axis. Shown numbers in inset mean the percentage of cells in upper right region of graphs. As positive control, an equivalent number of QD-labeled cells were added into the blood separately.

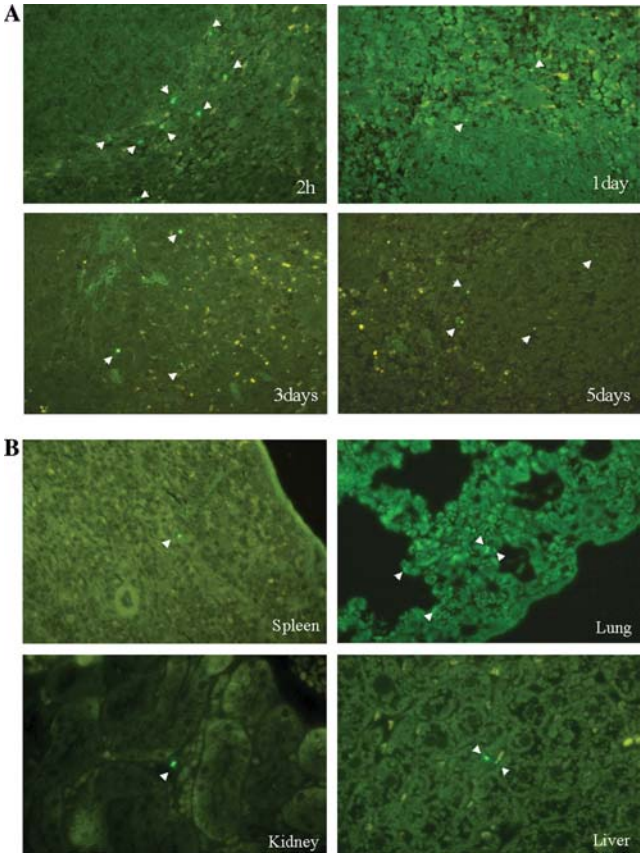

Fig. 7. QD-labeled EL4 was located into kidney, liver, lung, and spleen. (A) The fluorescence images of sliced spleen collected 2 h, 1 day, 3 days, and 5 days after administration ($n = 2$). (B) The fluorescence images of the kidneys, liver, lung, and spleen 7 days after administration. The cells were observed by fluorescence microscopy, as in Fig. 1. Arrowheads indicate the location of QD-holding cells.

accumulated in the splenic white pulp. In addition, QD-labeled cells could be detected in sections of the kidneys, liver, and lung in 7 days (Fig. 7B). The estimated value of QDs in each organ calculated in accordance with QDs contained in each section is shown in Table 1. This result suggests that approximately 20% of injected cells were present in those organs 7 days after injection.

Our results suggested that the QDs could be used as a cell-tracing marker, especially for that of the transplanted target cells. QDs could enable high luminescent labeling of target cells easily and these applications could also be applied to flow cytometric analysis. In this study, we performed the labeling of QDs in EL-4 lymphocytoma cell lines by cellular endocytotic pathways. This result indicated that it is possible to tag target cells only by adding albumin-conjugated QDs into the culture media. In addition, the injected QD-labeled cells were observed up to 5 days in this experiment. However, some of the QDs were eliminated from the cells after a long-time incubation time even in vitro, and the observed number of cells was smaller than expected; the number of cells observed 2 h after injection was quarter of the anticipated value and it decreased to 1/6 by next day. It was not elucidated in this research why QD labeled cells were disappearing from the body in these experiments, but we can presume three possibilities by our study. The first possibility was the attenuation of QDs from endosomes by cell division. As shown in Fig. 3, labeled QDs have already been detached in most of the labeled cells, even though a few QD-labeled cells remained for over a week in vitro. The fluorescence intensity of QD-labeled cell population was attenuated within first 24 h, but fluorescent attenuation ratio

Table 1
The estimated number of injected cells labeled by QDs contained in each organ at indicated days after injection

Time	Organ	QD-labeled cells in each section (cells/section)	Estimated cell numbers in each organ ($\times 10^4$ cells/organ)
2 h	Spleen	605 ± 133	41.2 ± 13.4 (4.1%)
	Kidney	Not detected	Not detected
	Liver	1.0 ± 1.0	0.58 ± 0.63 (0.06%)
	Lung	46.7 ± 18.0	18.6 ± 71.9 (1.86%)
3 days	Spleen	97.7 ± 27.7	6.64 ± 2.44 (0.66%)
	Kidney	0.33 ± 0.58	0.04 ± 0.07 (0.004%)
	Liver	2.0 ± 1.0	1.16 ± 0.68 (0.12%)
	Lung	47.3 ± 5.0	18.5 ± 2.15 (1.85%)
7 days	Spleen	72.0 ± 10.8	4.90 ± 1.24 (4.9%)
	Kidney	1.3 ± 0.6	0.15 ± 0.09 (0.15%)
	Liver	10.6 ± 2.1	6.17 ± 1.75 (6.1%)
	Lung	51.6 ± 11.0	20.1 ± 4.52 (20.1%)

QD-labeled cells including each organ section were counted ($n = 3$). The estimated cell numbers in each organ were the presumed value calculated from the volume of the organs and the number of QD-labeled cells contained in the sections. The data are presented as means ± standard deviation. The value in parentheses shows the percentages of the injected QD-labeled cells located in each organ.

became lower after more than 24 h culture. Moreover, QDs held in endosomes were gradually concentrated in a time-dependent manner. The distribution of QDs may incline toward the specific position in a cell, which resulted in that QD may be unevenly distributed to the two product cells at cell division. Probably QDs may be eliminated from injected cells in the same way in vivo. Furthermore, the influence by cell division in the mouse cannot be disregarded either. As shown in Fig. 6, injected EL-4 cells were grown after 5 days (*right graphs*) and detection of QD-labeled cells became difficult 5 days after injection (*left graphs*). Thus, cell labeling using QDs may tend to be influenced by cell division. The second possibility was exclusion of injected cells by the host–immune system. QDs holding cells may be vanishing more rapidly out of the blood circulation by some active pathways in the early stage after injection. As we surmised that QDs were excluded by the spleen function, QD-labeled cells were detected from the sections of the kidneys, liver, lung, and spleen. Approximately 20% of injected QD-labeled cells were accumulated in those four organs up to 7 days after injection. The last possibility was the loss of photoluminescent ability of the QDs. It was known that the fluorescence emitted from QDs was attenuated by depriving of electric charge on QDs. We assumed that the degradation of the surface of QDs in endosomes including QDs was due to a change of the pH as a consequence of activation of T cells. But as shown in Fig. 4, no significant fluorescent elimination was observed although QD-holding lymphocytes were stimulated with calcium ionophore A23187, PMA, and some lectins such as ConA and PHA.

Our approaches suggest that QDs can be traced using the injected cells for more than a week by quite easy techniques. In this approach, QDs had many more advantageous points than organic fluorophores. As shown in Fig. 5, the fluorescent intensity of QDs was approximately equal to that of the organic fluorophores in short-time exposure such as flow cytometry, but the fluorescence of QDs was superior to that of organic fluorophores in terms of the operating lifetime. QDs could be applied not only to cell labeling marker but also to real-time single-molecule bioimaging for several minutes in living cells, such as antigen–antibody reactions, the moment of viral infection, the movement of transcription in nuclei, and more. More improvements may be achieved by multicolor scanning using some QDs of different particle sizes [22].

At present, no damage or toxicity caused by injection of QD-labeled cells over individuals was observed although the adverse effects on the organisms by the QDs were anticipated, and cytotoxicity was observed at high concentration of QDs. Efforts to address this issue are being made in developing a different silicon based QD. Further miniaturization of the particle is anticipated and silicon-based QDs are expected to be much safer because neither cadmium nor selenium is used. Advanced surface treatment and improved introducing-methods of QDs were required to retain the QDs inside the cells for a longer and more stable duration. Nowadays, another introduction method that encapsulated individual nanocrystals in liposomes is being considered [23–25]. QDs could be used in both carrier or drug delivery systems and to assess the effects of treatment by combining this method and the other gene targeting therapy using the liposomes if a semi-permanent cell labeling technique was enabled in those methods [26]. Furthermore, a small tumor can be detected by specific wavelength infrared rays emitted from some QDs, as the QDs can be conjugated with biomolecules which can specifically recognize tumor cells in a similar way as antibodies and some lectins [27]. Visualization of QDs which flowed in the blood of the capillary vessels and which were accumulated in adipose tissue has already been reported using multiphoton microscopy [28].

The dynamics of QDs in this application is dependent on the albumin conjugating on the surface of the QDs. Further, it may be expected to conjugate QDs with various proteins or molecules for the carrier molecules of drug delivery system or cell specific marker. With the development of new surface treatments of QDs, it may soon become possible to make these possibilities come true using various methods to replace the conventional organic fluorophores that are currently being used.

Acknowledgments

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References

[1] S.J. Rosenthal, I. Tomlinson, E.M. Adkins, S. Schroeter, S. Adams, L. Swafford, J. McBride, Y. Wang, L.J. DeFelice, R.D. Blakely, Targeting cell surface receptors with ligand-conjugated nanocrystals, J. Am. Chem. Soc. 124 (2002) 4586–4594.

[2] B. Dubertret, P. Skourides, D.J. Norris, V. Noireaux, A.H. Brivanlou, A. Libchaber, In vivo imaging of quantum dots encapsulated in phospholipid micelles, Science 298 (2002) 1759–1762.

[3] G.T. Shubeita, S.K. Sekatskii, G. Dietler, I. Potapova, A. Mews, T. Basch, Scanning near-field optical microscopy using semiconductor nanocrystals as a local fluorescence and fluorescence resonance energy transfer source, J. Microsc. 210 (2003) 274–278.

[4] H. Xu, M.Y. Sha, E.Y. Wong, J. Uphoff, Y. Xu, J.A. Treadway, A. Truong, E. O'Brien, S. Asquith, M. Stubbins, N.K. Spurr, E.H. Lai, W. Mahoney, Multiplexed SNP genotyping using the Qbead system: a quantum dot-encoded microsphere-based assay, Nucleic Acids Res. 31 (2003) 43.

[5] X. Wu, H. Liu, J. Liu, K.N. Haley, J.A. Treadway, J.P. Larson, N. Ge, F. Peale, M.P. Bruchez, Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots, Nat. Biotech. 21 (2003) 41–46.

[6] W.C. Chan, D.J. Maxwell, X. Gao, R.E. Bailey, M. Han, S. Nie, Luminescent quantum dots for multiplexed biological detection and imaging, Curr. Opin. Biotech. 13 (2002) 40–46.

[7] H. Mattoussi, J.M. Mauro, E.R. Goldman, G.P. Anderson, V.C. Sundar, F.V. Mikulec, M.G. Bawendi, Self-assembly of CdSe–ZnS quantum dot bioconjugates using an engineered recombinant protein, J. Am. Chem. Soc. 122 (2000) 12142–12150.

[8] S.A. Weston, C.R. Parish, New fluorescent dyes for lymphocyte migration studies. Analysis by flow cytometry and fluorescence microscopy, J. Immunol. Methods 133 (1990) 87–97.

[9] A.B. Lyons, C.R. Parish, Determination of lymphocyte division by flow cytometry, J. Immunol. Methods 171 (1994) 131–137.

[10] M. Bruchez Jr., M. Moronne, P. Gin, S. Weiss, A.P. Alivisatos, Semiconductor nanocrystals as fluorescent biological labels, Science 281 (1998) 2013–2016.

[11] W.C. Chan, S. Nie, Quantum dot bioconjugates for ultrasensitive nonisotopic detection, Science 281 (1998) 2016–2018.

[12] X. Gao, W.C. Chan, S. Nie, Quantum-dot nanocrystals for ultrasensitive biological labeling and multicolor optical encoding, J. Biomed. Opt. 7 (2002) 532–537.

[13] E.R. Goldman, E.D. Balighian, H. Mattoussi, M.K. Kuno, J.M. Mauro, P.T. Tran, G.P. Anderson, Avidin: a natural bridge for quantum dot–antibody conjugates, J. Am. Chem. Soc. 124 (2002) 6378–6382.

[14] D. Gerison, F. Pinaud, S.C. Williams, W.J. Parak, D. Zanchet, S. Weiss, A.P. Alivisatos, Synthesis and properties of biocompatible water-soluble silica-coated CdSe/ZnS semiconductor quantum dots, J. Phys. Chem. 105 (2001) 8861–8871.

[15] I.L. Medintz, A.R. Clapp, H. Mattoussi, E.R. Goldman, B. Fisher, J.M. Mauro, Self-assembled nanoscale biosensors based on quantum dot FRET donors, Nat. Mater. 9 (2003) 630–638.

[16] E.R. Goldman, G.P. Anderson, P.T. Tran, H. Mattoussi, P.T. Charles, J.M. Mauro, Conjugation of luminescent quantum dots with antibodies using an engineered adaptor protein to provide new reagents for fluoroimmunoassays, Anal. Chem. 74 (2002) 841–847.

[17] K. Hanaki, A. Momo, T. Oku, A. Komoto, S. Maenosono, Y. Yamaguchi, K. Yamamoto, Semiconductor quantum dot/albumin complex is a long-life and highly photostable endosome marker, Biochem. Biophys. Res. Commun. 302 (2003) 496–501.

[18] J.K. Jaiswal, H. Mattoussi, J.M. Mauro, S.M. Simon, Long-term multiple color imaging of live cells using quantum dot bioconjugates, Nat. Biotech. 21 (2003) 47–51.

[19] H. van Dekken, A. Hagenbeek, J.G. Bauman, Detection of host cells following sex-mismatched bone marrow transplantation by fluorescent in situ hybridization with a Y-chromosome specific probe, Leukemia 10 (1989) 724–728.

[20] R. Rizzuto, M. Brini, P. Pizzo, M. Murgia, T. Pozzan, Chimeric green fluorescent protein as a tool for visualizing subcellular organelles in living cells, Curr. Biol. 5 (1995) 635–642.

[21] C. Kaether, H.H. Gerdes, Visualization of protein transport along the secretory pathway using green fluorescent protein, FEBS Lett. 369 (1995) 267–271.

[22] Y.T. Lim, S. Kim, A. Nakayama, N.E. Stott, M.G. Bawendi, J.V. Frangioni, Selection of quantum dot wavelengths for biomedical assays and imaging, Mol. Imaging 2 (2003) 50–64.

[23] A. Hayashi, T. Nakanishi, J. Kunisawa, M. Kondoh, S. Imazu, Y. Tsutsumi, K. Tanaka, H. Fujiwara, T. Hamaoka, T. Mayumi, A novel vaccine delivery system using immunopotentiating fusogenic liposomes, Biochem. Biophys. Res. Commun. 261 (1999) 824–828.

[24] T. Nakanishi, A. Hayashi, J. Kunisawa, Y. Tsutsumi, K. Tanaka, Y. Yashiro-Ohtani, M. Nakanishi, H. Fujiwara, T. Hamaoka, T. Mayumi, Fusogenic liposomes efficiently deliver exogenous antigen through the cytoplasm into the MHC class I processing pathway, Eur. J. Immunol. 30 (2000) 1740–1747.

[25] J. Kunisawa, T. Nakanishi, I. Takahashi, A. Okudaira, Y. Tsutsumi, K. Katayama, S. Nakagawa, H. Kiyono, T. Mayumi, Sendai virus fusion protein mediates simultaneous induction of MHC class I/II-dependent mucosal and systemic immune responses via the nasopharyngeal-associated lymphoreticular tissue immune system, J. Immunol. 167 (2001) 1406–1412.

[26] M. Harada-Shiba, K. Yamauchi, A. Harada, I. Takamisawa, K. Shimokado, K. Kataoka, Polyion complex micelles as vectors in gene therapy–pharmacokinetics and in vivo gene transfer, Gene Ther. 9 (2002) 407–414.

[27] M.E. Akerman, W.C. Chan, P. Laakkonen, S.N. Bhatia, E. Ruoslahti, Nanocrystal targeting in vivo, Proc. Natl. Acad. Sci. USA 99 (2002) 12617–12621.

[28] D.R. Larson, W.R. Zipfel, R.M. Williams, S.W. Clark, M.P. Bruchez, F.W. Wise, W.W. Webb, Water-soluble quantum dots for multiphoton fluorescence imaging in vivo, Science 300 (2003) 1434–1436.