Controlling the Direction of Kinesin-Driven Microtubule Movements along Microlithographic Tracks

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ABSTRACT Motor proteins are able to move protein filaments in vitro. However, useful work cannot be extracted from the existing in vitro systems because filament motions are in random directions on two-dimensional surfaces. We succeeded in restricting kinesin-driven movements of microtubules along linear tracks by using micrometer-scaled grooves lithographically fabricated on glass surfaces. We also accomplished the extraction of unidirectional movement from the bidirectional movements along the linear tracks by adding arrowhead patterns on the tracks. These "rectifiers" enabled us to construct microminiturized circulators in which populations of microtubules rotated in one direction, and to actively transport microtubules between two pools connected by arrowheaded tracks in the fields of micrometer scales.

INTRODUCTION

Using the chemical energy released by hydrolysis of adenosine 5′-triphospate (ATP), kinesin, a ubiquitous motor protein found in eukaryotic cells, moves along microtubules, tubular protein filaments of up to 100 μm long. Microtubules have an intrinsic polarity with kinesin moving toward the "plus" ends of microtubules. Vale et al. (1985) developed an in vitro system in which microtubules can be observed gliding over glass surfaces coated with kinesin in an ATP-dependent manner. A similar in vitro motility assay system in which actin filaments move over glass surfaces coated with myosin, another ubiquitous motor protein, was also developed (Kron and Spudich, 1986). However, these systems were not feasible for use as a micromachine device, partly because motor proteins were attached to the glass surfaces at random locations and microtubules or actin filaments moved in random directions on the two-dimensional surfaces. Thus, attempts were made to restrict the movements of microtubules or actin filaments along linear tracks, either by depositing tracks made of oriented poly(tetrafluoroethylene) films (Suzuki et al., 1995; Dennis et al., 1999), or fabricating microlithographic tracks (Suzuki et al., 1997; Nicolau et al., 1999) on glass surfaces. In vitro motility assay systems with a reversed geometry, in which particles absorbed with kinesin move along microtubules fixed to substrates, have also been subjected to improvements. These include alignment of microtubules on surfaces by a flow, such that cargo particles move in either direction along the aligned microtubules (Turner et al., 1995; Limberis and Stewart, 2000). However, in all these systems, movements occur in two directions along linear tracks, and such bidirectional movements are not useful in most applications. Therefore, extraction of unidirectional movements from these bidirectional movements seems to be the most important task in the development of microdevices powered by motor proteins. Recently, Stracke et al. (2000) succeeded in forcing microtubules to move in one and the same direction by applying mechanically induced flow fields. However, this method depends on continuous, external application of a flow, and the directionality disappeared when the flow was stopped. Therefore, we have decided to develop a simpler method in which the directionality of movements is determined intrinsically by the shape of microfabricated patterns produced by lithography.

Unfortunately, the previous systems for moving microtubules or actin filaments along linear tracks (Suzuki et al., 1997; Nicolau et al., 1999) had one drawback: moving filaments often ran off the tracks, and the number of filaments on the tracks gradually decreased over time. Thus, before devising a method to control the direction of movements, it was necessary to develop a lithographic process such that microtubule movements are efficiently restricted along the fabricated tracks, and the movements are maintained stably along these tracks. In this paper we show that the combination of two new technologies, i.e., a novel geometry of tracks for efficient restriction of microtubule movements along the tracks and the design of a pattern to force microtubules to move in one direction, enabled us to construct a system in which populations of microtubules move unidirectionally along linear tracks for hours.

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MATERIALS AND METHODS

Preparation of photomasks

Ti was deposited by spatter-coater onto quartz substrate to thickness of approximately 1500 Å. The patterns were drawn on an electron beam resist (SAL601, Shipley Microelectronics, Tokyo, Japan) layer, which was spin-coated on the Ti surface with electron-beam lithography. The electron beam exposure was performed with a scanning electron microscope

(S4500, Hitachi, Tokyo, Japan) equipped with a pattern generator (CPG1000, Crestec, Tokyo, Japan). After development, the nonmasked Ti surfaces with the resist were ion plasma-etched with SF_6 gas until UV light (~254 nm) could be passed through the etched surfaces.

Substrate preparation

SAL601 films on coverslips were prepared by spin-casting at 2000 rpm for 40 s, followed by baking at 105°C for 1 min on a hot plate. The resist-coated coverslip was irradiated with UV light (254 nm) through a photomask. The coverslip was baked again at 120°C for 2 min, developed in developer (Microposit Developer MF-312, Shipley) for 3 min, and then etched to remove the residues on the glass surface with a plasma reactor (O_2 flow rate at 150 ml/min, plasma power at 280 W) for 1 min. This etching process was necessary to selectively absorb kinesin onto glass surfaces.

Gliding assays

Gliding assays were performed using rhodamine-labeled microtubules (Hyman et al., 1991) and a fluorescence microscope (BX-FLA, Olympus, Tokyo, Japan) following standard methods (Vale et al., 1985), with some modification. Tubulin was purified from bovine brain (Shelanski et al., 1973; Weingarten et al., 1975). Kinesin was prepared from *Escherichia coli* expressing recombinant protein K560, dimeric human conventional kinesin with C-terminal truncation (Woehlke et al., 1997). Kinesin solution (5–20 μg/ml) dissolved in 0.1% Triton X100, 0.15 M of potassium acetate, 10 mM of Tris-acetate (pH 7.5), 4 mM of magnesium sulfate, 1 mM ethyleneglycoltetraacetic acid, 7 mM of 2-mercaptoethanol, 0.025 mg/ml casein was perfused into a flow chamber constructed using a resist-patterned coverslip, a glass slide and double-sided adhesive tapes, and was allowed to absorb onto the glass surfaces for 2 min. Note that our buffer contained a nonionic detergent (Triton X100) and an ionic strength that is higher than the published condition (Vale et al., 1985). If these reagents were absent, microtubule movements were no longer efficiently restricted to glass surfaces of the tracks, and a significant number of microtubules moved randomly over the surrounding resist surfaces.

Preparation of substrates with other resists

The photo-resist AZ5214 (Clariant, Tokyo, Japan) was spin-casted over glass surfaces. The resist-coated coverslip was irradiated with white light from a Hg lamp through a photo-mask followed by baking at 90°C for 90 s. It was then exposed to white light and developed for 1 min. The electron beam resists OBER1000 (Tok, Tokyo, Japan) and ZEP520 (Nippon Zeon, Tokyo, Japan) were spin-coated over Si substrates. The surfaces of the Si substrates were oxidized by soaking in a 1:1 mixture of H_2SO_4 and H_2O_2 for 10 min in advance to enhance the absorption of kinesin molecules to the surfaces. After spin-coating, the patterns were drawn on the resist surfaces with electron beam lithography at doses of 100–200 μCi/cm^2, then developed with a 1:1 mixture of methyl isobutyl ketone and isopropyl alcohol (for OBER1000) or with butyl acetate (for ZEP520) for 1 min.

Preparation of FITC-labeled kinesin

Kinesin dissolved in phosphate-buffered saline interacted with 1 mM of fluorescein isothiocyanate (FITC) for 20 min at 25°C. The reaction was stopped by adding sodium glutamate to a final concentration of 10 mM. The sample solution was immediately gel-filtered using Sephadex G-100. The kinesin fraction was dialyzed against kinesin stock buffer (10 mM Tris acetate, 25 mM potassium acetate, 4 mM magnesium chloride, 7 mM 2-mercaptoethanol, pH 8.0). The labeling stoichiometry of FITC to kinesin was 1.38 (FITC/kinesin). This labeling reaction deprived kinesin of its motor activity, probably because FITC attacked and modified loop12, a lysine-rich, major microtubule-binding region of kinesin (Woehlke et al., 1997).

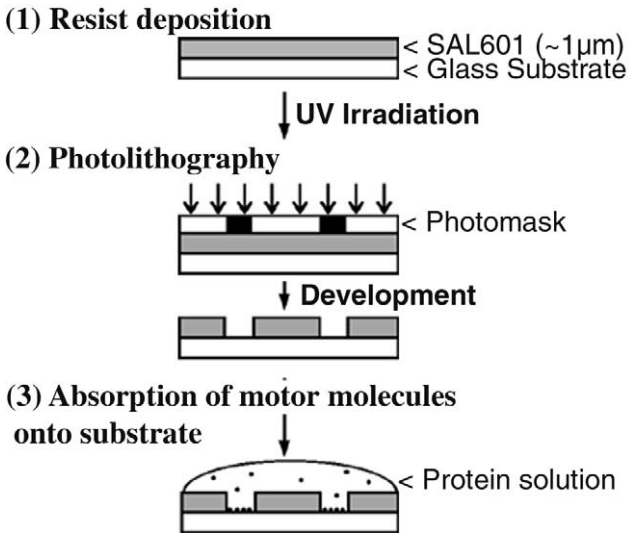

FIGURE 1 Process for the patterning of motor molecules onto lithographic surfaces. (*1*) Spin-coat the resist SAL601 on coverslips to approximately 1 μm in thickness. (*2*) Photolithography: Irradiate with UV light (wavelength 254 nm) passing through a photomask. (*3*) Treat the processed coverslips with a solution of kinesin. Under our conditions, kinesin was more efficiently absorbed onto exposed glass surfaces than to the surfaces of the resist.

RESULTS AND DISCUSSION

The tracks used in our experiments have a reversed geometry as compared with those used previously (Suzuki et al., 1997; Nicolau et al., 1999). Instead of elevating the tracks, the exposed glass surfaces on which microtubules move were depressed relative to the surrounding surfaces of the resist material (SAL601), so that the tracks are now bordered by the walls of the resist (Fig. 1). Selective absorption of motor protein onto exposed glass surfaces, but not to the surface of resist, was enhanced by including a nonionic detergent (Triton X-100) and a high ionic strength in the absorption buffer. The selective binding of kinesin to these surfaces and the effects of Triton X-100 in the absorption buffer are discussed in more detail below. Observation of microtubules gliding on surfaces prepared in this way demonstrated that most of the microtubules were moving along the tracks but not on the surfaces of resist (Fig. 2). When the leading tip of a microtubule moving over the glass surface bumped against the wall, the microtubule changed its direction of movement and either turned the direction similar to the reflection of light, or more often, moved along the wall. They rarely climbed up the wall and moved away from the track. Therefore, this method allowed us to efficiently and stably limit the kinesin-driven movement of microtubules to one dimension along a linear track.

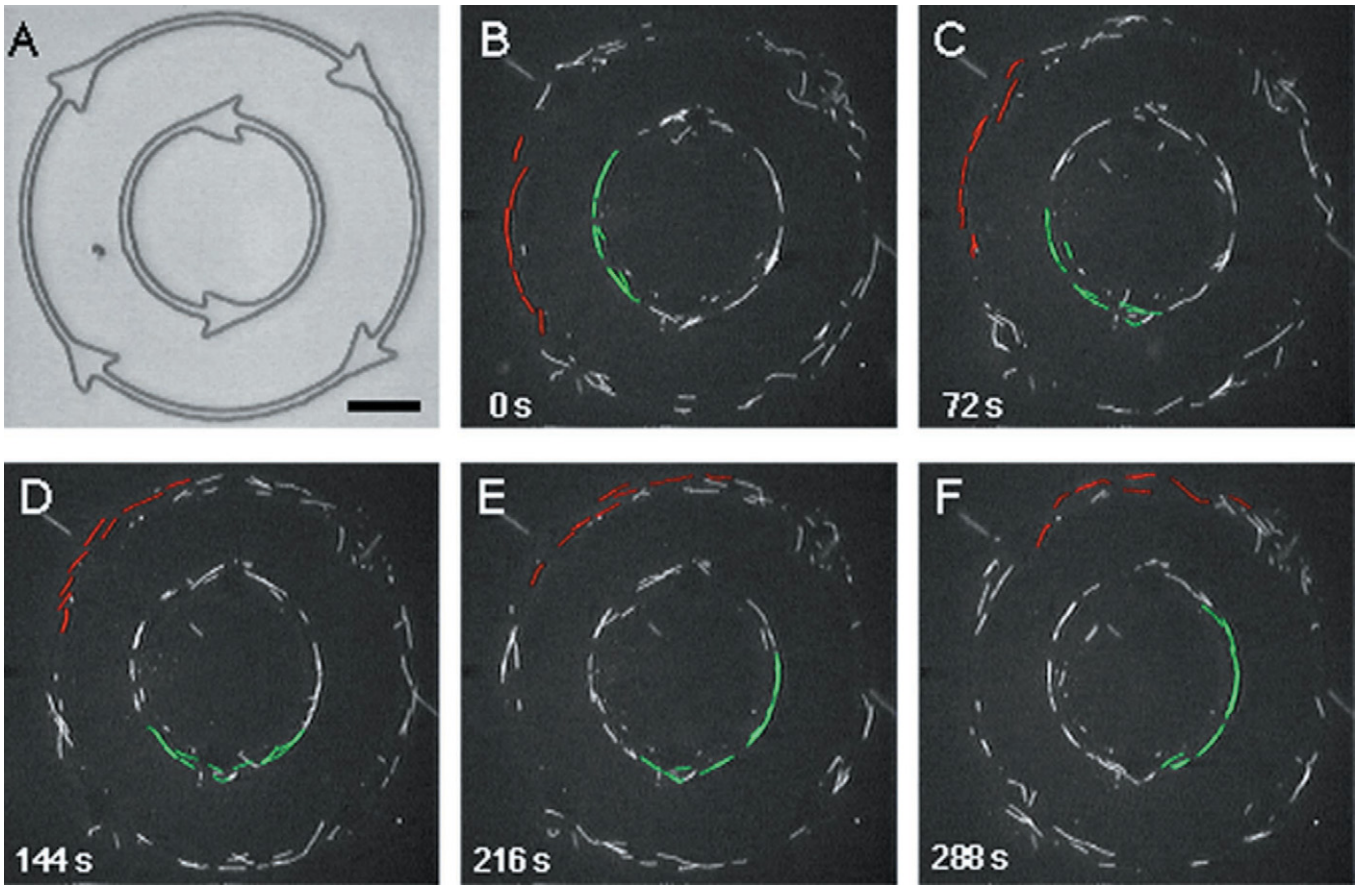

FIGURE 2 Unidirectional rotational movement of microtubules along circular tracks. The photographs represent snapshots of the movement of rhodamine-labeled microtubules taken at intervals of 72 s, 20 min after the addition of ATP (*B–F*). *A* shows an image of the transmission microscopy. Selected filaments were colored to allow tracking of groups of microtubules. Microtubules in the outer circle are moving clockwise (*red*), whereas those in the inner circle are moving counterclockwise (*green*). Scale bar, 20 μm. A movie of this movement can be seen at our web site (http://unit.aist.go.jp/genediscry/motility/biophysj/moviedl.html).

Our second goal was to control the direction of microtubule sliding. To achieve this, we added several arrowhead patterns along the linear tracks. Fig. 2 *A* shows one such example with two concentric circular tracks, each having the arrowhead patterns pointing in opposite directions. When the arrowheads were absent, microtubules moved in both directions, clockwise and counterclockwise, along each of the two circular tracks (not shown). In contrast, when arrowheads were added to the pattern, almost all the microtubules on the tracks, which were initially moving in both directions, started to move toward one direction within a few minutes after the addition of ATP. In nearly every case, microtubules in the outer circle moved clockwise whereas those in the inner circle moved counterclockwise throughout the observation periods of up to 2 h.

These unidirectional movements on the circular tracks were generated by the rectification of the bidirectional movements at the arrowhead patterns; that is, microtubules entering the arrowheads from the wrong direction often made a 180° turn and moved out in the correct direction. Examples of this behavior of microtubules in the arrowhead areas are shown in Fig. 3. Microtubules which entered from the correct direction (*left side* of the pattern illustrated in Fig. 3 *A*) passed through the arrowhead pattern undisturbed. In contrast, microtubules which entered from the wrong direction (*right side* of the pattern shown in Fig. 3 *B*) bumped against the left-side wall at the base of the arrowhead, changed the direction, and usually exited to the right by moving along the wall of the pattern (Fig. 3 *B*). As a result, the flow of microtubule movements changed from the left to right direction, and this is how the arrowheads function as rectifiers of the microtubule movement. It is also possible to design a number of other patterns having similar functions. To estimate the efficiencies of these patterns as rectifiers, we measured the probability of different patterns to invert movements from the wrong direction to the correct direction (p_{return}; Table 1). There was no observation of any microtubule moving in the correct direction to change its direction toward the wrong direction at the arrowheads. The values of p_{return} were, however, different depending upon

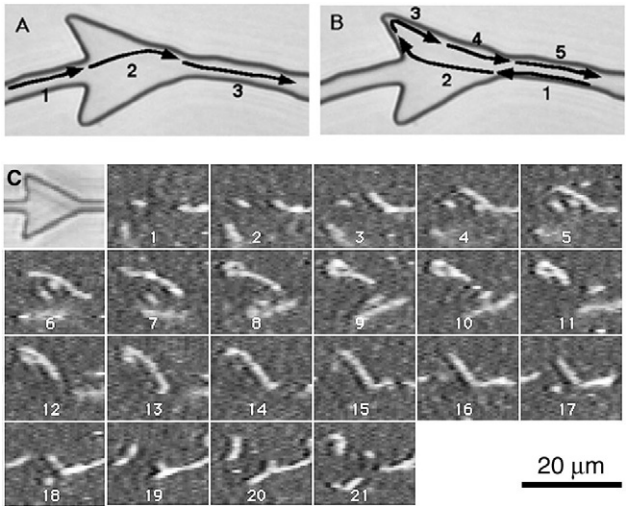

FIGURE 3 Arrowhead function as a rectifier of microtubule movements. *A* shows that a microtubule entering the arrowhead from the correct direction (from the *left side* entrance) passes through the arrowhead pattern. *B* demonstrates schematically how a microtubule entering the arrowhead from the wrong direction (from the *right side* entrance) makes a 180° turn and moves out in the correct direction. *C* is an actual sequence of fluorescence images showing turning of a microtubule at an arrowhead pattern. Images were captured with a SIT camera at a rate of 8 s/frame.

the patterns used. Among those tested, arrowhead-like patterns with barbs (pattern *A* in Table 1), such as those we used in the experiments shown in Figs. 2 and 3, gave the best results. The p_{return} for this pattern was >70%.

This unidirectional movement of microtubule along microfabricated tracks should enable us to actively transport materials at the micrometer scale. To explore this possibility, we prepared two square pools each with an area of 60·60 μm^2, in which microtubules moved freely in random directions, and connected these pools with three linear tracks each having four arrowheaded rectifiers (Fig. 4 *A*). Before

TABLE 1 Efficiencies of four different patterns as rectifiers

Pattern		Probability (p_{return}) of inversion (%)
A		73 ± 12 (n = 4)
B		51 ± 5 (n = 5)
C		35 ± 16 (n = 5)
D		26 ± 9 (n = 6)

The efficiency of each pattern as a rectifier was assessed by measuring the probability of inverting the direction of the microtubules movements in the pattern. p_{return} represents the probability of inverting the direction for microtubules entering the pattern from the wrong direction. p_{return} for patterns A and B are larger than their corresponding half patterns (C and D) approximately by a factor of two, respectively. The values are mean ± S.D. of data measured at multiple (n) sites for each pattern.

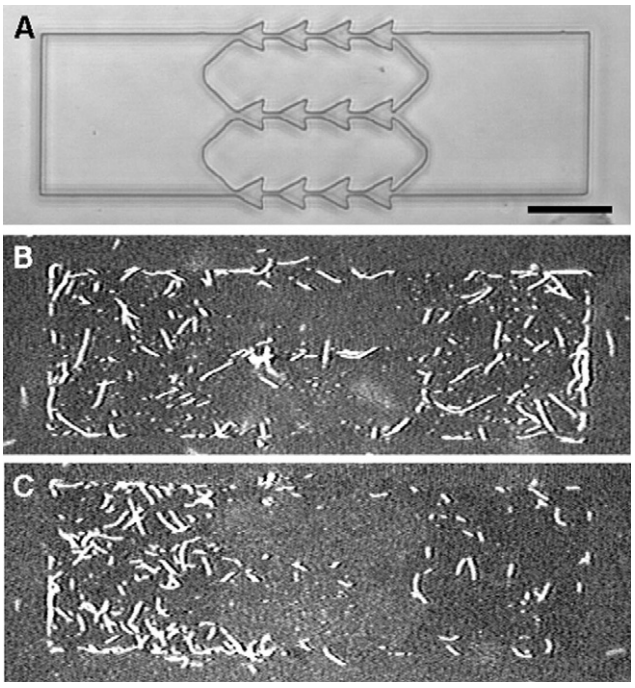

FIGURE 4 Active transport between two pools of micrometer scales. *A*, transmission micrograph; *B*, fluorescence image of rhodamine-labeled microtubules taken before ATP addition; and *C*, taken at 18 min after the ATP addition. Scale bar, 30 μm. A movie of this movement can be seen at our web site (http://unit.aist.go.jp/genediscry/motility/biophysj/moviedl.html).

ATP was added, the number of microtubules in the two pools were almost equal, reflecting similar kinesin densities in the two pools (Fig. 4 *B*). After ATP addition, the distribution was gradually shifted to the left pool, as directed by the arrows of the rectifiers. After 18 min, the number of microtubules accumulated in the left side pool increased to approximately 80% (Fig. 4 *C*). In theory, the fraction of microtubules in the left pool should be given by Eq. 1:

$$Y(t) = 1/(2.0 - p_{\text{return}}) + (Y(0) - 1/(2.0 - p_{\text{return}})) \cdot \exp(-(2.0 - p_{\text{return}}) \cdot K \cdot t) \quad (1)$$

where t is the elapsed time after the addition of ATP, K is a time constant determined by a number of factors including the sliding velocity, persistence of the movement, and the size and shape of the pools, and p_{return} is a combined efficiency of inversion. Because the efficiency of inversion at one arrowhead pattern was 0.73 (Table 1), the combined efficiency (for four sequential arrowhead patterns) should be 0.99 ($= 1 - (1 - 0.73)^4$). From Eq. 1, it is expected that the fraction of microtubules accumulated in the left pool approaches 99% at infinite time after the ATP addition. In reality, however, the fraction of microtubules in the left pool rapidly approached 80%, but increased only very slowly above that. We do not have a satisfactory explanation for this discrepancy. It is clearly not because the system had

reached an equilibrium at 80% because we never saw a microtubule moving from the left to the right pool. In contrast, we often saw some microtubules rotating around in fixed regions, which may be attributable to some irregularity of the surface or partial denaturation or heterogeneity (e.g., number of protofilaments) of the microtubule. These rotating microtubules in the right pool would have little chance to enter the tracks and move to the left pool, and we suspect this might explain part of the discrepancy.

One might suspect that shorter microtubules are sorted less efficiently, and therefore, do not accumulate as efficiently as longer ones in the left pool. To test this possibility, we measured the length of microtubules in the two pools at 18 min after the addition of ATP. Surprisingly, the length distributions of microtubules were not significantly different between the two pools, with the mean lengths ± SD of 4.6 ± 3.0 μm (*left pool*) and 3.9 ± 2.7 μm (*right pool*), respectively. This length distribution should be affected by two factors, one being the efficiency of inversion in the arrowheads and the other being the probability of spontaneous inversion within the linear parts of the tracks connecting the pools. Therefore, these data indicate that neither of the probabilities of these two inversion events is significantly affected by the length of microtubules. However, because we did not score microtubules shorter than 1 μm, we cannot exclude the possibility that extremely short fragments of microtubules are sorted less efficiently.

Under our condition for sliding assay, the movements of microtubules were observed only on exposed glass surfaces of the tracks, but not on the surrounding SAL601 resist surfaces. One likely hypothesis for these highly restricted movements is that kinesin is selectively absorbed onto the glass surfaces, but not onto the SAL601 surfaces. To test this hypothesis, we estimated the affinities of kinesin molecules to each of the two surfaces using FITC-labeled kinesin (Fig. 5). When 0.1% Triton X-100 was not included in the kinesin absorption buffer, the densities of kinesin molecules, as judged from the fluorescence intensities, on the resist and the glass surfaces were almost the same. When unlabeled kinesin was absorbed onto surfaces with tracks in the absence of 0.1% Triton X-100, movements of microtubules occurred on both SAL601 and glass surfaces, and moving microtubules often climbed up the wall made of the resist so that the restricted movements along the tracks were not attained. In contrast, when Triton X-100 was included, the densities of kinesin molecules on SAL601 surfaces were decreased to approximately 50% compared with that on glass surfaces. This demonstrates that Triton X-100 selectively decreases relative affinities of kinesin toward the resist surfaces, and should be part of the reason for the restricted movements along the tracks. However, decreasing the kinesin density on the resist surfaces to 50% is not sufficient to explain the efficient exclusion of movements from these surfaces, because even a single active kinesin molecule is able to drive continuous movement of a microtubule (Howard et al., 1989). We suspect that most of the kinesin molecules on the SAL601 surfaces were absorbed to the surface in a manner in which they could not interact

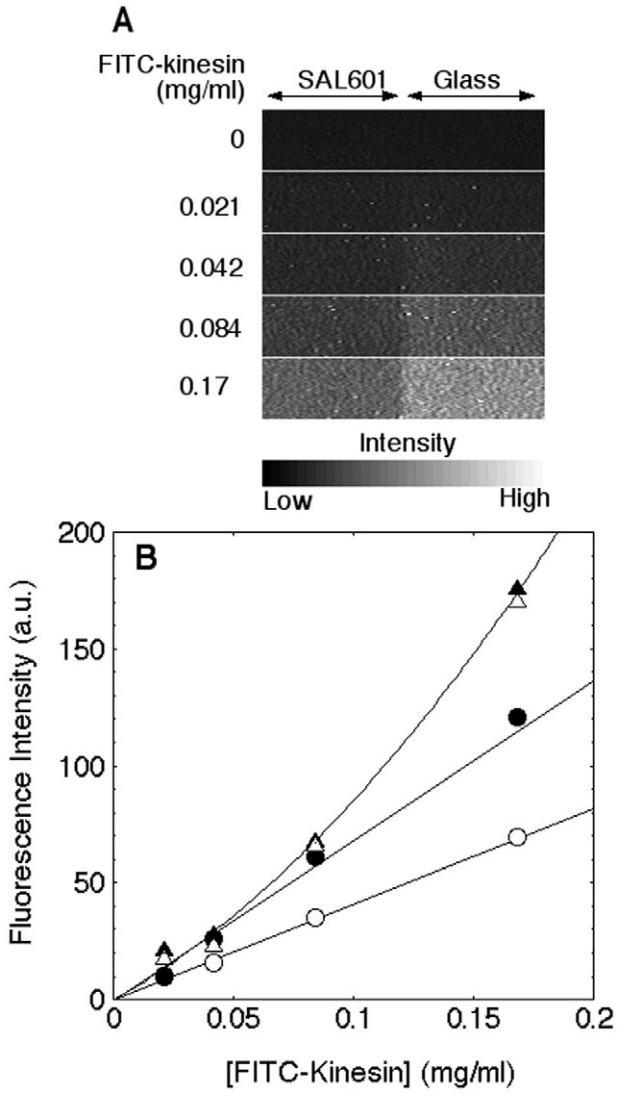

FIGURE 5 Affinities of kinesin for glass and resist surfaces assayed using FITC-labeled kinesin. Affinities of kinesin to the surfaces were estimated indirectly using FITC-labeled kinesin. Solutions of FITC-kinesin dissolved in the kinesin absorption buffer (described in Materials and Methods), with or without Triton X-100, were infused into flow chambers at room temperature. After 2 min, the flow chambers were washed twice with the same absorption buffer without FITC-kinesin, and the buffer was exchanged to the normal assay buffer. Fluorescent images of surfaces of the coverslips were then observed using a fluorescent microscope equipped with a SIT camera. *A* shows fluorescence images of square areas covering the border between the SAL601 and exposed glass surfaces prepared in the presence of Triton X-100. *B* shows fluorescent intensities of the surfaces plotted against the concentration of FITC-kinesin in the absorption buffer. Glass surfaces prepared in the presence of Triton X-100 (*solid circles*), SAL601 surfaces prepared in the presence of Triton X-100 (*open circles*), glass surfaces prepared in the absence of Triton X-100 (*solid triangles*), and SAL601 surfaces prepared in the absence of Triton X-100 (*open triangles*).

TABLE 2 Characterization of four resist materials as a material of the wall of the tracks

Resist	Climbing up	Selectivity of absorption	Type	Notes
SAL601	Rare	Good	Negative electron beam and photo	Possible to make many copies photolithographically on glass surfaces.
				Necessary to remove the residues on glass surface after development.
				Usually used as an electron beam resist.
AZ5214	Rare	Good	Negative photo	Brightly fluorescent, and is not compatible with the observation of rhodamine-labeled microtubules.
OBER1000 (PMMA)	Often	Excellent	Positive electron beam	Very low affinity for kinesin.
				Optimization of lithographic conditions is required to prevent climbing up of microtubules.
				Possible to draw patterns narrower than 200 nm.
ZEP520	Often	Good	Positive electron beam	Same as above except for a higher affinity for kinesin.

Four resist materials were characterized in terms of suitability to use as a material of the track's walls, and two important features for restricting the microtubule movements within patterns are summarized here. They are whether microtubules do not "climb up" the resist wall and run away when they bump against the wall, and whether active kinesin molecules are selectively absorbed onto the glass surface, but not onto the resist surface. Here, we refer to kinesin molecules that are able to bind and move microtubules as active. The relative densities of active kinesin molecules were estimated indirectly by measuring the densities of microtubules bound to the resist surfaces in the absence of ATP. On the whole, SAL601 was most preferable among the four resists with rare climbing and running away, and we used this material for all experiments in this study. With regard to the selective adsorption. OBER1000 was superior to the three other resists. Although active kinesin molecules were preferentially absorbed onto the glass surface rather than onto the surfaces of the three resists, SAL601, AZ5214 or ZEP520, small amounts of active kinesin molecules were absorbed onto these resists, as demonstrated by the fact that a few microtubules were bound to the resist surfaces before the addition of ATP. This was not a major problem in this study, because those microtubules initially present on SAL601 surfaces rapidly diffused away into the assay buffer upon addition of ATP. In contrast, very few microtubules were found on OBER1000 surfaces even before the addition of ATP, suggesting that very few active kinesin molecules were absorbed onto the surface of this resist.

productively with microtubules. Binding of kinesin to surfaces resulting in inactivation of its motor activities has been experienced when kinesin solutions without protein additives were applied to glass surfaces (Howard et al., 1989). For more general and reproducible applications of this technique, it is warranted to develop methods to attach active molecular motors to exposed surfaces relying on specific binding with a high affinity, such as polyhistidine-tag binding to Ni surfaces or Ni (II) ions chelated over surfaces with NTA-SAM (Sigal et al., 1996; Soong et al., 2000), or biotin-tag binding to surfaces coated with streptavidin (Hengsakul et al., 1996; Mooney et al., 1996; Ruiz-Taylor et al., 2001).

We tested three other resist materials, AZ5214, OBER1000, and ZEP520, to find out whether they can substituted for SAL601 in terms of efficiently restricting movements of microtubules within the tracks (Table 2). The characteristics of the photo-resist AZ5214 were similar to those of SAL601, except that this resist was brightly fluorescent when excited by green light and was not compatible with the observation of the movements of rhodamine-labeled microtubules. The two electron beam resists, OBER1000 and ZEP520, behaved similarly in our assay. Microtubules moving along the tracks often climbed up the walls and moved away when the microtubules bumped against the walls made of either of these two resist materials. Scanning electron microscopic observation revealed that the walls made from SAL601 were steeper ($\sim 80°$) than those of OBER1000 prepared under our current conditions ($<20°$). Thus, we speculate that optimizing parameters of the lithographic process to prepare steeper walls is needed before these resist materials are used in similar applications. Construction of overhangs might also help to restrict microtubule movements within the tracks made of these resists, although such structures were not necessary for grooves made of SAL601.

Technology already exists to attach foreign materials to microtubules using the avidin-biotin system (Kuo and Sheetz, 1993; Meyhofer and Howard, 1995) or antimicrotubule antibodies (Wada et al., 2000). With the combination of such attachment methods, this novel technology to control the direction of microtubule movements by molecular motors in vitro should find its application in a number of fields. For example, it may become possible to attach a cogwheel to microtubules moving around circular tracks to create micrometer-scaled rotators, or to use microtubules as carriers for unidirectional transport of reaction materials or products in chemical or biochemical plants on chips.

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