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Dissection of the angle of single fluorophore attached to the nucleotide in corkscrewing microtubules

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ABSTRACT

Direct dissection of the angles of single fluorophores under an optical microscope has been a challenging approach to study the dynamics of proteins in an aqueous solution. For angle quantifications of single substrates, however, there was only one report (Nishizaka et al., 2014) because of difficulties of construction of experimental systems with active proteins working at the single-molecule level. We here show precise estimation of orientation of single fluorescent nucleotides bound to single tubulins that comprise microtubule. When single-headed kinesins immobilized on a glass surface drive the sliding of microtubules, microtubules show corkscrewing with regular pitches (Yajima et al., 2005 & 2008). We found, by using a three-dimensional tracking microscope, that S8A mutant kinesin also showed precise corkscrewing with a 330-nm pitch, which is 13% longer than that of the wild type. The assay with the mutant was combined with a defocused imaging technique to visualize the rotational behavior of fluorescent nucleotide bound to corkscrewing microtubule. Notably, the defocused pattern of single TAMRA-GTP periodically changed, precisely correlating to its precession movement. The time course of the change in the fluorophore angle projected to the *xy*-plane enabled to estimate both the fluorophore orientation against microtubule axis and the precision of angle-determination of analyses system. The orientation showed main distribution with peaks at~40°, 50° and 60°. To identify their molecular conformations, the rigorous docking simulations were performed using an atomic-level structure modeled by fitting x-ray crystal structures to the cryo-electron microscopy map. Among isomers, 2′-*O*-EDA-GDP labeled with 5- or 6-TAMRA were mainly specified as possible candidates as a substrate, which suggested the hydrolysis of TAMRA-GTP by tubulins.

1. Introduction

Techniques to visualize biological molecules in an aqueous solution have been developed with the progress of single molecule biophysics. We have so far made various efforts to tackle this subject with the development of optical microscopes [1–4]. Additional approach is the detection of orientation of probes, which enabled to address critical features of proteins directly. Localization of each molecule can be determined as 'point spread function' of the image of a fluorescent probes under an optical microscope, and has helped to understand mechanisms of various molecular machinery at the molecular level. Example of this are the rotational motion of a filamentous structure [5] or a rotary motor [6], the tilting of the lever part of myosin motor [7], the angle change in a single α-helix of immobilized protein [2], and so on. In despite of these contributions, one important aspects has been lacking in visualizations of biomolecules: the angle detection of substrates bound to enzymes. How substrates are oriented in specific catalytic sites is one of the most important questions to understand mechanisms of enzymes, and this point is always highlighted in atomic models in X-ray crystallography to explain how they work as efficient catalysts. At the single molecular level in contrast, there was only one report to detect the angle and binding-release process of substrates [8], and

Abbreviations: MT, microtubule; ATP, adenosine-tri-phosphate; PSF, point spread function; iTIRFM, isotropic total internal reflection fluorescence microscope.

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thus new evolutionary progresses have been awaited. We here address this point using *in vitro* sliding assay of microtubules (MT) driven by single-headed kinesins. We previously reported that surface-immobilized single-headed kinesins have the ability to drive stable corkscrewing of MT with a pitch independent of its helical structure [9,10]. In the present study, we attempted to establish the method to measure the absolute angle of fluorescent nucleotide that binds to tubulin by the use of this corkscrewing characteristic.

2. Materials and methods

2.1. Microscopy

We used an inverted microscope (Ti-E, Nikon Instruments) equipped with a × 100 objective (Plan Apo TIRF, NA 1.49, Nikon Instruments). The position of the objective was stabilized with the commercial 'perfect focus system' after the calibration of the dial adjustment with piezoelectric element (Physik Instrumente). For visualization of single fluorophores, a green laser beam (wavelength of 532 nm; Chukosha) was introduced into a microscope (TE2000E, Nikon Instruments) to construct isotropic TIRFM, which had been developed by our research group, with hollow cone illumination using a diffractive diffuser (D074, Frankfurt Laser Company), custom-made dichroic mirror to keep laser polarization after reflection (Chroma) and two emission filters (NF03-532E & NF01-532U, Semrock). The basic designing and construction of isotropic TIRFM were previously described [11,12]. For more details, see Supplementary Methods.

2.2. Defocused imaging

Each defocused image acquired through EMCCD camera was analyzed 3D steerable filters as an image formation model [13]. For more details, see Supplementary Methods.

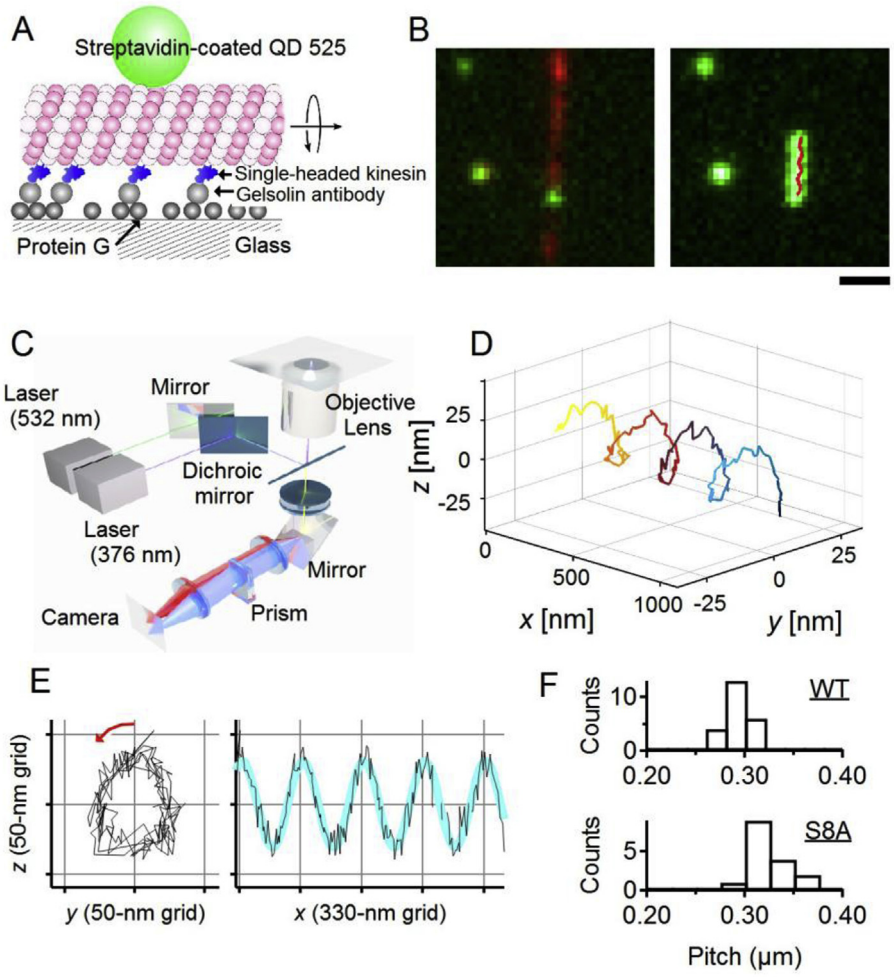

Fig. 1. 2D and 3D tracking of quantum dots biding to MT that slides on single-headed surface-immobilized kinesin mutant. (*A*) Schematic of the *in vitro* microtubule (MT) sliding assay with quantum dots (QD525). Single-headed kinesin was genetically fused to a large protein, gelsolin, and anchored to the glass surface through its antibody and protein G. (*B*) A merged fluorescence image of a rhodamine-labeled microtubule (*left*, red) and QD525 (*left*, green). 2D tracking of the center of the QD (*right*, red line) clearly showed its sinusoidal movement, which indicates corkscrewing of MT. Scale bar, 1 μm. (*C*) Schematic of 3D tracking of QD. QD and rhodamine were excited by green and UV laser, respectively. Single image of QD was split into two components of light (red and blue) by wedge prism. Relative displacements of split image of QD in sequential images directly tells motions along optical axis. The whole optics and microscope were set into a compartment, in which the temperature was controlled to ±0.1 °C, to avoid any drifting motion of the sample stage. (*D*) An example of 3D plot of a single QD attached to a sliding microtubule driven by single-headed S8A kinesin for 15 s with intermittent color code 'yellow → blue'. (*E*) An example of *yz*- (*left*) and *xz*- (*right*) plots of corkscrewing motion. (*F*) Histograms of pitches of the wild type (*top*; $n = 20$) and that of S8A mutant (*bottom*; $n = 16$). The pitch became longer by introducing the mutation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)