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Development of Dual-Color Simultaneous Single Molecule Imaging System for Analyzing Multiple Intracellular Trafficking Activities*

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Abstract— **Intracellular trafficking is a critical process for cell physiology. Previous extensive studies employing biochemical and molecular biological approaches have provided qualitative information about intracellular trafficking, but we have little quantitative information due to technical limitations of these assays. We therefore developed a novel method for quantifying intracellular trafficking based on single molecule imaging with Quantum dot (QD) fluorescent nanocrystals and quantitatively described the trafficking properties of some recycling proteins. We herein first describe how to label intracellular molecules with QD which has no cell permeability and how to quantify intracellular trafficking, and then we detail the development of a novel experimental system allowing multi-color simultaneous single molecule imaging for analyzing the relationships of intracellular trafficking activities among multiple molecules having distinct trafficking properties. Finally, we document how we confirmed the reliability of our system by simultaneously analyzing the intracellular movements of two recycling protein, GLUT4 glucose transporter and transferrin receptor. Since impairment of intracellular trafficking has critical etiological roles in various late-onset diseases such as type 2 diabetes, our novel imaging system may be a powerful tool for developing next-generation biomedical devices for diagnostics and medical treatment based on intracellular trafficking.**

I. Introduction

Intracellular trafficking is one of the fundamental cellular systems, and dysfunction/impairments of these processes are directly related to various disorders [1]. Conventionally, localization, expression levels and regulatory factors of intracellular trafficking molecules have been extensively studied with biochemical and molecular biological assays. These assays have provided us with qualitative information about intracellular trafficking, but intracellular behavioral characteristics of the trafficking molecules themselves and their regulatory mechanisms remain largely unknown due to technical limitations of the assays used to analyze the collective behavior of numerous molecules. In order to understand the fundamental cellular systems, quantitative descriptions of intracellular trafficking activities are necessary.

Therefore, we previously developed a novel approach based on intracellular single molecule imaging with Quantum dot (QD) fluorescent nanocrystals, characterized by fluorescence which is extremely bright and stable compared to that of traditional fluorescent molecules like fluorescent proteins [2-4]. With this approach, we succeeded in quantifying the trafficking activities of certain recycling proteins such as the glucose transporter GLUT4, which mediates insulin- and exercise-facilitated glucose uptake, and transferrin receptor (TfR), which mediates iron uptake into cells via binding with its ligand transferrin. These studies revealed that there are at least two components of intracellular GLUT4 movement, and that the ratios of various components can be altered by various physiological/pathological conditions. On the other hand, we have as yet found no significant changes in TfR movement as far as we examined.

These results were based on population studies, *i.e.* independent imaging of each molecule. However, considering biomedical applications, trafficking activity should be precisely and directly compared among multiple molecules in the same cells. This can be achieved by multi-color simultaneous single molecule imaging. Therefore, we herein developed a novel imaging system allowing such imaging, and then confirmed the reliability of the system by analyzing intracellular movements of GLUT4 and TfR.

II. Experimental Methods

All experiments were performed in murine 3T3L1 fibroblasts. QD-conjugated Fab fragment of anti-myc antibodies were prepared with Fab Preparation Kit (Pierce) and QD-Antibody Conjugation Kit (Invitrogen). Biotinylated transferrin and QD-conjugated streptavidin were purchased from Invitrogen. Imaging experiments were performed with a home-made microscope consisting of an inverted microscope (IX71, Olympus), an electron-multiplying charge-coupled device (EMCCD) camera (iXon 887, Andor Technology), a Nipkow disk confocal unit (CSU10, Yokogawa), and an oil-immersion objective lens (UPLSAPO100×O, Numerical Aperture of 1.4, Olympus) (see Fig. 3(a)). QDs were excited at 532 nm (0.7 mW) and the fluorescent images were acquired at 20 frames per second for 15 seconds (300 frames). Determination of the position and movement of individual QD fluorescence was performed by G-Count (G-Angstrom) with a two-dimensional Gaussian fitting within an 8×8 pixel region-of-interest.

III. Results

A. QD Labeling of Intracellular Molecules

To track single molecule movements within cells, we intended to label intracellular interest molecules with QD fluorescent nanocrystals. However, since QD itself has no cell permeability, it is impossible to label intracellular molecules employing only extracellular administration. Therefore, to

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label intracellular typical recycling proteins GLUT4 and TfR, we developed a strategy for labeling as follows. For labeling of GLUT4, we used cells exogenously expressing exofacially myc-tagged GLUT4 (myc-GLUT4-ECFP) (Fig. 1 (a)) and the QD-conjugated Fab fragment of anti-myc antibodies (Fig. 1 (b)). First, the cells were treated with 5 nM QD-conjugated anti-myc antibodies for 1 h to bind cell surface myc-GLUT4-ECFP. Then, the cells were extensively washed to remove excess (unbound) QD-conjugated antibodies, followed by an additional incubation for at least 3 h for allowing the labeled proteins to undergo internalization. The QD-labeled transferrin was prepared by mixing 10 nM biotinylated transferrin and 10 nM QD-conjugated streptavidin in the presence of an excess (100×) of the amount of biotin (1 μM) needed to achieve a single transferrin per QD (Fig. 1 (c)) [5]. For cellular labeling, the cells were treated with the QD-labeled transferrin for 15 min. We previously found that 1) the most fluorescent particle contained a single QD, 2) QD-labeled proteins behave similarly with endogenous proteins and 3) very sparsely labeled proteins are representative of entire protein pool [2]. Employing these approaches, we were able to observe intracellular individual GLUT4 or TfR as QD fluorescence (Fig. 1 (d)).

B. *Quantification of Intracellular Movement*

To track the movement of individual molecules, we next determined the position of individual QD fluorescent spots and then connected the detected dots (Fig. 2 (a) and (b)). We typically tracked 40–70 particles per cell, and such very sparse labeling is important for accurate single particle tracking. We quantified the movements with two distinct parameters. The first was the velocities which were calculated by linear fit of the displacement during 4 frames (Fig. 2 (c)). We constructed "velocity distributions" representing the fraction of movement having the identified speed. The second was diffusion coefficient maps which were constructed as follows. First, we calculated the mean-square displacement (MSD) of individual molecules, which represent the average square distance a given particle traveled, with the following equation:

$$MSD(n\Delta t) = 1/(N-n)\cdot\sum_{i=1}^{N-n}(p_{i+n}-p_i)^2 \qquad (1)$$

where N, n, Δt and p are the total number of positions measured, the measurement index going from 1 to N–1, the time interval between two consecutive image sequences and positions of the molecule, respectively [6]. In two dimensional movements, the slope of MSD *versus* $n\Delta t$ plots is proportional to four times of the diffusion coefficient of the molecules (Fig. 2 (d)). Therefore we calculated the diffusion coefficient of the molecule by fitting the first 10 time-points of the MSD *versus*

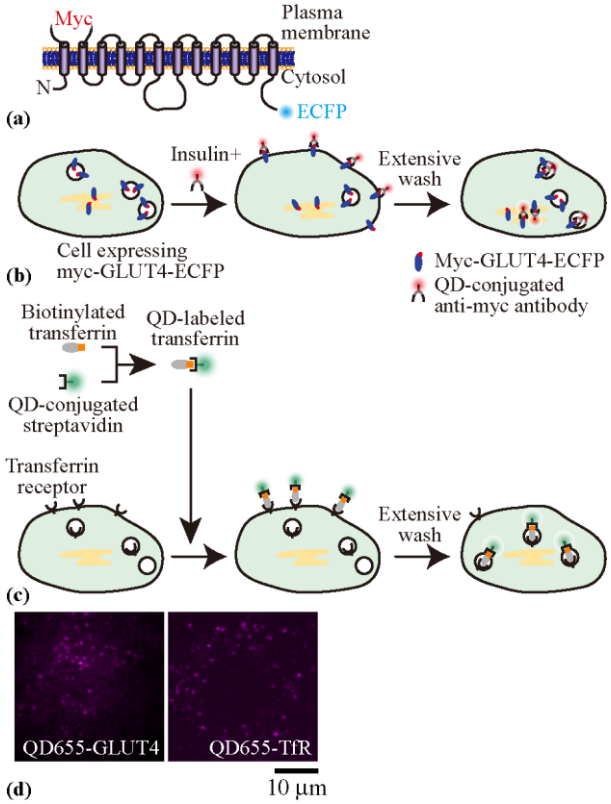

Figure 1. Strategies for QD labeling of GLUT4 and transferrin receptor. (a) Structure of exofacially myc-tagged GLUT4. (b and c) Labeling protocols of mycGLUT4 (b) and transferrin receptor (c) with QD. (d) Snapshots of QD fluorescence in cells treated with QD655-conjugated anti-myc antibodies (left) or QD655-labeled transferrin (right).

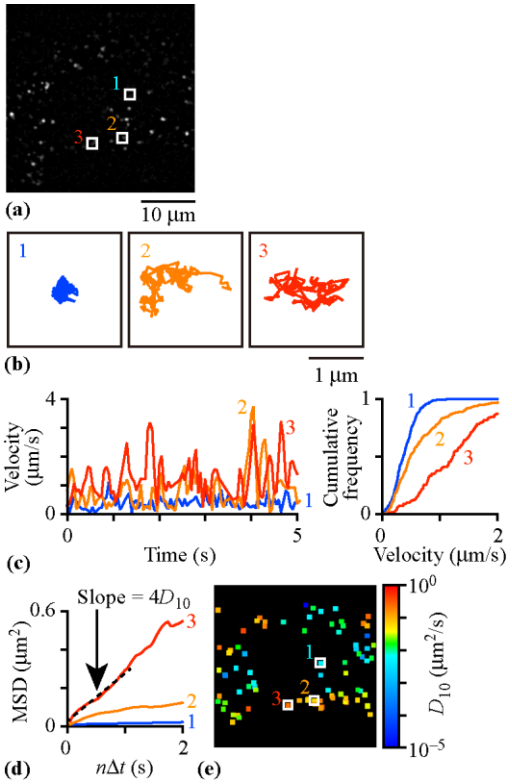

Figure 2. Quantification of intracellular movement based on single molecule nanometry. (a) Snapshot of QD fluorescence in cells treated with QD655-conjugated anti-myc antibodies. (b) Trajectories of three particles shown in (a). (c) Velocity versus time plots (left) and cumulative velocity distributions (right) of three particles shown in (b). Note that rightward shift of the distribution (right) represents increases in the number of fast-moving molecules. (d) MSD curves of three particles shown in (b). As shown in (2), the slope of each curve represents four times of diffusion coefficient of the molecule. (e) Diffusion coefficient map. Each dot represents an individual molecule, and a pseudo-color coding displayed on the right is used to represent the diffusion coefficients of the molecules.

$n\Delta t$ plots with

$$MSD(n\Delta t) = 4D_{10}n\Delta t + C \qquad (2)$$

where D_{10} and C are the diffusion coefficient of the first 10 time-points and instrumental noise, respectively. Finally, diffusion coefficient maps were constructed based on D_{10} values (represented in color) and the positions of the particles (Fig. 2(e)).

C. *Development of an Experimental System for Multi-color Simultaneous Intracellular Single Molecule Imaging*

All available QDs have broad excitation and narrow fluorescent spectrum [7], *i.e.* QDs are suitable for multi-color imaging and therefore analyzing multiple intracellular trafficking activities simultaneously by using two or more QDs having distinct fluorescent spectrum excited at single wavelength. We first developed a novel imaging system for simultaneous imaging of two distinct QDs by installing emission splitting optics on previously developed home-made microscope consisting of a Nipkow disk confocal unit and an ultra-sensitive EMCCD camera (Fig. 3 (a)). This optics system consists of a dichroic mirror for separating the fluorescence of the two QDs, a movable lens for compensating aberrations (Fig. 3 (b)). All mirrors can adjust the reflection angles for projecting the two separated fluorescent images into a single EMCCD camera. With this imaging system, we were able to observe optical images with two distinct wavelengths derived from the identical focal plane simultaneously with single EMCCD camera (Fig. 3 (c)). For fine alignment of the horizontal position between the two images having different wavelength, we daily took a bright-field image of a test pattern (Fig. 3 (c)). The horizontal position was first roughly aligned by adjusting the reflection angle of the mirrors in the emission splitting optics system before each experiment, and then precisely aligned manually by pixel shift.

D. *Simultaneous Imaging of Multiple Trafficking Molecules*

For dual-color simultaneous imaging, we tried various combinations of QDs for labeling of intracellular GLUT4 and TfR respectively, and chose QD565 and QD655 because of their brightness, *i.e.* we mainly used QD655-conjugated Fab fragment of anti-myc antibodies for labeling of exogenously expressed myc-GLUT4-ECFP and streptavidin-QD565 for labeling of TfR by using transferrin-biotin. We also confirmed that the reverse combination of QD (*i.e.* QD565-conjugated anti-myc antibodies for labeling of myc-GLUT4-ECFP, streptavidin-QD655 for TfR labeling) showed similar behavior (not shown). The fluorescence of these two QDs can be divided by a FF593-03 dichroic mirror. With these QDs, we next attempted to simultaneously observe the two distinct trafficking molecules. The labeling strategy was the same as shown above except for sequential labeling of myc-GLUT4-ECFP and TfR, *i.e.* myc-GLUT4-ECFP was first labeled, and TfR labeling was performed during washing of the QD-conjugated anti-myc antibodies. By this strategy, we successfully observed the fluorescence of these two distinct QDs simultaneously (Fig. 4 (a)).

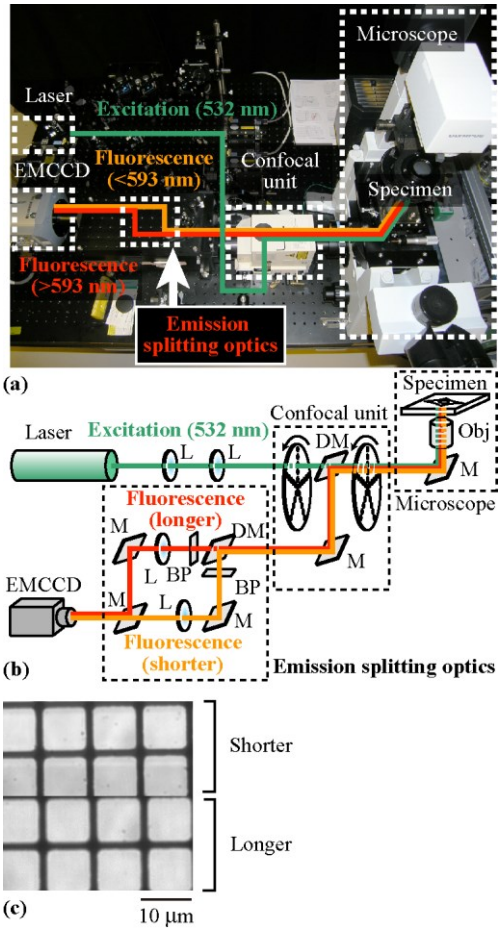

Figure 3. Experimental system for dual-color simultaneous single molecule imaging. (a and b) Photograph (a) and diagram (b) of imaging system used. Green line represents excitation light path, and orange and red lines represent emission light paths. Note that optical paths are simplified. An emission splitting optics system was used for dividing the fluorescence of two distinct QDs and projecting this fluorescence into a single EMCCD camera. (c) An image of a test pattern of a square lattice (10 μm). The upper and lower halves are images with shorter and longer wavelength, respectively.

We previously found that, by population studies, 1) intracellular movements of GLUT4 and TfR were very similar in control (native) cells and in contrast 2) exogenous expression of sortilin, a Vps10p family sorting receptor that is involved in intracellular sorting between endosomes and *trans*-Golgi networks, selectively generated static (restricted) movement of GLUT4 but not TfR [3]. We therefore decided to take advantage of these characteristics to confirm the reliability of our novel imaging system. As expected, the movements of GLUT4 and TfR were very similar in control cells (Fig. 4 (b) and (c)), while GLUT4 movement was selectively reduced in cells expressing sortilin (Fig. 4 (d) and (e)), indicating that our novel imaging system should be reliable and can analyze intracellular movement of these two proteins simultaneously. In cells expressing sortilin, we found that GLUT4 has a tendency to be localized in more inner regions compared to TfR, consistent with our previous results by immunofluorescent observations [3].

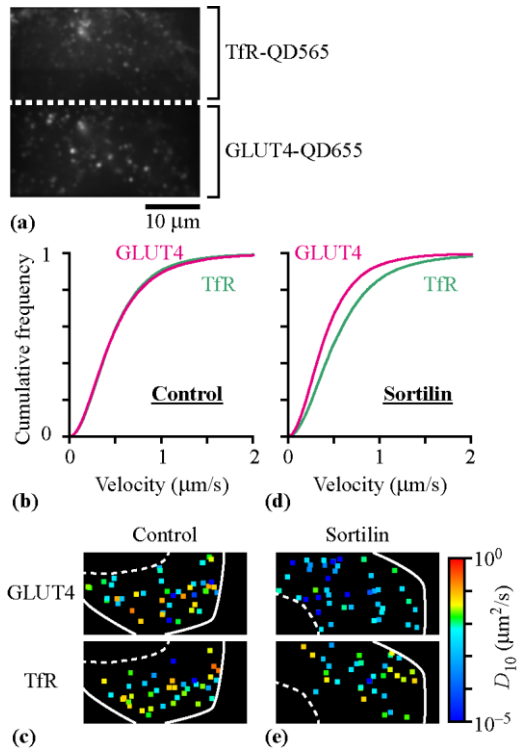

Figure 4. Simultaneous tracking of two distinct trafficking molecules. (a) Snapshot of QD fluorescence in cells treated with QD655-conjugated anti-myc antibodies and QD565-labeled transferrin. (b–e) Velocity distributions (b and d) and diffusion coefficient maps (c and e) of GLUT4 (magenta in (b) and (d), upper in (c) and (e)) and TfR (green in (b) and (d), lower in (c) and (e)) in control cells (b and c) and cells exogenously expressing sortilin (d and e). In velocity distributions, the data were obtained from at least 3 cells. The movement of GLUT4 and TfR in cells expressing sortilin was significantly different (p<0.01). In diffusion coefficient maps, a pseudo-color coding displayed on the right is used to represent the diffusion coefficients of the molecules.

IV. Conclusion and Future Plans

Movements of intracellular molecules have traditionally been monitored by using classic fluorescent molecules like fluorescent proteins. However, such observations can only detect collective behaviors derived from numerous molecules. Since the processes of intracellular trafficking are extremely complex involving heterogeneous intracellular elements including organelles, vesicles and tubulovesicular structures, such collective analyses cannot dissect the trafficking processes into discrete experimentally traceable steps. In contrast, our approach based on single molecule imaging successfully performed such dissection by taking advantage of the capacity to track individual molecules. We herein reliably developed a novel imaging system allowing us to simultaneously analyze intracellular trafficking behaviors of multiple molecules having distinct trafficking properties. This approach has allowed, for the first time, to provide a multilateral analysis of intracellular movement properties of various molecules such as mobility, localization and populations in cells by taking fluorescent images for only 15 seconds. Such analyses are anticipated to provide us with comprehensive understanding of intracellular trafficking activities.

Insulin and exercise facilitates translocation of GLUT4 from intracellular storage compartment to the plasma membrane [8]. Given the importance of insulin- and exercise-dependent GLUT4 translocation for maintaining healthy glucose homeostasis, it is clear that a detailed understanding of the GLUT4 trafficking events could have major implications for our knowledge about normal physiology. In addition, under insulin resistance, a characteristic of type 2 diabetes, insulin-dependent GLUT4 translocation was markedly impaired, and it had been widely considered that insulin resistance is caused by defects in insulin signaling. In this regard, we previously revealed the behavioral nature of GLUT4 trafficking and the functional aspects of insulin [2]. Importantly, we also revealed detrimental derangements in GLUT4 behaviors under certain insulin resistant condition, strongly suggesting that such "sorting/trafficking defects" also have critical etiological roles in insulin resistance [3]. We suppose that there are equilibriums of intracellular trafficking activities that can be finely adapted to various cellular circumstances, and the resulting transitions of the equilibriums are involved in various cellular responses. Differences in GLUT4 behavior between presence and absence of sortilin (Fig. 4 (b–e)) is one typical example of the transition. By comparing with appropriate control molecules such as TfR, comprehensive analyses of intracellular trafficking activities with our novel imaging system would contribute to reveal such transitions in trafficking activities involved in various physiology and pathophysiology.

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