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Single Molecule FRET Combined with Defocus Imaging

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1. Introduction

Fluorescence resonance energy transfer (FRET) is a physical process where the excitation energy of a donor molecule is transferred to a neighboring acceptor molecule via dipole-dipole interaction when the donor and acceptor molecules approach within a few nano meters. FRET efficiency depends on an intermolecular distance between the donor and acceptor molecules. Therefore FRET is widely used to measure distances between specific positions of biomolecules or proteins at a nano scale and clarify their dynamics.

FRET efficiency is dependent on not only an intermolecular distance but also relative orientation between the donor and acceptor molecules. The relative orientation in FRET is characterized by an orientation factor ($\varkappa$). So far an averaged value ($\kappa^2 = 2/3$) has been used as an orientation factor in FRET measurements where the molecules are randomly oriented. In order to measure the intermolecular distances accurately, relative orientation between the donor and acceptor molecules should be taken into account.

In this presentation, we demonstrate accurate measurements of intermolecular distances between donor and acceptor molecules at a single molecular level by combining FRET measurements with defocus imaging for measuring molecular orientations (emission dipole moments) of both donor and acceptor fluorescence molecules.

2. Experiment

Cy3 (donor) and Cy5 (accepter) fluorescence molecules that are connected with double stranded DNA (14 bp) were used for single molecule FRET measurements. To immobilize the motion of the molecules, the molecules were embedded in polyvinyl alcohol. A Nd:YAG laser (532 nm) was used for FRET measurement and determination of Cy3 orientation, while a He-Ne laser (633 nm) was employed for determination of Cy5 orientation. Fluorescence emitted by the molecules was separated into Cy3 and Cy5 fluorescence channels by a dichroic mirror. The both fluorescence signals were imaged with an EMCCD camera.

3. Results and Discussion

Figure1 (a) and (b) show the fluorescence patterns of Cy3 and Cy5 molecules in defocus imaging, respectively. Clear asymmetric fluorescence patterns were obtained. The dark lines in the patterns correspond to the directions of dipole moments. The direction of emission dipole moments of Cy3 and Cy5 molecules were rigorously determined by comparing these experimentally obtained fluorescence patterns with the calculations based on dipolar fluorescence radiation. We found that Cy3 and Cy5 molecules were oriented each other with 61 degrees.

Figure2 (a) and (b) show that the fluorescence images of the Cy3 molecules before and after the paired Cy5 molecules were bleached, respectively. It can be seen that the fluorescence intensity of the target molecule was decreased because FRET was terminated due to the Cy5 bleaching. FRET efficiency can be determined by the ratio of the fluorescence intensities from Cy3 molecules in the presence and absence of Cy5 molecules. It should be noted that we purposely bleached Cy5 molecules to make a situation of absence of Cy5 molecules. FRET efficiency was found to be 0.12.

An intermolecular distance between Cy3 and Cy5 molecules was calculated to be 5.7 nm from FRET efficiency and the relative molecular orientation ($\kappa^2 = 0.24$). This value is in good agreement with a value determined by the number of bases of DNA (5.5 nm). In contrast, the intermolecular distance was found to be 6.8 nm if the relative molecular orientation was not considered ($\kappa^2 = 2/3$). These results imply that accurate measurements of intermolecular distances were realized by taking the relative molecular orientation into account.

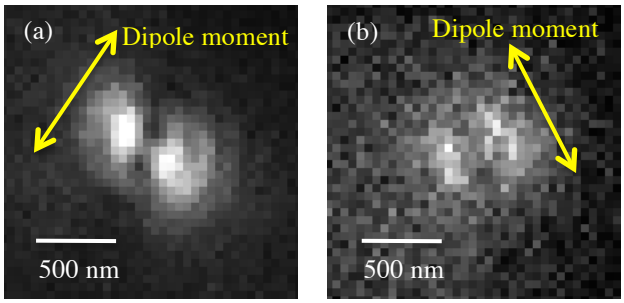

Fig.1 Fluorescence intensity patters of (a) Cy3 and (b) Cy5 in defocus imaging (ΔZ = 1.0 μm).

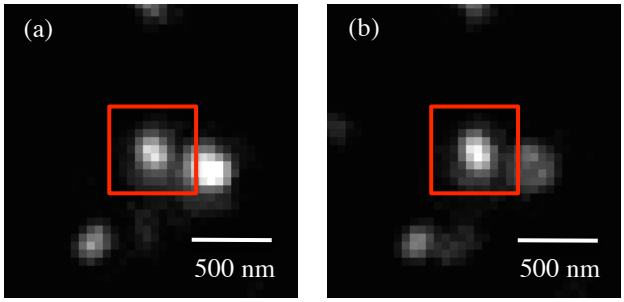

Fig.2 Fluorescence images of Cy3 (a) before and (b) after Cy5 bleaching. The red squares indicate positions of the target molecule.