

Linear-zero mode waveguides for single-molecule fluorescent observation of nucleotides in kinesin-microtubule motility assay

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Abstract

Single-molecule fluorescent microscopy is a key tool to investigate the chemomechanical coupling of microtubule-associated motor proteins, such as kinesin. However, a major limitation of the implementation of single-molecule observation is the concentration of fluorescently labeled molecules. For example, in total internal reflection microscopy, the available concentration is of the order of 10 nM. This concentration is much lower than the concentration of adenosine triphosphate (ATP) *in vivo*, hindering the observation of nucleotides hydrolyzed by motor proteins. Here, we provide a method for the use of single-molecule fluorescent microscopy in the presence of ~500 nM of fluorescently labeled nucleotides. To achieve this, a device equipped with nanoslits is used to confine excitation light into its slits as an expansion of zero-mode waveguides (ZMWs). While conventional ZMWs, equipped with apertures with a diameter smaller than the wavelength of light, are not compatible with filamentous objects, our linear-ZMWs enable the usage of filamentous objects, such as microtubules, while suppressing background noise from labeled molecules diffusing outside of the nanoslits. An experiment using linear-ZMWs demonstrated the successful exploration of the interaction between kinesin and ATP using single-molecule fluorescent microscopy.

Keywords: single-molecule; fluorescent microscopy; nano-slit; chemomechanical coupling.

1 Introduction

The mechanism of kinesin motility has been investigated for many years, with various distinct findings having been reported. As a result, its translocation and adenosine triphosphate (ATP) hydrolysis are widely considered to be tightly coupled. A variety of experimental techniques have been developed for the study of the ATP hydrolysis cycle in kinesin, including a stopped-flow method and/or the usage of ATP analog molecules [1, 2, 3].

Among the experimental methods developed to investigate the complicated mechanisms of biomolecules, single-molecule fluorescent microscopy is among the most powerful to directly observe their reaction processes. For example, the distributions of the transition time of t-RNA bound to ribosomes have been measured from individual phenomena, which is not possible by the observation of an ensemble of multiple molecules [4].

In single fluorescent molecule microscopy, the concentration of fluorescently labeled molecules is a major limitation because the high concentration of fluorescent molecules prevents the detection of individual signals. Zero-mode waveguides (ZMWs) have been used in single-molecule fluorescent observations to overcome the concentration limit and achieve the use of fluorescent molecules with concentrations an order of magnitude higher than in conventional total internal reflection microscopy (TIRFM) [5]. However, the device is not compatible with the observation of linear motor proteins because a filament (10–100 μm) on which motor proteins move is larger in length than the size of the holes (~ 100 nm) of ZMWs. Herein, we describe the fabrication of linear-ZMWs and motility assay methods, allowing for the observation of individual binding/unbinding of ATP to kinesins. The concentration of fluorescently labeled ATP used in this method (500 nM) represents a ~ 10 -fold increase compared to previously reported methods [6].

2 Materials

2.1.1 Fabrication of linear zero-mode waveguides (LZMWs)

Nano-slit structures in an aluminum thin layer are formed on borosilicate substrates as linear-ZMWs using electron-beam lithography and lift-off processes. The width of the slits was set to 100 nm. During the optimization of the lithography conditions, the width could be reduced to 80 nm.

1. Substrate cleaning: Soak borosilicate coverslips (24×24 mm) (No. 1; Matsunami Glass Ind., Ltd., Japan) in a beaker filled with a mixture of 27%

ammonium hydroxide, 30% hydrogen peroxide, and deionized water (volume ratio: 1:1:5) for 10 min at 75°C, as illustrated in Fig. 1–1. After cleaning, rinse the coverslips with deionized water and dry with a stream of nitrogen gas. Bake cleaned coverslips for dehydration at 200°C for 20 min on a hotplate.

2. HMDS coating: Spin-coat a drop of hexamethyldisilazane (HMDS) solution (OAP; Tokyo Ohka Kogyo Co. Ltd., Japan) to coverslips using a spin-coater for 5 s at 1000 rpm and 30 s at 5000 rpm. Bake the coverslips at 90°C for 5 min to enhance resist adhesion by modifying the coverslip surface with siloxane (Fig. 1–2).
3. Resist coating: Spin-coat electron beam (EB) resist (ma-N 2403; Micro Resist Technology GmbH, Germany) and bake coverslips at 90°C for 5 min to cure, leaving the resist with a thickness of 300–350 nm (Fig. 1–3) (see Note 1).
4. Spacer 300 coating: After cooling the coverslips to room temperature (RT) for 1 min, spin-coat Spacer 300Z (Showa Denko K.K., Japan) for 5 s at 1000 rpm for 40 s at 5000 rpm as a charge-dissipating agent (Fig. 1–4).
5. EB patterning: Draw a linear-ZMW pattern to the resist by an EB writer (F7000S-KYT01; ADVANTEST, Japan) at 300 $\mu\text{C}/\text{cm}^2$ (Fig. 1–5). Soak each coverslip in deionized water for 40 s to remove the Spacer 300 film and bake at 100°C for 2 min.
6. Development: After cooling, develop an unexposed resist in CD-26 (Rohm and Haas Electronic Materials LLC, USA) for 70 s (Fig. 1–6). After rinsing in deionized water, dry the coverslips (see Note 2). Ash the surface in oxygen plasma for 5 s to remove organic debris using a plasma etcher (power: 50 W, pressure: 10 Pa, mass flow: 10 sccm; FA-1) (SAMCO, Japan).
7. Aluminum deposition: deposit a 100-nm thick aluminum layer using thermal deposition equipment (VPC-260FI; ULVAC, Japan) (Fig. 1–7).
8. Lift-off process: Soak the coverslips in acetone for 15 min with ultrasonic agitation to remove the remaining resist and form nano-slit patterns (Fig. 1–8). Then, rinse the coverslips in deionized water and dry them using a nitrogen gas stream. Check whether resists are removed; if not, soak the coverslips again for 5–10 min in acetone with ultrasonic agitation.

2.2 Kinesin

A kinesin construct consisting of truncated human kinesin head (1–465 amino residues),

EGFP at the C-terminal, His-tag at the N-terminus, and biotin tag at the N-terminus was used. The protein was expressed in *Escherichia coli* and purified according to the standard protocol for His-tagged proteins [7].

2.3 Microtubules

Tubulins are purified from porcine brains using a previously reported method [8]. Briefly, $\sim 5 \text{ mg mL}^{-1}$ of tubulin solution is stored in liquid nitrogen. Fluorescently-labeled tubulin and biotinylated tubulin are prepared by labeling with succinimidyl ester-conjugated tetramethylrhodamine (A2000; Thermo Fisher Scientific, USA) and succinimidyl ester-conjugated biotin (B30010; Thermo Fisher Scientific), respectively. All tubulin concentrations were adjusted to approximately 5 mg mL^{-1} .

In the observation of a common microtubule motility assay, fluorescently labeled microtubules are generally used by copolymerizing fluorescently labeled tubulin and non-labeled microtubules. In linear-ZMWs, because their structure suppresses excitation light, the fluorescent signals emitted by microtubules are weaker than those in a conventional TIRFM experimental setup. Therefore, in this protocol, quantum dots were conjugated to microtubules, which were co-polymerized by mixing non-labeled, fluorescently labeled, and biotinylated tubulins to obtain sufficient fluorescence intensity to observe them in linear-ZMWs (see Note 3).

2.3.1 Microtubule polymerization procedure

1. Mix non-labeled tubulin, fluorescently labeled tubulin, and biotinylated tubulin at a volume ratio of 17:2:1.
2. Add 1 mM GTP and 1 mM MgSO_4 (final concentrations) and incubate for 30 min at 37°C .
3. Mix polymerized microtubule solution ($9 \mu\text{L}$) with $0.5 \mu\text{L}$ of $1 \mu\text{M}$ Qdot 585 streptavidin conjugate (Q10113MP; Thermo Fisher Scientific) and incubate for 15 min at RT.
4. Add $0.5 \mu\text{L}$ of 1 M biotin to passivate excess binding sites of streptavidin on Qdots.

2.4 Optical system

An IX71 inverted microscope (Olympus, Japan) with epifluorescence or in TIRFM mode

is used, as illustrated in Fig. 2a. A blue diode-pumped solid-state (DPSS) laser (473 nm) (DPBL-9050; Suwtech, China) is used to excite EGFP fused to kinesin and Q-dots conjugated to microtubules, and a red DPSS laser (640 nm) (ORBIS 640; Coherent, USA) is used to excite Alexa Fluor 647-labeled ATP (A22362; Thermo Fisher Scientific). Laser sources are modulated using custom LabVIEW software (National Instruments, USA) via a data acquisition device (NI USB6221; National Instruments) with duty cycles of 60% for the 473 nm laser and 30% for the 640 nm laser at 50 Hz to suppress photobleaching. Light from a red laser source was linearly polarized by a polarization filter (SPF-30C-32; SIGMA KOKI, Japan), and the polarization direction is aligned parallel to the direction of the slits of linear-ZMWs at the objective lens by rotating a half-wave plate (WPQ-6328-2M; SIGMA KOKI). An excitation light whose polarization direction is parallel to the slit forms an evanescent field inside the slits of linear-ZMWs (Fig. 2b). Otherwise, the excitation light penetrates through the slits and creates higher background noise, disrupting single-molecule observation.

The microscope is equipped with a 100 $\times$ objective lens (numeral aperture, 1.4) (UPLAPO100XOHR; Olympus) and a dichroic mirror (Di01-R405/488/560/635-25x36; Semrock, USA). Images are split according to wavelength using image splitting optics (W-View Gemini, Hamamatsu, Japan) equipped with a dichroic mirror (FF640-FDi01-25x36; Semrock) and band-pass filters (ET525/50; ChromaTechnology, USA; BLP-01-635R; Semrock). An EM-CCD camera (iXon 897; Andor Technology, UK) is used to capture fluorescent images through a 2 $\times$ extender lens (TS-EX-20; Sugitoh Co., Ltd., Japan) to enhance the accuracy of position estimation from fluorescent images.

3 Methods

3.1 Aluminum surface blocking by PVPA

To prevent protein adhesion on aluminum, passivate linear-ZMWs using polyvinyl phosphoric acid (PVPA). Protein adsorption is selectively blocked by PVPA covalently bonded to an aluminum surface [9].

1. PVPA treatment: heat the prepared device on a hotplate set to 80°C. Drop 0.1% water solution of PVPA on the surface to fully cover the aluminum surface of the device. Place another coverslip on the device to prevent the PVPA solution from drying out, and incubate for 2 min.
2. Washing: Place the device in a DIW in a beaker and rinse gently for 30 s. Rinse the device again in another fresh DIW beaker and dry gently using a

hand blower.

3. Resist curing: Bake the substrate on a hotplate set to 100°C for 5 min to form a covalent bond between aluminum and phosphate.
4. Flow-chamber construction: Cut parafilm into 5-mm-wide slips and create a flow chamber by placing two pieces of the slips parallel on the device substrate.

3.2 Motility assay

Kinesin, microtubules, and the reagents necessary for motility observation were introduced into a flow chamber for single-molecule observation (Fig. 3).

1. Prepare an anti-fade cocktail (Table 1) and use for ~30 min while maintaining on ice (4°C) to avoid the deterioration of the reducing reagents and oxygen scavenging system (glucose oxidase, catalase, and glucose).
2. Fill the chamber with 5 mg/mL of casein solution in BRB 80 buffer (80 mM PIPES, 1 mM MgCl₂, and 1 mM EGTA) to cover the glass surface and incubate for 5 min.
3. Introduce the His-tag kinesin solution and incubate for 5 min. Wash the flow chamber twice with 15 µL of an anti-fade cocktail to remove excess kinesin.
4. Mix the anti-fade cocktail with Qdot-labeled microtubules, unlabeled ATP, and labeled ATP to obtain a motility assay cocktail (Table 2). Use a concentration of unlabeled ATP and labeled ATP of 5 µM and 500 nM, respectively, for the simultaneous observation of ATP and kinesin.

Table 1. Anti-fade cocktail. The final concentration is the value after mixed with microtubule and ATP

Reagent	Stock concentration	Volume (µL)	Final concentration
Trolox	10 mM	20	2 mM
Casein	20 mg mL ⁻¹	10	2 mg mL ⁻¹
Glucose oxidase	21.6 mg mL ⁻¹	1	216 µg mL ⁻¹
Catalase	3.6 mg mL ⁻¹	1	36 µg mL ⁻¹
Taxol	4 mM	1	40 µM

Dithiothreitol	1 M	2	20 mM
Pluronic	10%	1	0.1%
F127			
Glucose	1 M	2.5	25 mM
BRB80	—	51.5	—
Final	—	90	—

Table 2. Example of motility assay cocktail

Reagent	Stock concentration	Volume (μL)	Final concentration
Anti-fade cocktail	—	13.5	—
ATP	150 μM	0.5	5 μM
AlexaFluor 647	15 μM	0.5	500 nM
labeled ATP			
Microtubules	~5 mg mL	0.5	—

3.3 Image alignment

To analyze the spatial co-localization of kinesin and fluorescently labeled ATP, the two image fields needed to be aligned. The positions of the two images for Qdot and labeled ATP signals (512×256 pixels) were calibrated by positions of TetraSpeck beads (T7280; Thermo Fisher Scientific) nonspecifically attached to a coverslip surface in each image. For each movie of the simultaneous observation of kinesin, microtubules, and labeled ATP, TetraSpeck bead images were captured in the corresponding spectral channels, including 10 or more bead positions. The point spread function centers were obtained from the images to generate a polynomial fitting function to map the correspondence of the positions between the two channels. For each movie sequence, the kinesin positions were obtained from the fluorescent images by low-pass filtering and binarization. ATP molecules in the nanoslits were observed in the image sequence. The precise positions of the spots were obtained by fitting each spot with a 2D Gaussian function. The detection

and position estimation of bright spots were implemented using the Image J plugin, TrackMate. The positions were aligned by the polynomial fitting function, and ATP molecules colocalized with kinesin were counted for the analysis of kinesin chemo-mechanical functionality.

4 Notes

1. The resist thickness and edge shape are critical for a successful lift-off process. If the resist thickness is insufficient or the developed shape is not sharp enough, the aluminum on the patterned resist cannot be removed. A thickness of 300–350 nm is recommended to remove the aluminum layer sharply at the boundary of the resist edges.
2. Rinsing and blow-drying must be implemented with extreme care, since resist patterns after development collapse easily by fluid shearing force or surface tension.
3. The Q-dots used contain cadmium and the use of gloves is recommended when handling them. Solutions containing Q-dots should be discarded by absorbing with paper and handled according to the corresponding regulations.

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Figures

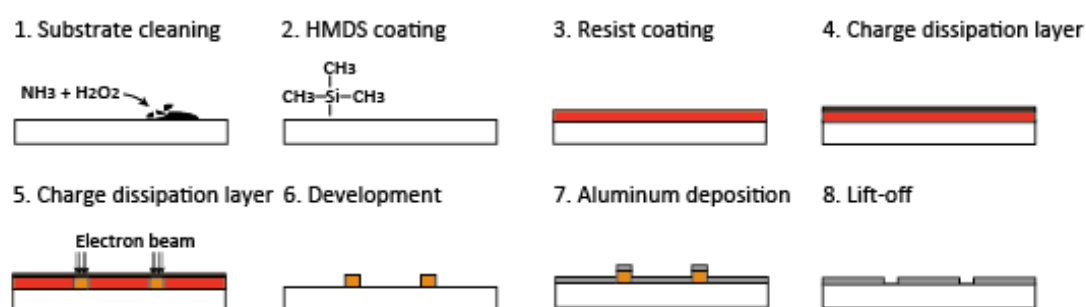


Fig. 1 Schematic illustration of the fabrication process. (1) Coverslips are cleaned in a mixture of NH_4OH and H_2O_2 . (2) HMDS spin-coated on coverslips to covalently bond $\text{Si}(\text{CH}_3)_3$ group. (3) Electron-beam resist (ma-N2403) is spin-coated with 300–350 nm of thickness. (4) Espacer 300 is spin-coated to suppress charging up during electron beam lithography. (5) Slit patterns are drawn by an electron beam. (6) After development, the resist remains where slits are constructed. (7) An aluminum layer is thermally deposited on the device coverslips. (8) The remaining resists are removed, accompanied by the removal of the aluminum layers to form nano-slits.

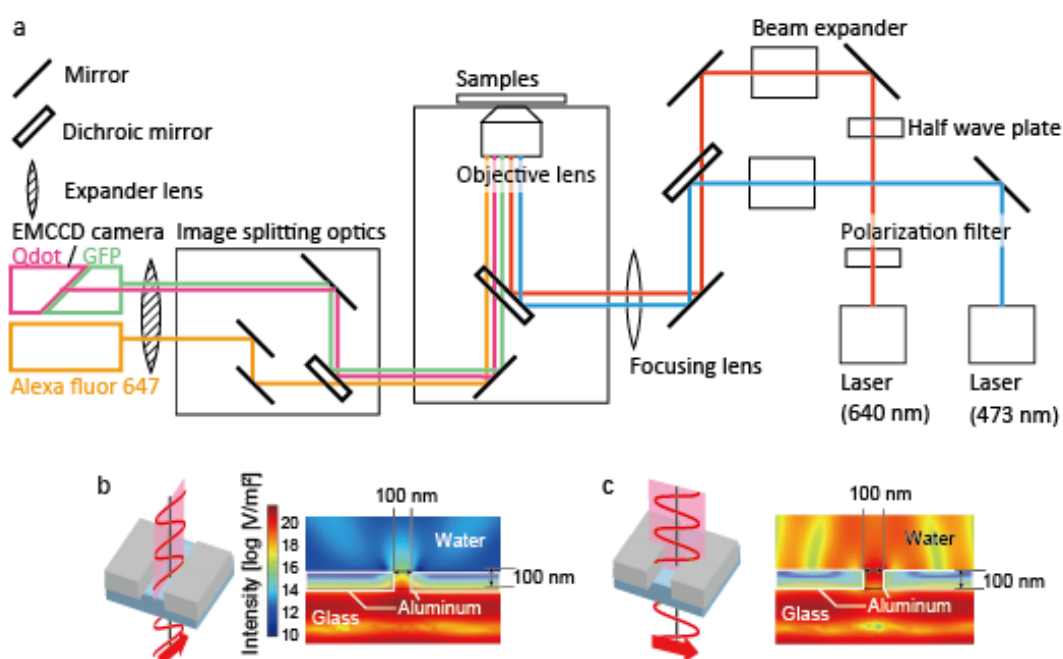


Fig. 2 Schematic illustration of the optical system. (a) AlexaFluor 647 labeled ATP are excited by a 640 nm laser, while GFP, Qdots, and TAMRA are excited by a 473 nm laser. Two laser lights are regulated by mirrors, expanders, dichroic mirrors, and a focusing lens to illuminate samples through an objective lens. Fluorescent lights are captured by an EM-CCD camera through a dichroic mirror, bandpass filters, and a 2× extender lens. The fluorescent lights from the dyes are split by a dichroic mirror and filtered by a bandpass and a long-pass filter. (b) Excitation light whose polarization direction is parallel to the slits is contained in the slits, while excitation light with polarization direction orthogonal to the slits propagated through the slits. Reprinted with permission from [6]. Copyright (2018) American Chemical Society.

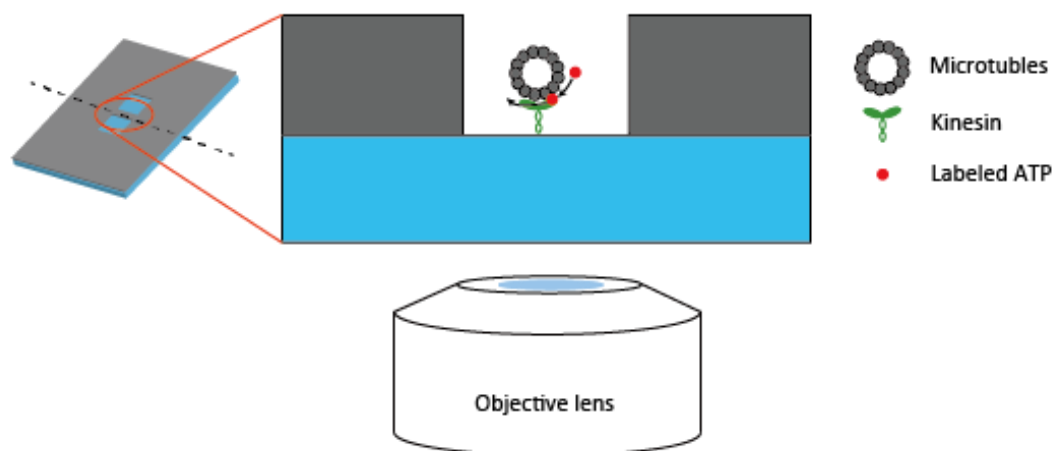


Fig. 3 Illustration of the experimental setup. Linear-ZMWs are fabricated around the center of the coverslips, as shown on the left-hand side. After the addition of the reagent, microtubule motility by kinesin in linear-ZMWs, as shown in a cross-sectional image on the right-hand side, and turn-over of labeled ATP is observed.