In situ velocity control of gliding microtubules with temperature monitoring by fluorescence excitation on a patterned gold thin film

T Nakahara, J Ikuta, H Shintaku, H Kotera and R Yokokawa
Department of Micro Engineering, C3, Kyoto daigaku-Katsura, Nishikyo-ku, Kyoto 615-8540, Japan
E-mail: ryuji@me.kyoto-u.ac.jp

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Abstract
Microtubule (MT) gliding on a kinesin-coated surface is a promising nanoactuator to manipulate nanomaterials in microfluidic environments. However, controllability of motors with respect to velocity, direction, and lifetime has been challenging for engineering purposes. Here, we used fluorescence excitation to control the MT velocity on a photolithographically patterned gold surface. The excitation wavelength was selected to match that used for the observation of MTs. Since a resistance temperature detector (RTD) was integrated on the assay substrate on which kinesin motors were coated, in situ temperature monitoring was implemented. Compared with the velocity of gliding MTs on the bare glass surface, the velocity increased by 1.8-fold on the gold-coated surface with the increase of temperature of 10.4 °C, which was caused by irradiance of 13.5 W · cm⁻². We achieved repetitive velocity control, which was solely caused by the increase of temperature, i.e., irradiation energy. This key technology development enables reversible and localized velocity control of MT gliding, which can be easily integrated in nanosystems driven by kinesin motors.

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1. Introduction

Kinesin is a motor protein that moves on microtubule (MT) filaments by hydrolysing adenosine triphosphate (ATP) [1]. Transport of intracellular cargoes such as endosomes [2, 3] and mitochondria [4] is one of its essential functions in vivo, and is well organized in terms of velocity, direction, and selective attachment and detachment of cargoes [5, 6]. Due to kinesin size and highly efficient energy conversion [7], researchers in bionanotechnology fields have investigated kinesin as a candidate nanoactuator to manipulate target nanomaterials or molecules in microfluidic environments. Once tubulins have polymerized to MTs, MT gliding can be easily observed on a kinesin-coated surface in vitro. Kinesin and tubulin are purified by conventional methods after expression in E. coli [8] and porcine brain [9]. Recently, those proteins became available for purchase along with optimal assay buffer solutions [10]. However, the reconstituted kinesin-MT system in vitro retains motility but not functionality with regard to organized directional and controlled transport and velocity as seen in vivo. Therefore, such functionalities of reconstituted kinesin-MT system need to be realized for the engineering use in microfluidic environments. To address such problems, many methods have been reported [11–36], mostly focusing on MT gliding direction and velocity.

Directional control of gliding MTs has been extensively studied since the late 1990s. Conventional kinesin (kinesin-1) motility directs to the plus end of MTs, which is a faster growing end than the minus end in MT polymerization. Conversely, the minus end becomes a leading tip of MTs when they glide on a kinesin-coated surface. Since the tip is exposed to thermal fluctuation, the MT gliding direction is random without a governing method [11]. There are several methods to define the direction, which are based on micro/nano structures [12–14], electric field [15–17], magnetic field [18], and fluid shear force [19–22]. Design flexibility of photolithography to fabricate structures at the micrometre scale shed light on engineering control by predefined tracks. As far as tracks are narrow enough to prevent U-turns of MTs, they keep gliding in one direction. Therefore, random motility of numerous MTs can be controlled by rectifier structures [13]. Electrophoretic force is induced on negatively charged MTs by an external electric field, providing active control by switching the field directions [15, 16]. Molecular design to attach magnetic particles on a tip of MT also enables directional motility of MTs in a magnetic field [18]. Applying a fluid shear force to gliding MTs is another method to orient MT polarities in one direction, because MTs gliding upstream are removed or redirected by the applied force. Compared with a conventional flow cell [19], the large force generated in a microfluidic channel oriented over 90% of MTs in the designated direction [21]. With the help of external factors, the gliding direction is now controllable in a reconstituted system.

Another essential technique is the velocity control. Focusing on ATP hydrolysis cycles, relations among temperature [23, 24], ATP concentration, and buffer condition [25] have been extensively studied. Additional engineering approaches for modulating gliding velocity are electric fields [26] and fluid shear force [20]. Recently, chemical controls have been also incorporated, such as photoisomerizable monolayer [27], thermoresponsive polymer [28], electrically switched polymer surface [29], and ATP analogues [30]. Compared with these methods, complete on-off operation of motors is realized by UV irradiation to caged compounds such as ATP [31, 32], peptides [33, 34], and microfluidic components in combination with hexokinase [35]. Although the reported methods successfully control the MT gliding velocity, their area for velocity control is limited to flow cells or microstructures in the
millimetre range, because the entire fluidic environment in a flow cell needs to be changed in any method. For example, releasing caged materials achieves relatively high temporal resolution of ∼1 s when switching on motors; however, flushing the solution is necessary to eliminate the released materials to reduce the MT velocity [32]. When temperature control was adopted, it was also conducted for the entire flow cell [24]. To the best of our knowledge, reasonable spatial and temporal resolutions of ∼100 μm and ∼1 s, respectively, have been reported to alter the rotation speed of F1-ATPase by a laminar flow in branched microfluidic channels [36, 37]. However, none of the above-mentioned methods has achieved such a high spatiotemporal resolution to control kinesin motors toward a functional nanosystem, which necessitates local and arbitrary velocity control. Therefore, a method to regulate the velocity at high spatiotemporal resolution is in high demand.

Here, we propose a method to modulate the velocity of MTs gliding on microfabricated gold patterns by varying the exposure irradiance of fluorescent excitation light (figure 1). Since the temperature on the gold-coated surface is locally elevated by the heat transfer effect, only MTs in the irradiated gold area are accelerated, whereas the other MTs maintain a constant velocity. Not only the acceleration but also deceleration can be easily controlled by changing the irradiance. Besides, single-mask photolithography enables the integration of the gold-patterned area for temperature control and a resistance temperature detector (RTD) adjacent to the area, which provides an in situ temperature monitoring tool during the assay. We found that the velocity modulation is purely caused by the local temperature control. Our method enabled us to control the velocity and observe MTs simultaneously in the selectively irradiated area, which could be a key technology in building kinesin-driven nanosystems.

2. Materials and methods

2.1. Microfabrication and flow cell construction

We designed a gold-coated region and a bare glass region on a substrate, in which each region had an RTD at the centre to monitor the temperature (figure 2(a)). Due to chemical inertness, biocompatibility, and appropriate temperature coefficient (∼0.0034 K⁻¹), we selected gold as both the sensing and heater material. Four-wire RTD eliminates contact resistances and other resistances caused by lead wires. Fabrication started with cleaning a glass substrate...
(76 mm × 26 mm, No. 1 thickness, Matsunami, Osaka, Japan) in Piranha solution (H$_2$SO$_4$: H$_2$O$_2$ = 3:1) at 80 °C for 5 min. Additional cleaning was conducted using oxygen plasma for 10 min (20 Pa, 100 sccm, 100 W; RIE 10NR, Samco, Kyoto, Japan). Chromium (20 nm) as the adhesion layer and gold (100 nm) were thermally deposited and patterned by UV lithography using a photoresist (S1813, Rohm and Haas Electronic Materials, Philadelphia, PA, USA). After hard baking at 120 °C for 5 min, gold and chromium layers were etched using 3-fold diluted aqua regia (HCl:HNO$_3$ = 3:1) and a commercialized chromium etchant (S-24, Sasaki Chemical, Kyoto, Japan), respectively. Residual photoresist was removed in Piranha solution and dried to construct the flow cell. In our experimental setup, since the assay conditions might be affected by the total amount of assay solution due to thermal dissipation, thickness-defined double-sided tape (50 μm in thickness, 400P50, KGK, Saitama, Japan) was employed to prepare the flow cell with a top coverslip (18 mm × 18 mm, No. 1 thickness, Matsunami, Japan). This resulted in flow cell size of 10 mm in width, 18 mm in length, 50 μm in height, and total volume of ∼3 μL (figure 2(b)).

2.2. Characterization of RTDs

We adopted four-wire RTDs for higher accuracy and reliability than those of two-wire RTDs. One pair of wires connected to a resistive sensor was used to supply a constant current of 1.0 mA by a low-voltage source meter (2401, Keithley, Cleveland, OH, USA), and the other pair was used to measure the voltage across the RTD using an electrometer (6517B, Keithley, USA) as shown in figure 2(c). Each fabricated RTD was calibrated to minimize the measurement error that might be caused by the variation of the RTD wire width and thickness, which was not avoidable even though we used the same photomask, gold deposition, and etching conditions. A first calibration was performed to obtain the relationship between the resistance of the RTDs and temperature by immersing the RTDs in a water bath (SM-05R,
Taitec Corp., Saitama, Japan). The calibration range for the temperature was between 20 °C and 50 °C with 5 °C intervals. Although the water bath had a feedback temperature control, we monitored the water temperature using a commercialized sensor. A second calibration was performed to relate temperature and irradiance when the gold-coated and bare glass areas were excited with various fluorescence intensities, which was controlled using an incorporated six-step iris (3%, 6%, 12%, 25%, 50%, 100%; U-HGLGPS, Olympus, Tokyo, Japan), which corresponds to the irradiance of 0.4 W · cm\(^{-2}\), 0.6 W · cm\(^{-2}\), 1.5 W · cm\(^{-2}\), 3.6 W · cm\(^{-2}\), 7.2 W · cm\(^{-2}\), and 13.5 W · cm\(^{-2}\), respectively. The irradiance was calibrated using a power meter (PM-247 A, Neoark, Tokyo, Japan).

2.3. Protein preparation

Tubulin was purified from porcine brain through two assembly-disassembly cycles and phosphocellulose chromatography [38]. A part of tubulin was labeled with tetramethyl rhodamine (TMR; C1171, Invitrogen, Carlsbad, CA, USA) to generate labeled tubulin [39]. TMR-labeled MTs were prepared by polymerizing unlabeled and labeled tubulin (5:1) for 30 min at 37 °C. Polymerized MTs were stabilized by adding 20 μM paclitaxel (T1912, Sigma-Aldrich, St. Louis, MO, USA). The kinesin construct used for the MT gliding assay consisted of human kinesin (amino acid residues 1–573) with an N-terminal histidine tag purified as previously described [40]. Protein concentrations were determined using the Bradford method with bovine serum albumin as the standard.

2.4. In vitro motility assay with RTDs

The MT gliding assay was performed in a flow cell prepared as described above and mounted on an inverted microscope (IX73 Olympus, Japan). In addition, RTD wires were also connected before starting the assay. Unless otherwise stated, all proteins were prepared in BRB80 buffer solution (80 mM PIPES, 1 mM EGTA, 1 mM MgCl\(_2\), pH 6.8). A kinesin (50 μg · mL\(^{-1}\)) solution containing 0.1 mg · mL\(^{-1}\) casein flowed into the flow cell and was incubated for 5 min. Then, the MT solution (0.03 mg · mL\(^{-1}\)) was introduced and incubated for 5 min. After replacement of the solution with an assay buffer containing 1 mM ATP and oxygen scavenging system (0.03 mg · mL\(^{-1}\) catalase, 0.2 mg · mL\(^{-1}\) glucose oxidase, 20 mM DTT, 25 mM glucose, 1% 2-mercaptoethanol), the measurement of the velocity was started under a given exposure condition controlled using the incorporated six-step iris for 30 s each.

Repetitive accelerations and decelerations were switched with a neutral density (ND) filter (transmittance of 5%, Sigma Koki, Saitama, Japan) and without the filter (transmittance of 100%), which correspond to 0.7 W · cm\(^{-2}\) and 13.7 W · cm\(^{-2}\), respectively. The filter was mounted for switching in a filter wheel (99A041, Ludl, Hawthorne, NY, USA) incorporated with an inverted microscope (IX71 Olympus, Japan). The exposure time with each filter was 20 s and automatically changed to the next filter. Temperature monitoring and the timing of changing the ND filters were synchronized using a custom-written LabVIEW routine (National Instruments, Austin, TX, USA).

The MT velocities on a bare glass surface without RTDs were measured as the control. A flow cell made of two coverslips was prepared, and the motility assay was conducted as described above. Then, the flow cell was placed in a temperature-controlled chamber (INUB-ONICS-F1-MX, Tokai Hit, Shizuoka, Japan). The temperature was set at 22.5 °C, 26.0 °C,
29.0 °C, and 33.0 °C, which was in the temperature range that could be achieved by the light excitation.

Fluorescent images of MTs were captured using the inverted microscope equipped with a 100× oil objective (NA 1.3) and a complementary metal-oxide semiconductor (CMOS) camera (ORCA-Flash4.0 V2, Hamamatsu, Shizuoka, Japan) and charge-coupled device (CCD) camera, which were set for microscope IX73 and IX71, respectively. Images were stored every 2 fps for the velocity measurement and 10 fps in the velocity modulation experiment using a recording software (HCImage, Hamamatsu, Japan). The acquired images and videos were processed using the softwares ImageJ (NIH, Bethesda, MD, USA), MATLAB (MathWorks, Natick, MA, USA), and FIESTA [41] to measure the velocity of the MTs. The measurement of the velocity was performed by tracking the end of the gliding MTs. The tracking time was 20 s for each experiment.

3. Results and discussion

3.1. RTDs and its characterization

An overview of the fabricated substrate is shown in figure 3(a). Four electrode pads patterned on the left-hand side were connected to the RTD located at the centre of the gold-coated area. The other four electrode pads on the right-hand side were connected to the RTD in the bare glass area. The RTD wires are visualized as white lines and surrounded by the gold-coated area in the enlarged image in figure 3(b). In contrast, only the RTD wires were captured in the bare glass area in figure 3(c). The width of the wire was reduced to 4 μm from 5 μm in the original design, and the RTD occupied an area of 30 μm × 35 μm. Since the excitation area corresponds to the observation area of 265 μm in diameter when a 100× objective is used, not only one RTD but also the surrounding area fitted to the field of view, thus enabling the evaluation of the velocity of the MT with real-time temperature monitoring.

For the first calibration, the resistances of the RTDs located in the gold-coated and bare glass areas are plotted against the temperature values in figure 4(a). Both resistances were proportional to the temperature with significant positive correlation ($r^2 > 0.99$). Although the
The graph shows a difference in the resistance values between the RTDs in two areas, the difference was not attributed to the difference between gold and glass areas but to the variation of the sensor size as described in section 2.2. For the second calibration, we measured the resistance of the RTDs that could be translated to the temperature using the data reported in figure 4(a), when the excitation light power was modulated. The RTDs in both areas obviously exhibited linear relationships in figure 4(b) ($r^2 > 0.99$), and significant difference in the temperature between the two areas. When the excitation power was set at the maximum value (i.e., 13.5 W · cm$^{-2}$), the measured temperatures were 33.2 ± 1.2 (mean ± standard deviation [SD]) °C in the gold-coated area and 24.6 ± 1.1 °C in the bare glass area. Here, the differential from the initial room temperature was 10.4 ± 1.2 °C in the gold-coated area, while it was only 2.1 ± 1.0 °C in the bare glass area. The difference was apparently caused by the absorbance of gold, which resulted in the increase of the temperature. In our two-step calibrations, even with variation of the RTD size, we found proportional relationships among resistance, temperature, and excitation power. Therefore, each RTD was calibrated for the following experiments.

3.2. Velocity modulation of MTs

As shown in figure 5(a), MTs gliding was observed by fluorescence at the maximum excitation of 13.5 W · cm$^{-2}$. The average velocities under this condition were 0.56 ± 0.07 μm · s$^{-1}$ ($N = 20$) and 0.44 ± 0.05 μm · s$^{-1}$ ($N = 20$) on the gold-coated and bare glass surfaces, respectively. The difference in velocity resulted in travel distance of 2.5 μm after 20 s (figure 5(a)).

The velocities of the MTs in the two areas against the excitation light power are plotted in figure 5(b). The results were normalized using the initial velocity, $V_0$, obtained from the MT gliding experiment at the irradiance of 0.4 W · cm$^{-2}$ to avoid dependences on protein or flow-cell batches. The velocity increased 1.8-fold on the gold-coated surface, when the excitation was modulated from 0.4 W · cm$^{-2}$ to 13.5 W · cm$^{-2}$. However, the increase was only 1.1-fold on the bare glass area. This result was also statistically supported by the Tukey–Kramer test that indicated a significant difference in the velocity on the gold-coated surface ($p < 0.05$) and nonsignificant difference on the bare glass area ($p > 0.05$). Moreover, given that the velocity increase was proportional to the excitation power, we could estimate the velocity on the gold-coated area, $V/V_0$, that could be obtained at a certain irradiance, $x$, using the equation $V/V_0 = 6.3 \times 10^{-2} x + 0.96$, which was obtained by the least squares method applied to the experimental data of figure 5(b).
Figure 5. Relationship between gliding velocity and irradiance. (a) Sequence fluorescent images of MT gliding on gold and glass area, respectively. The difference in the distance after 20 s was 2.5 μm. (b) Normalized gliding velocity against irradiance on the gold-coated and bare glass areas.

Figure 6. Evaluation of the temperature dependence. (a) Normalized gliding velocity against temperature in the gold-coated area and bare glass area in the temperature controller. (b) Arrhenius plot of (a).
To examine if the velocity increase was purely due to the increase of the temperature, we compared the velocities on a bare glass surface placed in a temperature-controlled chamber. The normalized velocities for the two conditions are plotted in figure 6(a). The two regression lines shown in the graph were tested using the t-test, and no significant difference was found. This supported the finding that the velocity control within the exposure range of 0.4–13.5 W · cm$^{-2}$ was achieved by modulating the temperature from 22.8 °C to 33.2 °C. The Arrhenius plot of the data reported in figure 6(a) is shown in figure 6(b). Since we normalized the velocity, the Arrhenius equation was expressed as $V/V_0 = \exp(-E_a/RT)$ and the y-axis as $\ln (V/V_0)$, where $V$ is the MT velocity, $E_a$ is the activation energy, $T$ is the temperature, and $R$ is the gas constant. Activation energies were measured as 45 kJ · mol$^{-1}$ on the gold-coated surface and 41 kJ · mol$^{-1}$ on the bare glass surface in the temperature-controlled chamber. Previous studies using MT gliding assays reported 65 kJ · mol$^{-1}$ (<27 °C) \cite{24} and 53 kJ · mol$^{-1}$ \cite{42} driven by multiple kinesins, and 50 kJ · mol$^{-1}$ \cite{23} driven by a single kinesin. The slight difference in our activation energies from the reported values can be explained in two ways. One is that, in our study, kinesin might have been less damaged than in the previous studies, which lowered activation energies. Since kinesin deactivates at high temperature, our temperature control was performed within 30 s for each temperature, resulting in assay time of 3 min for a total of six steps, which was short enough to reduce the damage. The deactivation of kinesin at high temperature is also noted by Kawaguchi et al \cite{43}. The other is the difference in kinesin species and constructs. Although the reported values are for bovine \cite{24, 43} or Drosophila \cite{42} kinesins, human kinesin consisting of only 1–573 amino acid residues was used in our study.

As shown in figure 7, in situ repetitive velocity control was achieved by switching between the minimum exposure of 0.7 W · cm$^{-2}$ (blue-coloured area) and the maximum exposure of 13.7 W · cm$^{-2}$ (red-coloured area). Velocities were switched sequentially: 0.53 ± 0.03 μm · s$^{-1}$, 1.01 ± 0.03 μm · s$^{-1}$, 0.55 ± 0.06 μm · s$^{-1}$, 0.96 ± 0.07 μm · s$^{-1}$, and 0.58 ± 0.06 μm · s$^{-1}$ at each minimum and maximum exposure, respectively, which was clearly observed under the fluorescent microscope (see the supplementary movie, stacks.iop.org/MRX/1/045405/mmedia). The method demonstrated repetitive velocity control on the gold-patterned area without losing kinesin activity. In contrast, we did not find the switching property in the bare glass area because the velocity was almost the same for the increase of irradiance. Velocities were: 0.66 ± 0.04 μm · s$^{-1}$, 0.66 ± 0.04 μm · s$^{-1}$, 0.60 ± 0.03 μm · s$^{-1}$, 0.65 ± 0.04 μm · s$^{-1}$, and 0.62 ± 0.05 μm · s$^{-1}$ at each minimum and maximum exposure, respectively.
4. Conclusion

In this study, we present a method for controlling MT velocity using fluorescence excitation and a gold-patterned substrate. To measure the change in temperature, we fabricated a thermal sensor on a substrate by patterning the gold layer. The fabricated sensors showed linear resistance properties in relation to irradiance. The maximum change in temperature was $10.4 \pm 1.2 ^\circ C$ at an irradiance of $13.5 \ W \cdot cm^{-2}$. The results of the velocity measurements showed that the gliding velocity was modulated in the localized target area on the gold, achieving a maximum change of 1.8-fold. In addition, we demonstrated repetitive velocity control on the gold-patterned area. These data implied that our system might be useful for various molecular applications.

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