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Research Article

Intracellular imaging of targeted proteins labeled with quantum dots

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ABSTRACT

We developed a new method for imaging the movement of targeted proteins in living cancer cells with photostable and bright quantum dots (QDs). QDs were conjugated with various molecules and proteins, such as phalloidin, anti-tubulin antibody and kinesin. These bioconjugated QDs were mixed with a transfection reagent and successfully internalized into living cells. The movements of individual QDs were tracked for long periods of time. Phalloidin conjugated QDs bound to actin filaments and showed almost no movement. In contrast, anti-tubulin antibody conjugated QDs bound to microtubules and revealed dynamic movement of microtubules. Kinesin showed an interesting behavior whereby kinesin came to be almost paused briefly for a few seconds and then moved once again. This is in direct contrast to the smoothly continuous movement of kinesin in an *in vitro* assay. The maximum velocity of kinesin in cells was faster than that in the *in vitro* assay. These results suggest that intracellular movement of kinesin is different from that in the *in vitro* assay. This newly described method will be a powerful tool for investigating the functions of proteins in living cells.

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Introduction

Recent development of imaging techniques for single molecules provides detailed information of the molecular mechanism of proteins [1–4]. However, the mechanism underlying the function of these molecules within the cells is still not known. Many studies of cell biology are utilizing organic fluorophores and fluorescence proteins, such as Cy3 and GFP, as probes to visualize single protein molecules, and have been very successful [5,6]. Single molecule observation of these fluorophores, however, is available only for short periods of a few seconds due to their rapid photo bleaching. Thus, development of a new technique to image single molecules of targeted proteins for long periods of time is crucial in order to investigate the detailed mechanisms of the proteins in living cells. The bright and photostable quantum dots (QDs) are useful for detecting the behavior of biomolecules in living cells for long periods of time [7–11], 1 h or longer [10]. The positions of bright fluorescence spots were determined with more than one nanometer accuracy for the long periods, when the QDs are excited with very bright light [12,13]. Thus, the direct labeling of biomolecules in a living cell with QDs is important for the future advances of biological and medical research [12,14,15].

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Internalization of the QD-bioconjugates across the cell membrane into cytoplasm of living cells is very challenging for intracellular imaging. Several methods for the internalization of QD-bioconjugates have been tested, electroporation [16], liposome fusion [17,18], microinjection [17] and cell penetrating polymers and peptides [19,20]. These methods, however, could internalize QDs in aggregates [17,18]. To image single molecules labeled with QDs in living cells, the bioconjugated QDs need to be delivered in a monodispersion and have increased targeting efficiency. Recently, osmotic lysis of pinosomes was used for QD internalization [21]. This method is simple but has sometimes resulted in damage of cellular functions [22].

Our method using the lipid transfection has been widely used for DNA transfection in cell biology [23] to deliver QDs into cells without damaging cellular functions. We successfully delivered the bioconjugated QDs and imaged them internally using QDs conjugated with phalloidin, anti-tubulin antibody and kinesin as excellent examples of small molecule, antibody and enzyme, respectively (Fig. 1). Here, the interesting dynamics of microtubule and the intracellular-specific behavior of kinesin were discovered for the first time. Therefore, the methods in the present study will be a powerful tool to image other cellular components as well as motor proteins using photostable QDs as a probe.

Materials and methods

Preparation of phalloidin–QD-bioconjugate

For the specific labeling of actin filaments, phalloidin molecules were conjugated to QDs. Amino functionalized PEG coated QDs 655 (Qdot® nanocrystals, Invitrogen Co.), where the number indicates the emission wavelength, were cross-linked to amino functionalized phalloidin (Amino Phalloidin, Alexis Biochemicals) by Bis (sulfosuccinimidly) suberate (BS3, Pierce Chemicals). BS3 is a homobifunctional *N*-hydroxysuccinimide ester (NHS ester) that reacts efficiently with primary amino groups. The procedure to make phalloidin conjugated QDs is described as follows: 0.03 mg of BS3 was added to 50 μl of 4 μM QD solution and incubated for 30 min at room temperature (~25 °C). Excess cross-linker was removed by NAP-5 column (Amersham Biosciences) with PBS (pH 7.4). 6 μg of amino-phalloidin was then added to the solution, and incubated for 2 h with gently mixing at room temperature. To remove the free phalloidin, a 100 kDa ultrafiltration filter (Nanosep, Pall Life Sciences) was used according to manufacturer instructions. The phalloidin–QDs were resuspended in phosphate buffered saline, PBS (pH 7.4) and stored at 4 °C. The final concentration of phalloidin–QDs was determined by measuring the absorbance of the conjugate solution.

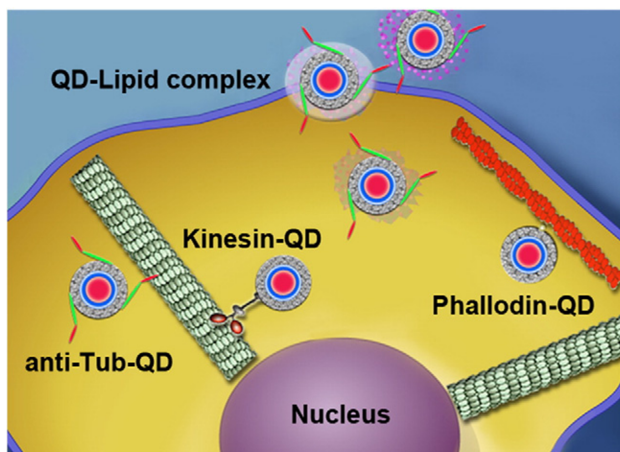

Fig. 1 – A schematic diagram of our intracellular imaging technique. QD–lipid complexes were internalized by the fusion of cellular membrane. Various bioconjugated QDs entered into cytoplasm and the bioactivities were retained in the cells. Phalloidin–QDs and anti-tubulin–antibody–QDs specifically bound to the actin filaments and the microtubules, respectively. Kinesin-QDs moved on the microtubules.

Antibody–QD conjugate

To label microtubules in the living cells, QDs were conjugated to a monoclonal antibody against microtubules. Amino functionalized PEG coated QD 655 (Qdot® nanocrystals, Invitrogen Co.) was linked to anti-bovine α-tubulin mouse monoclonal antibody (isotype IgG1, Molecular Probes). Qdot® Antibody Conjugation Kit (Qdot® nanocrystals, Invitrogen) was used according to manufacturer instructions. The anti-tubulin–QDs were suspended in PBS (pH 7.4) and stored at 4 °C. The final concentration of anti-tubulin–QDs was determined by measuring the absorbance of QDs in the conjugate solution.

Kinesin–QD conjugate

Kinesin-1, 560 amino acids with biotin-tag at C-terminal, from mouse (KIF-5a) was linked to QDs via the streptavidin–biotin reaction. Streptavidin functionalized PEG coated QD 655 (Qdot® nanocrystals, Invitrogen) was conjugated to C-terminal biotinylated kinesin. 5 μl of 1 μM QD solution was diluted by 5 μl of PBS (pH 7.4). Then 30 μl of 1 μM kinesin solution was added to the QD solution and incubated for 20 min at room temperature. The kinesin–QDs were stored at 4 °C.

Internalization of QD-bioconjugates into living cells

Assemblies of QD–lipid complexes were prepared as follows. The bioconjugated QD solution was diluted with 97 μl of serum-free L-15 medium (GIBCO, Invitrogen Co.) to 5 nM, and then 3 μl of lipid reagent (FuGENE® HD, Roche Ltd.) was added directly into the medium containing the diluted QD-bioconjugates. The solution was tapped for mixing, and incubated for 20 min at room temperature. For treatment with the QD–lipid complex, human breast cancer cells, MDA-MB-231 (ATCC Co.), were grown in a glass-bottom dish with L-15 medium containing 10% FBS. The cells were washed with FBS-free L-15 medium, and the culture medium was then exchanged with 1 ml of the FBS-free L-15 medium. The QD–lipid complex was added to the cells (with final concentration of 0.5 nM) and incubated for 3 h. After 3 h of incubation, the cells were washed with PBS buffer to remove the non-binding QD–lipid complex, and then 2 ml of L-15 medium with 10% FBS was added. Observations commenced 2 h after this final addition.

All internalization procedures were performed at 37 °C in an incubator and observed at 35 °C on the microscope except in the QD–kinesin conjugates experiment. In this latter case the QD–kinesin–lipid complex incubation in cells was performed at 27 °C

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