MERIT Self-directed Joint Research

Dynamic 3D behavior of dynein using DXT

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Abstract : We measured the minute behavior of microtubule-interacting dynein between ATP and Apo states using Diffracted X-ray Tracking (DXT) method. We confirmed that 3 to 6 times larger dynamic motion (θ: 0.79 mrad, χ: 1.26 mrad) coexisted between 50 to 100 μs time resolution. This dynamics must correspond to the motion of the linker of dynein on the phase transition between ADP and Apo states, which shows a precious finding of dynein as 3 dimension dynamics.

1. Introduction of authors

Yoshimi Kinoshita: Specialty is motor proteins in biophysics. She has focused on the mechanics of human cytoplasmic dynein. In this study, she designed and adjusted the materials enough to measure its minute behavior.

Yufuku Matsushita: He has been tackling the physical measurement of local solution structure for the supersaturated solution. In this study, he was in charge of the measurement setup and data analysis of DXT.

2. Background and purpose

Cytoplasmic dynein, 'dynein', is a motor protein, which transports cargos along microtubules in the cell with the energy of ATP hydrolysis (Fig.1(A)). The field of motor proteins has been dramatically improved by many biochemical experiments, especially fluorescent-microscopy imaging and optical tweezers system as single molecule tracking techniques since 1990s. These methods lead 2-dimensional behavior detection of proteins (nm-precision), due to the low accuracy of vertical position to the substrate. Dynein causes the large conformational changes in the process of the power stroke[1]. Moreover, the conformations of dynein both in ADP and Apo state, that are post power stroke states, differ a bit from the crystal structure studies (Fig.1(B)) [2, 3]. However, these minute dynamical changes

have not been observed yet.

Here, we used the diffracted x-ray tracking (DXT) method for tracking the dynamic 3D behavior of dynein. We utilized and tracked gold nanocrystals (20-50 nm size), and detected the trajectories of single diffraction spots by x-ray irradiation with ultrafast time resolution (μs) and high position precision (pm). So far, dynamic molecular motion and protein-protein interaction have been reported by DXT method, such as the single-molecule motion of immunogenic MHC II protein[4], ligand-induced motion of membrane protein nAChR[5], and ATP dependent rotational motion of group II chaperonin[6].

In this study, we demonstrated the dynamic 3D behavior of dynein between ADP and Apo states by DXT method. The dynein motor domain, which consists of the most essential part including ATP binding sites, bound with gold nanocrystal at the N-terminal of dynein. We fixed dynein on the microtubule and monitored the rotational motion of nanocrystals at low ADP concentration.

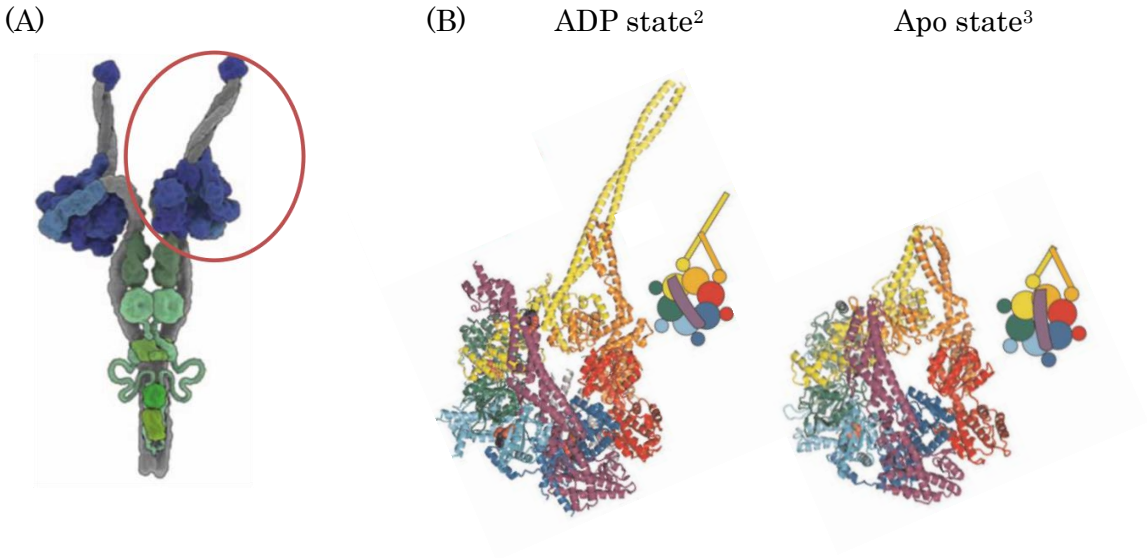


Fig.1 (A) Representation of full-length dynein (revised from ref[7]). Dynein consists of multiple domains. Motor domain (shows as red circle) is essential for its motility. (B) The minute different conformations of dynein motor domain in ADP and Apo states in crystal studies (revised from ref[8]). Microtubule binding domain exists at the tip (not displayed in figures). The N-terminal tip of the linker (the upper side, colored purple) must move a bit, comparing two structures.

3. Materials and methods

3.1 Pre-experiment with microscope

We built human dynein motor domain construct, whose molecular weight is ~384 kDa, and a biotin-carboxyl carrier protein was inserted at its N-terminal. We expressed it with Baculovirus system, purified and confirmed its motility. Microtubules were purified from bovine brain, and were labeled with rhodamine only for fluorescent imaging observation.

Before DXT experiment, we prepared the samples and adjusted these conditions. To observe the fluorescent images and scattered images of samples by microscope, we used cover glasses as the substrate, instead of polyimide film[8].

We established the sample between two cover glasses as follows (Fig.2(A)). With NHS ester on the surface, the glasses were treated with air plasma cleaner for 3 minutes, and were incubated with amino silane for 30 minutes and 50 mg/ml BS^3 overnight at room temperature. We incubated microtubules (~2 μM as dimer tubulin) for longer than 5 hours, blocked with 2 mg/ml casein for 5 minutes, reacted with dynein-colloid (mixing 20 nM dynein plus 50pM avidin-coated gold colloid (30nm in diameter) with avidin-biotin reaction) for 5 minutes and observed images at 20 μM ADP at room temperature. Firstly, we confirmed that no steric clash happens to interact with microtubules and dynein-colloid with samples floating in the chamber (Fig.2(B)). Then, we observed the images that gold colloids stand along the microtubules (Fig.2(A),(C)). Based on these conditions, we conducted DXT experiments.

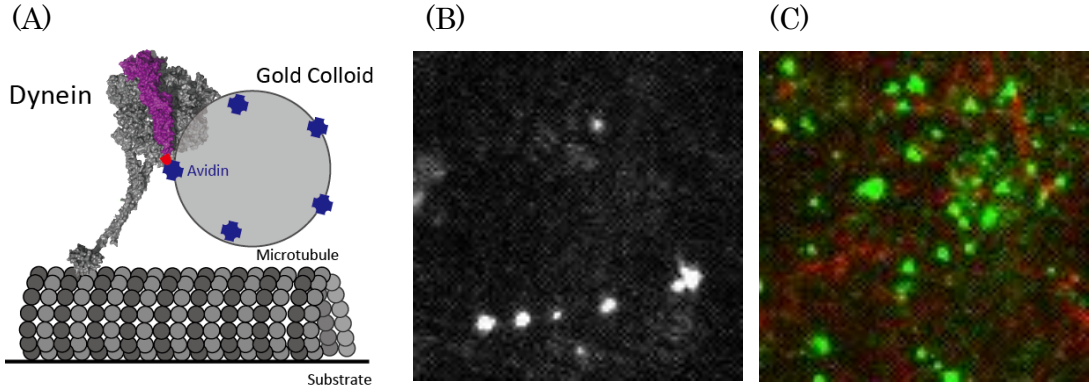


Fig.2 (A) The schematic diagram of microscopy observation (dynein pdb: 3VKH). (B) The scattered image of gold colloids along microtubules with floating from the substrate. (C) The superposed image of microtubules (fluorescent image, colored red) and gold colloid (scattered image, colored green). (B,C: the full scale of image is 19.3μm×19.3μm.)

3.2 Sample preparation for DXT

3.2.1 Avidin-coated gold nanocrystals

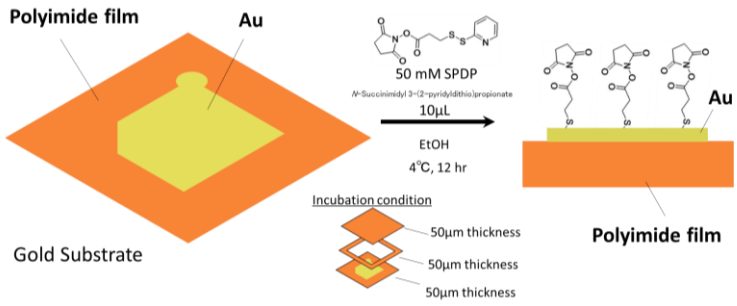
Gold nanocrystals (0.015 pM) were fabricated by dissolving three KCl (111) substrates in the buffer (25 mM PIPES, 25 mM K-acetate, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.2). The size of gold nanocrystals was estimated 20 - 80 nm from AFM observation. Gold nanocrystals were incubated with 2mg/ml NeutrAvidin overnight at 4°C, and the pellet (centrifuged 7,200×g, 2 minutes×2) was replaced to the buffer for excluding the free avidin[9].

3.2.2 Chamber preparation

Polyimide films were coated with gold by vapor deposition, and were incubated with 50 mM SPDP for over 12 hours at 4°C. After this process, samples (~5 μl) were reacted by sandwiching films and free samples were washed by the buffer or were removed by air duster (Fig.3(A)).

After treating SPDP, we fixed microtubules (~2 μM as dimer tubulin), 2 mg/ml casein for blocking the substrate surface, and 20 nM dynein for 5 minutes each at room temperature. We incubated gold nanocrystals for long time before observation. After we add 20 μM ADP, we developed the sample-coated film holder and fixed it to the apparatus (Fig.3(B)).

(A)



(B)

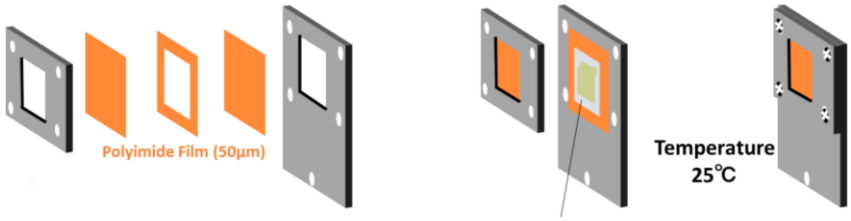


Fig.3 (A) The treatment of the substrate. (B) The fixing of the film holder after sample adjustment.

3.3 Diffracted X-ray Tracking; DXT

DXT is the single molecule measurement method to detect two-axis rotational motion (tilting precision θ: 0.18 mrad, and twisting precision χ: 0.79 mrad) of a gold nanocrystal in the solution by irradiating white x-ray with the energy widths (14.0 - 16.5 keV). The schematic diagram of the gold nanocrystal-labeled dynein motion on the microtubule was shown in Fig.4. We conducted experiments at SPring-8 BL40XU with the time resolution of 100, 50, 25 μs/frame (total: 10 ms) on December 2015 and June 2016.

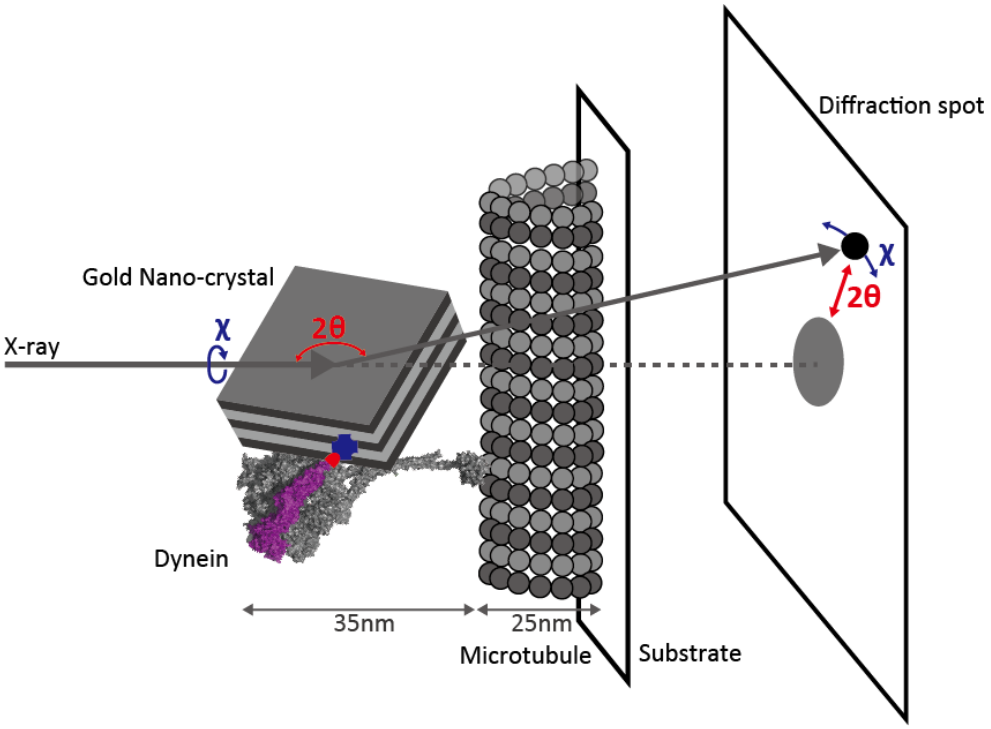


Fig.4 The schematic diagram of gold nanocrystal-labeled dynein motion on the microtubule.

4. Results and discussion

Time resolved traces of x-ray diffraction spots from gold nanocrystals labeled on individual dynein protein are shown in the Fig. 5. The amount of traces from 100, 50, 25 μs/f conditions are 5936, 2538, 1692 respectively. Although the observable maximum angle of θ direction according to Bragg's law is approximately 29 mrad, traces do not exceeded 25 mrad even under these three different conditions. On the other hand, one of the traces had been reached approximately 100 mrad in χ direction. The dynamic range of the χ direction is not limited by Bragg's law and it is able to observe 6.28 rad (360°) as a maximum angle. In this study, we handled 0.2 ms as a maximum time scale in order to perform statistical processing.

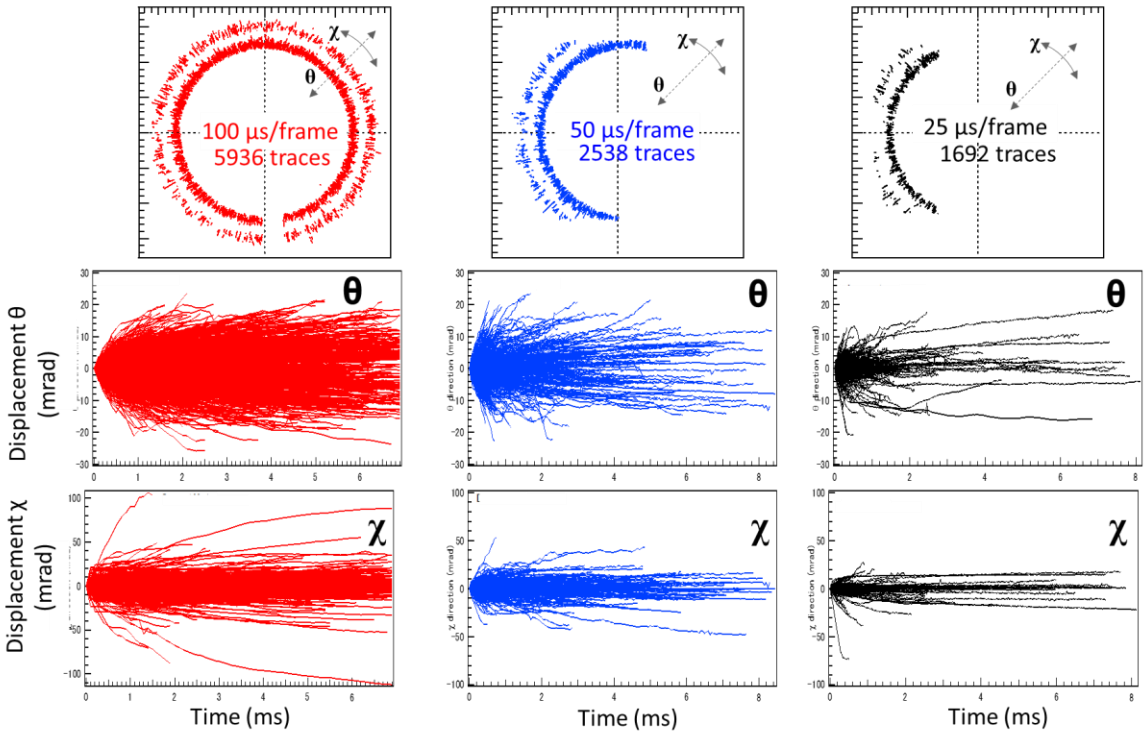


Fig. 5 Traces of diffraction spots by DXT measurement in θ and χ direction with the lapse of time.

Fig. 6 show the Means Square displacement (MSD) of gold nanocrystals during 0.2 ms by each time scale, as well as the each MSD was fitted by single

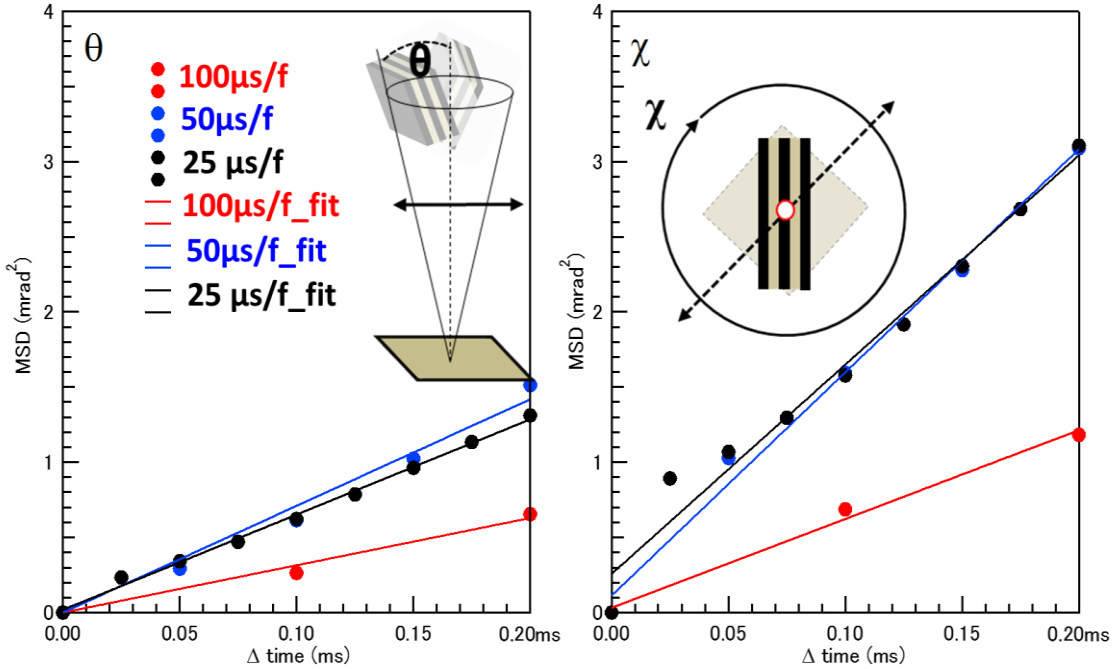


Fig. 6 Mean Square displacement (MSD) in θ and χ direction for each time resolved condition.

dimensional diffusion equation (1)[10].

$$MSD = y + 2Dt \qquad (1)$$

y, D, and t, are intercept, diffusion constant (mrad²/ms) and time (ms) respectively. All of the fitting results are shown in the Table 1.

		D ***[mrad²/ms]***	***Y*** ***[mrad²]***
θ direction	100 μs	1.57 ± 0.20	0.00 ± 0.05
	50 μs	3.55 ± 0.28	0.00 ± 0.07
	25 μs	3.17 ± 0.07	0.02 ± 0.02
χ direction	100 μs	2.96 ± 0.28	0.03 ± 0.07
	50 μs	7.43 ± 0.39	0.11 ± 0.10
	25 μs	6.97 ± 0.41	0.26 ± 0.10

Table 1 Fitting results for each Mean Square displacement in θ and χ direction

From the above results, only 100 μs/f time resolved condition remarked small values of diffusion constant D in both of the directions, compared with the other two conditions. In addition, it is confirmed that the intercept value y was increased as the time resolution becomes higher. This tendency is considered as the motion of the gold nanocrystals on the dynein molecule is required a higher time resolution measurement as sub-micro second order to analyze the complex dynamics of dynein molecule in detail. In this reports, we will discuss the difference between 100 and 50 μs time resolved condition to pick up the slightly difference of the transitional dynamics of Apo and ADP state in the several tens-micro second time scale.

In order to analyze the difference of the time resolved condition, we demonstrated the Log-normal distribution of rotational angles in θ and χ in Fig. 7. From the results, the distribution of 25 and 50 μs/f condition are shifted to right side compared with the 100 μs/f condition. In particular, the tendency is appeared

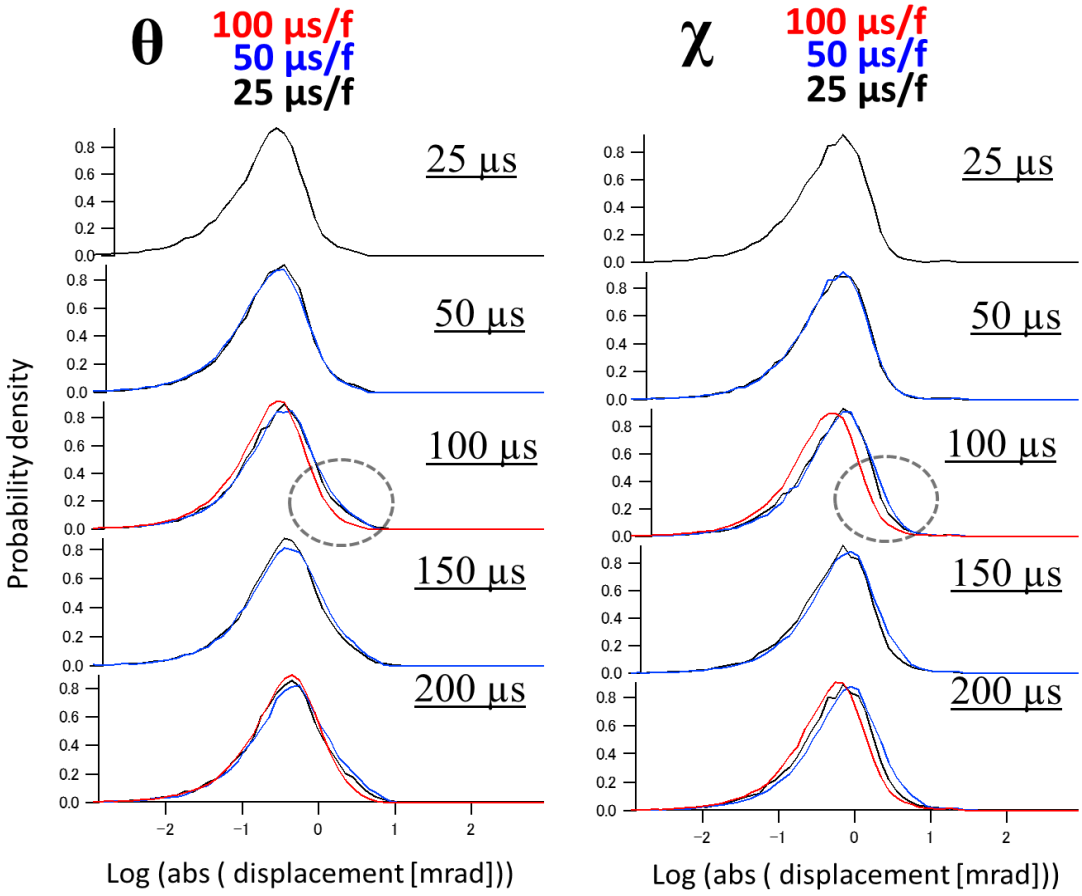


Fig. 7 Log-normal distribution in θ and χ direction in each time resolved condition.

strongly in χ direction. It is considered that dynamic ranges do not covered the gold nanocrystals dynamics in 100 μs/f condition in spite of the difference between 50 and 25 μs/f condition had not been remarkable changed. Based on the results, it is expected that a characterized motion of dynein molecules in Apo and ADP transition is appeared in 100 – 50 μs time scale.

Finally, in order to demonstrate the stereographical dynamic behavior of dynein molecule, we tried to calculate the two-dimensional histogram produced by integrated process of log-normal distribution in θ and χ direction for each axis. Fig. 8 (A) and (B) indicate the histograms of 100 and 50 μs/f condition. In addition, Fig. 8 (C) show the subtracted histogram by subtracted 100 μs/f condition from 50 μs/f condition. Peak positions of two-dimensional histogram of 50 and 100 μs/f in both direction are, θ: 0.28 mrad χ:0.38 mrad and θ:0.29 mrad χ: 0.57 mrad respectively. Subtracted histograms show that the peaks are separated as remarkably as θ: 0.20 mrad, χ: 0.25 mrad in 100 μs/f (Red peak) condition and θ: 0.79 mrad, χ: 1.26 mrad in 50 μs/f condition (Blue peak).

From the discussion, we demonstrated that the dynamics of gold nanocrystals on dynein molecule in 50 μs/f condition is 3 and 6 times larger than that in 100 μs/f in θ and χ direction respectively. This is concluded that the several tens μs time scale is likely to be a notable time order for the transitional dynamics

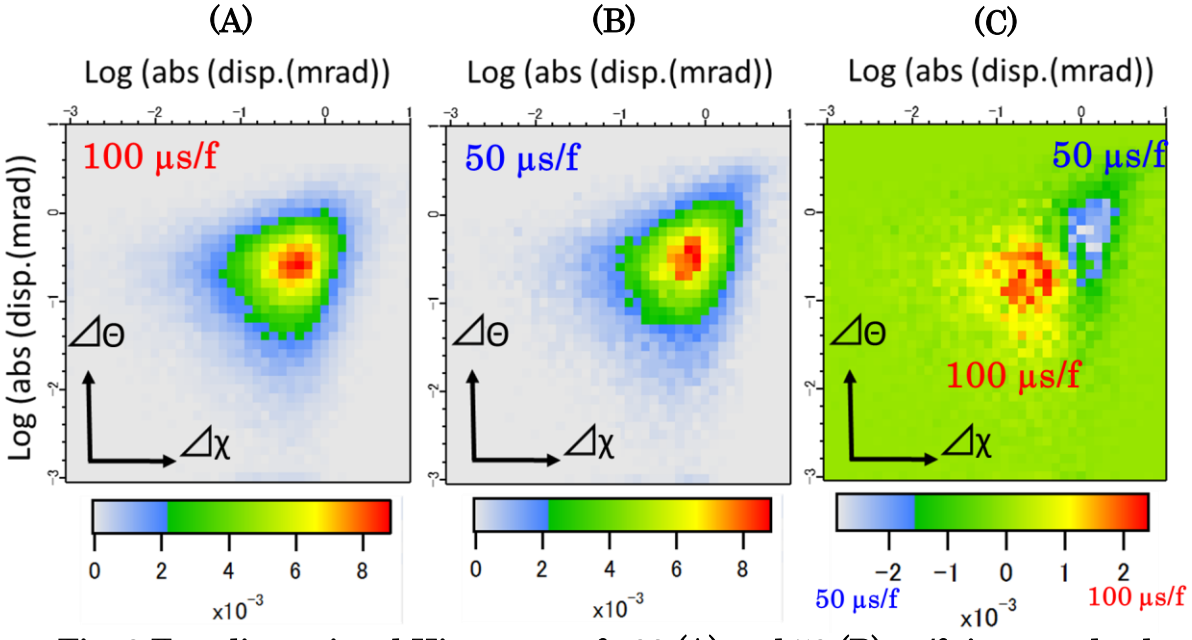
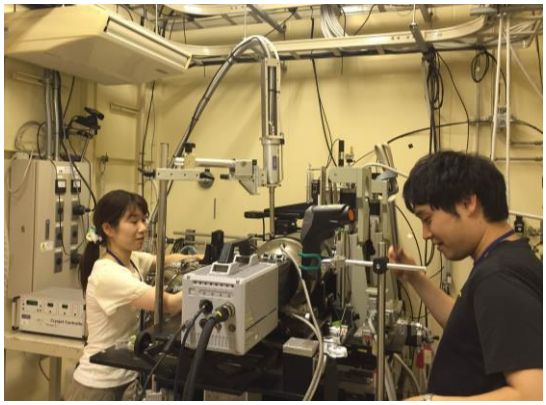


Fig. 8 Two-dimensional Histogram of 100 (A) and 50 (B) μs/f time resolved condition. Subtracted two-dimensional histogram from 100 and 50 μs/f condition

of the linker of dynein in ADP and Apo state. For the next study, we would like to come to grips with trying to observe the experiment with higher time resolution, modifying wide dynamic ranges and varying gold nanocrystal size.

5. Summary

In this self-directed joint research, we succeeded in measuring dynamic 3D behavior of single molecule of dynein motor domain on the microtubules between ADP and Apo states by DXT in less than 50μs time resolution. We expect that our study will contribute to understand the mechanism of dynein, which is known to have the notably complex mechanics comparing to other proteins. When the further detailed molecular mechanics are pursued by DXT, we also expect to have significant impact on single molecule biophysics to clarify the complicated conformational changes of motor proteins (especially dynein, kinesin and myosin). In the future, we would like to examine the dynamics of dynein by changing the position of label gold nanocrystal, ADP concentration and the time resolution.



Experiment at SPring-8 BL40XU.
(left) : Kinoshita, (right): Matsushita

6. Acknowledgements

We would like to special thanks our supervisors, prof. H. Higuchi, prof. Y.C. Sasaki, prof. K. Hashimoto, prof K. Nozaki and prof. H. Noji for their great supports and advises. We would also thank Sasaki group for conducting our experiments during their beam time, assistant prof. K. Ikezaki in Sasaki lab for discussing the DXT results, and Dr. H. Sekiguchi in RIKEN SPring-8 center, for advising DXT measurement. We would thank MERIT program for providing the valuable collaborative research.

7. References

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