LINEAR ZERO MODE WAVEGUIDES FOR THE STUDY OF CHEMO-MECHANICAL COUPLING MECHANISM OF KINESIN

Kazuya Fujimoto[1], Yuki Morita[1], Ryota Iino[2], Michio Tomishige[3], Hirofumi Shintaku[1] Hidetoshi Kotera[1] and Ryuji Yokokawa[1]
[1]Kyoto University, Kyoto, JAPAN
[2]Institute for Molecular Science, Okazaki, JAPAN
[3]The University of Tokyo, Tokyo, JAPAN

ABSTRACT

We developed Linear Zero Mode Waveguides (LZMWs) for simultaneous observation of kinesin displacement and ATP hydrolysis cycle under higher concentration of fluorescently labeled ATP than that used in conventional total internal reflection microscopy (TIRFM). Single-molecule fluorescent microscopy is a promising method to analyze correlation between the mechanical displacement and hydrolysis of ATP by a kinesin molecule. However, the concentration of fluorescently labeled ATP was limited up to 10 nM in TIRFM, thus available data from experiment was limited. Proposed method enables to use ten times higher concentration of fluorescently labeled ATP by confining excitation lights in LZMWs, which are nano-slits with 100 nm in width.

INTRODUCTION

Motility of kinesin-1

Kinesin-1 (hereafter referred to as kinesin) is a class of motor proteins that drives anterograde transport of intracellular cargos on microtubules (MTs), which is a filament like polymer consisted of tubulin dimers. Since kinesin was discovered, its biochemical functions in eukaryotic cells and biophysical mechanisms have been intensively studied.

Today, it is a common understanding that kinesin is a homo-dimeric motor protein that processively moves on a MT by alternate 8-nm steps of their heads, which synchronizes with a hydrolysis of ATP [1]. Coupling between timing of mechanical displacements and ATP hydrolysis cycle, including ATP binding, phosphate release and adenosine diphosphate (ADP) release, have been intensively studied utilizing the single molecule microscopy. So far, it have been revealed that binding affinity between kinesin heads and MT varies depending on attachment, hydrolysis of ATP or release of ADP. Recently, researchers are focusing to the mechanism that processive motility is regulated by ATP hydrolysis cycle [2]. However, it is still unclear that when ATP binds to kinesin and when ADP detaches from kinesin in the cycle. Understanding these timing will answer a question how a kinesin avoids to dissociate from a MT during its steps.

Use of fluorescently labeled ATP to observe the ATP attachment to or detachment from kinesin is promising to elucidate a temporal relationship between mechanical displacement and ATP hydrolysis cycle of kinesin. Direct observation of the interaction between motor proteins and fluorescent nucleotide has been reported in actin-myosin system[3]. However, the concentration available for single molecule fluorescent observation is limited to order of 10 nM in TIRFM, resulting in low efficiency of ATP attachment/detachment observation.

Proposing method

In the past decade, Zero Mode Waveguides (ZMWs) have been developed and widely used to observe single fluorescent molecules with concentration in the order of 1 μM. ZMWs confine the excitation light into apertures with ~100 nm in diameter fabricated on a metal thin layer, resulting in reduction of excitation volume. Visualization of single protein polymerization shows the capability of ZMWs [4].

In this paper, we propose the use of LZMWs, which is an expansion of conventional ZMWs to linear tracks with ~100 nm in width (Fig. 1). Use of LZMWs for observation of actin filaments was once reported but only myosin motility was observed [5]. Our goal is to establish a simultaneous observation of kinesin and higher concentration of fluorescent ATP at single molecule toward further understanding of the kinesin mechanism.

Achievements

LZMWs were designed referring the distribution of the excitation light calculated by the finite element method (FEM) and fabrication process were established by optimizing conditions of the electron beam lithography (EBL) and lift-off process. Methods for selective passivation of LZMWs and microtubule introduction into LZMWs were developed to realize kinesin motility assay.

Simultaneous fluorescent observation of kinesin and ATP was tested by TIRFM and then implemented in LZMWs. Observation results proved the feasibility of the

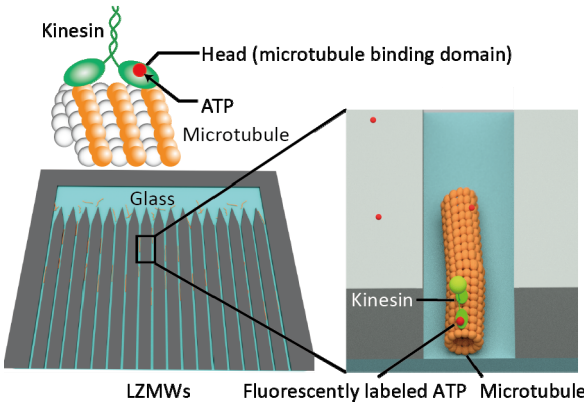

Figure 1: Schematic of the experimental system. MTs and kinesin are introduced into LZMWs with ~100 nm of width. Kinesin motility on MTs and attachment/detachment of fluorescently labeled ATP were observed by inverted microscope.

simultaneous observation with ~ten times higher concentration of fluorescent ATP in LZMWs.

EXPERIMENTS

FEM simulation

The excitation light distributions in LZMWs were calculated using COMSOL (COMSOL Inc.). Calculation was done with a 2D cross sectional model because the field can be regarded as uniform along LZMWs. The excitation light polarized parallel to or orthogonal to LZMWs to evaluate the effect of polarization direction of the excitation light. Width of LZMWs was also varied from 100 to 200 nm. The wave length of the excitation light was set as 640 nm for all calculations.

LZMWs Fabrication

LZMWs was fabricated by EBL and lift off process. Borosilicate glass cover slips were cleaned in ammonium hydroxide and then coated with ma-N2403 and ESPACER 300 to prevent charge up. LZMWs pattern were lithographed by EBL equipment (ELS-F125HS, ELIONIX) and developed in CD 26 (Dow Electronic Materials). Then 100 nm thickness of aluminum layer was deposited on coverslips by thermal deposition. Resists were lifted off by sonicating substrates in acetone for 10 min to form nano-slits. Residuals were cleaned by plasma etching for 30 s.

Aluminum surface of LZMWs was selectively passivated as shown in Fig. 2c [6]. LZMWs were incubated in 2% poly (vinyl phosphonic acid) (PVPA) aqueous solution at 90°C for 2 min to modify aluminum surface of LZMWs to be a protein repellant surface. They were annealed on a 90°C hot plate for 10 min after rinsing in DIW.

Kinesin motility assay in LZMWs

Kinesin motility assay was implemented in flow chambers constructed from LZMWs and coverslips. They were heated Parafilm. The flow chamber was first filled with a kinesin solution and incubated for 5 min. Then 2.7 mg mL^{-1} MT solution was introduced to be attached on the kinesin-coated glass surface. Gliding assay buffer containing 10 mM ATP and 20 mM $MgSO_4$ was then introduced to initiate MT motility toward LZMWs. MTs density in LZMWs reached to the highest value after 3 min of gliding. MTs were then immobilized by treatment with 0.1% glutaraldehyde for 3 min to cross-link MTs and kinesin on the surface. Finally, excess glutaraldehyde was neutralized by introducing a 0.1 M Glycine solution.

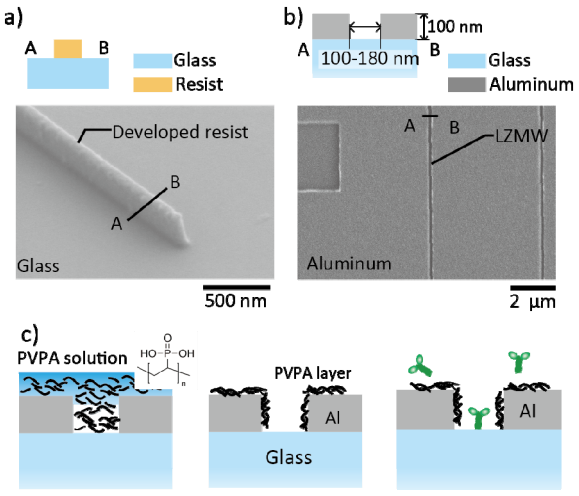

Figure 2: Fabrication and surface modification. a) Scanning electron microscope (SEM) image of EB resist after development. b) SEM image of fabricated LZMWs after Al deposition and lift off process. c) Selective passivation of Al surface by PVPA treatment.

Qdot-kinesin was prepared by mixing Qdot 525 and AviTag kinesin at the 1:2 molar ratio. Qdot-kinesin and Alexa Fluor 647 ATP were diluted in motility buffer containing anti-fade cocktail (140 mM βMe, 20 mM DTT, 21.6 mg mL^{-1} glucose oxidase, 3.6 μg mL^{-1} catalase, 100 mM glucose and 2 mM trolox) for motility assay. This motility buffer containing 1 nM of Qdot-kinesin, 1 μM ATP and 250 nM of Alexa Fluor 647 ATP was introduced into the flow cell for the simultaneous observation.

Simultaneous fluorescent observation

Qdot 525 and Alexa Fluor 647ATP were excited by 404 nm laser (CUBE 404, Coherent) and 640 nm laser (OBIS 647, Coherent), respectively. Fluorescent signals from samples were observed by an inverted microscope (IX71, OLYMPUS) equipped with a ×100 objective lens and a stabilized stage. Dual color fluorescent image was split by a dichroic mirror in Dual View (Photometrics) and separately focused on an EMCCD (iXon DU 897, Andor). Objective lens TIRFM was used to confirm a simultaneous observation of fluorescent samples without LZMWs.

Image analysis

All images were taken and stored at a PC via capturing software (Andor iQ, Andor). Image processing was carried out by Image J (NIH) and Matlab 2015b (Math works). 2D Gaussian function was fitted to the intensity profile of Qdot-kinesin to obtain precise position. Step-wise displacements of Qdot-kinesin was detected by a least chi-square algorithm kindly provided by Dr. Kerssemakers (Delft University).

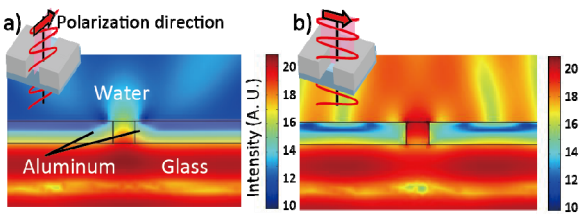

Figure 3: Results of FEM simulation. a) Distribution of light intensity with the excitation light polarized parallel to LZMWs. b) Distribution of light intensity with the excitation light polarized orthogonal to LZMWs. Width and depth of LZMW was 100 nm for both simulation.

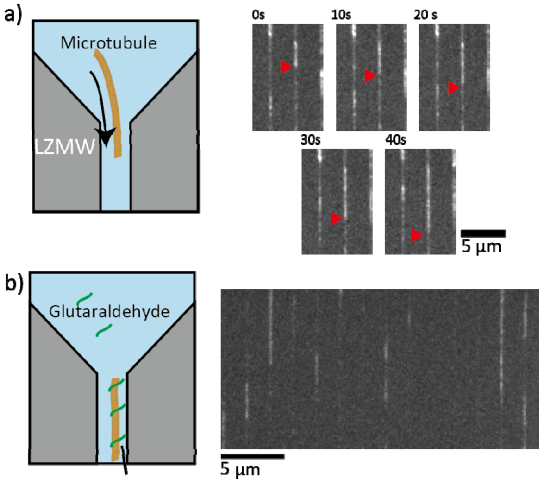

Figure 4: MT introduction and immobilization in LZMWs. a) MTs were transported by kinesin coated on the glass surface. b) Introduced MTs were immobilized by 3 min of glutaraldehyde treatment.

RESULTS AND DISCUSSIONS

Simulation of excitation light distribution in LZMWs

Fig. 3 illustrate simulation results of excitation light intensities in LZMWs as a color distribution. When the excitation light polarizes in a direction parallel to LZMWs, it was confined in LZMWs (Fig. 2a). On the other hand, the excitation light polarized orthogonal to LZMWs penetrated LZMWs, and thus is not suitable for single molecule observation (Fig. 3b).

The effect of LZMW width was evaluated by intensity of the excitation light in the direction orthogonal to the substrate surface at the center of the LZMWs (polarization direction was parallel to the LZMWs in these calculations). In LZMWs with 100 nm of width, the excitation light intensity at 100 nm away from the surface attenuate to 1/100 of the intensity at surface. On the other hand, in LZMWs with 200 nm in width, excitation light intensity at 100 nm was around half of the intensity at the surface. Whereas absolute intensity at 50 nm away from the surface was ten times higher than that in 100 nm wide LZMWs.

Better confinement of the excitation light in narrower LZMWs enables the use of higher concentration of ATP. Contrary, strong excitation in wider LZMWs is required to detect discrete step of kinesin. Regarding this trade-off in LZMW width, LZMWs were designed with 150–200 nm of width to reconcile detection of discrete Qdot-displacement and of single molecule fluorescent at high concentration as possible.

Microtubule introduction

Fig. 4 show introduction and immobilization of MTs in LZMWs. Before the introduction, MT density in LZMWs was almost zero, because the area of the LZMW surface is 1/20 of whole surface of the device, making the chance that MTs randomly attach to LZMWs small. Once motility buffer containing ATP was introduced, MTs started to glide into LZMWs (Fig. 4a). The complete immobilization of MTs were also confirmed after glutaraldehyde treatment (Fig. 4b).

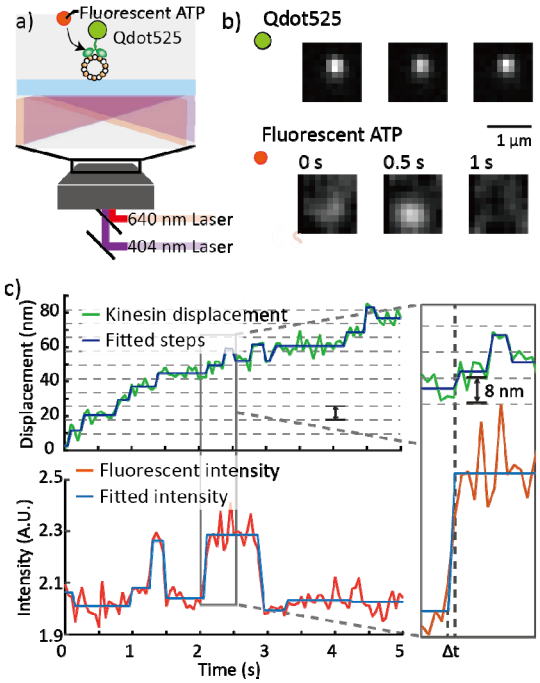

Figure 5: Simultaneous observation of Qdot-kinesin and Alexa Fluor 647 ATP by TIRFM. a) Dual color TIRFM observation of Qdot 525 and Alexa Fluor 647. Individual Qdot spots and Alexa Fluor 647ATP spots were observed. b) Plot of discrete steps of kinesin and fluorescent intensity of Alexa Fluor 647 ATP. Δt is a smallest time differences between kinesin step and abrupt increases of fluorescent intensity.

Kinesin and fluorescent ATP observation by TIRFM

Individual fluorescent spots were observed in both of Alexa Fluor 647 channel and Qdot-525 channels (Fig. 5ab). One-step bleaching of fluorescent spots in Alexa Fluor 647 channel evidenced that individual Alexa Fluor 647 ATP molecule was observed (data not shown).

Functionality of Alexa Fluor 647 ATP as a substrate of kinesin was evaluated from velocities of kinesin at various concentrations of Alexa Fluor 647 ATP and non-labeled ATP. Velocities were fitted by Michaelis-Menten equation to obtain parameter K_m, which reflects affinity between the enzyme and the substrate, and V_{max} reflects maximum speed of hydrolysis. K_m was 209 µM and V_{max} was 316 nm s^{-1} for Alexa Fluor 647 ATP, whereas 136 µM and 523 nm s^{-1} for non-labeled ATP. From these results, it is confirmed that Alexa Fluor 647 ATP serves as a substrate of kinesin-1 with about a half of activity compared to non-labeled ATP. This result agrees to a previous report [7].

Qdot-kinesin moving on immobilized MTs were captured from simultaneous observation images. The velocity of Qdot-kinesin with 1 µM ATP was 5-10 nm s^{-1}, which is suitable for step detection (in Fig. 5c). An representative displacement of Qdot-kinesin over time is plotted and discrete 8 nm steps were detected from the plot in Fig. 5c. In addition, time course of fluorescent intensity taken from 5 × 5 pixel in the Alexa Fluor 647 ATP channel image at the position corresponding to the Qdot-kinesin position (Fig. 5c). To evaluate temporal relationship between discrete steps of Qdot-kinesin and abrupt

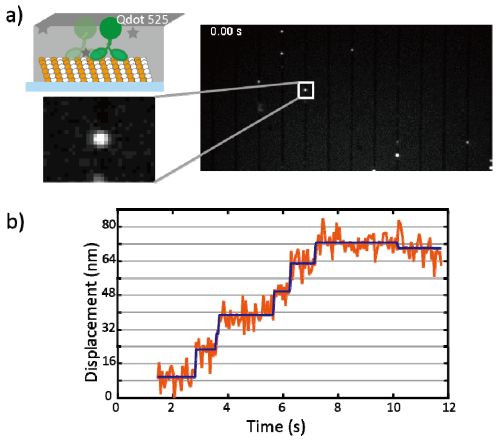

Figure 6: Simultaneous observation of labeled kinesin and ATP. a) Qdot-kinesin motility along MTs in LZMWs was observed. b) 8 nm discrete steps were detected from Qdot-kinesin trajectory.

increases of fluorescent intensity, For each kinesin step, Δt was calculated as smallest time difference between the step and increases of fluorescent intensity of Alexa Fluor 647 ATP (Fig. 5c). Δt calculated from multiple steps distributed around 0 ms, indicating that Qdot-kinesin and Alexa Fluor 647 ATP are available for visualization of attachment of ATP and mechanical step of kinesin.

Simultaneous observation in LZMWs

Qdot-kinesin and Alexa Fluor 647 ATP were simultaneously observed in LZMWs. Qdot-kinesin moving in LZMWs were observed, showing kinesin motility was not inhibited by LZMWs (Fig. 6a). Time course displacement of Qdot-kinesin at 1 μM of non-fluorescent ATP and 250 nM of Alexa Fluor 647 ATP is plotted in Fig. 6b (orange). Although fluorescent intensity of Qdot was weaker than the intensity observed in TIRFM at the same excitation power, resolution of position determination was high enough to detect discrete 8 nm steps (Fig. 6b, blue).

Individual spots of Alexa Fluor 647 ATP were also observed with 250 nM of concentration, which is 25 times higher than the concentration available in TIRFM (Fig. 7a). Time course of fluorescent intensity is plotted (Fig. 7b), showing an S/N ratio enough to detect a single molecule fluorescence. These results exhibit feasibility of the simultaneous observation of Qdot-kinesin displacement and attachment/detachment of Alexa Fluor 647 ATP.

Conclusion

In this paper, we proposed a simultaneous observation of single molecule in LZMWs to study the temporal relationship between mechanical displacement of kinesin and attachment to or detachment from kinesin. LZMWs were designed based on calculated excitation light distributions. Optical system, image processing procedures and motility assay in LZMWs were established. From kinesin motility assay in LZMWs, 8-nm step of kinesin and single molecule detection of Alexa Fluor 647 ATP at the concentration of 250 nM were exhibited. These results prove that proposed experimental method in LZMWs is promising for exploring further understanding of kinesin and applicable for other motor proteins.

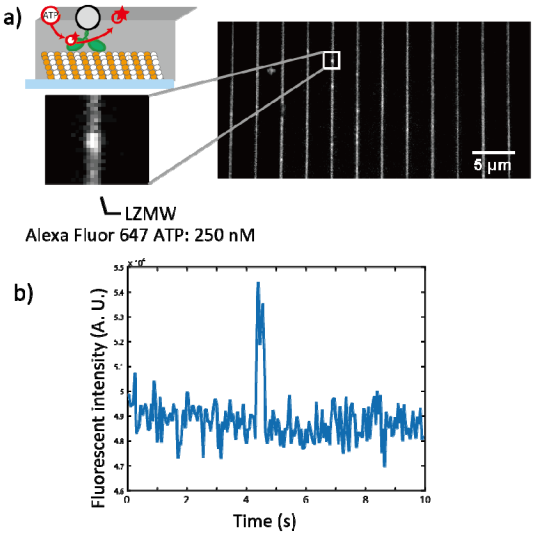

Figure 7: Fluorescent observation of Alexa Fluor 647 ATP. a) Individual spots were observed in 250 nM of fluorescent ATP. b) Time course fluorescent intensity taken from 5 × 5 area in Alexa Fluor 647 ATP image. Fluorescent signal is found between 4 s and 5 s in the plot.

ACKNOWLEDGEMENTS

This work is supported by KAKENHI 16K13644 and Kyoto University Nano Technology Hub in "Nanotechnology Platform Project" sponsored by Ministry of Education, culture, sports, science and technology (MEXT), Japan.

REFERENCES

[1] N. Hirokawa and Y. Tanaka, *Exp. Cell Res.*, vol. 334, no. 1, pp. 16–25, May 2015.

[2] J. O. Andreasson, B. Milic, G.-Y. Chen, N. R. Guydosh, W. O. Hancock, and S. M. Block, *Elife*, vol. 4, Apr. 2015.

[3] T. Komori, S. Nishikawa, T. Ariga, A. H. Iwane, and T. Yanagida, *Biophys. J.*, vol. 96, no. 1, pp. L04-6, Jan. 2009.

[4] S. Uemura, C. E. Aitken, J. Korlach, B. a Flusberg, S. W. Turner, and J. D. Puglisi, *Nature*, vol. 464, no. 7291, pp. 1012–7, 2010.

[5] M. W. Elting, S. R. Leslie, L. S. Churchman, J. Korlach, C. M. J. McFaul, J. S. Leith, M. J. Levene, A. E. Cohen, and J. A. Spudich, *Opt. Express*, vol. 21, no. 1, pp. 1189–202, Jan. 2013.

[6] J. Korlach, P. J. Marks, R. L. Cicero, J. J. Gray, D. L. Murphy, D. B. Roitman, T. T. Pham, G. a Otto, M. Foquet, and S. W. Turner, *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 4, pp. 1176–81, 2008.

[7] S. Verbrugge, B. Lechner, G. Woehlke, and E. J. G. Peterman, *Biophys. J.*, vol. 97, no. 1, pp. 173–82, Jul. 2009.

CONTACT

*K. Fujimoto, tel: +81-075-383-3687,
fujimoto.kazuya.2m@kyoto-u.ac.jp