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Unidirectional Transport of Kinesin-Coated Beads on Microtubules Oriented in a Microfluidic Device

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ABSTRACT

We have established an orientation technique of microtubules and evaluated their polarities by the movement of kinesin-coated beads quantitatively in poly(dimethyl siloxane) (PDMS) channels. More than 95% of beads moved to the desired direction; this indicates almost all the microtubules were functionally oriented including plus and minus polarities. The technique is essential for fabricating a bio-hybrid nanotransport system, in which the kinesin–microtubule system combined with microfluidic structures provides a driving mechanism in an aqueous environment: once microtubules are immobilized inside a microfluidic channel, kinesin-coated objects can be transported on the designated pathways without any liquid manipulation.

We have quantitatively evaluated the unidirectional movement of kinesin-coated beads on oriented microtubules in a microfluidic channel. The orientation process is an essential step in establishing a transportation system powered by a biomotor system composed of kinesin and microtubules. The fluid force by a buffer flow orients microtubules during the in vitro gliding assay and they are chemically immobilized by glutaraldehyde. We have successfully carried beads by kinesin motors on oriented microtubules unidirectionally. The transportation system is inspired by intracellular transport mechanisms in living organisms.

The kinesin−microtubule system is focused here among several nanoscale transport mechanisms in cells. Motor molecule, kinesin, has the dimension of 80 nm in total length.[1] One of cytoskeletal filaments, microtubule, has the size of 25 nm in diameter and 10−30 μm in length. Kinesin transports a cargo such as a vesicle on microtubules in cells by hydrolyzing adenosine 5′-triphosphate (ATP); this transportation mechanism allows the cell to handle molecules that are captured by surface receptors or synthesized in it.

The biochemical reconstruction of the kinesin-microtubule system has been studied over decades. It has proved that genetically engineered kinesin is able to move on microtubules in vitro; those proteins are purified separately by different methods. When a micro/nanoscale bead instead of a biological cargo is coated with kinesin in the reconstructed system, the bead can be transported on microtubules immobilized on a glass surface. Therefore, our final goal is to establish the hybrid nanotransport system that is modeled after the one in cells by integrating the kinesin−microtubule system and a microfluidic device.[2−3] The system will handle not only micro/nanoscale objects but also target molecules immobilized on the object.

One of advantages in integrating the biomotor-based transport system to a microfluidic system is the ability to handle smaller numbers of molecules than conventional micro total analysis systems (μTAS) in which dissolved molecules or a cluster of beads are carried by liquid flow. The target molecules will be transported on microtubules and concentrated at the destination for further reaction and detection processes.

Another advantage is to miniaturize μTAS from micrometer to nanometer scale. The liquid manipulation by pressure becomes drastically difficult when the channel size is decreased from micrometer to submicrometer scale. This prevents us from downsizing a channel even though the fabrication technique can realize a nanoscale channel. If a microtubule is placed in a narrow channel, kinesin can transport target molecules through the channel without manipulating the surrounding liquid.

When carrying the object to the designated direction efficiently in such an artificial nanotransport system, it is

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necessary to control the moving direction. This can be realized by orienting microtubules to the direction and immobilizing them during the reconstruction of the biological transportation system in a microfluidic device, because the processive movement of conventional kinesin directs to the plus end from the minus end of microtubules. In cells, the target molecules are concentrated in a vesicle at the minus end of self-oriented microtubules and carried to the other end. In an engineering system, the target molecules may be encapsulated in liposomes or attached selectively on beads; they are transported by kinesin immobilized on the carriers along a track composed of oriented microtubules. Thus, we can directly handle target molecules in stead of their solution.

The unidirectional transportation using biomolecular motors is an essential component of the hybrid system but poses a challenging problem in practice. This is because microtubule polarities cannot be visualized without fluorescent labels after the polymerization in vitro.[4] Two approaches have been actively studied: one is to utilize the in vitro gliding assay. Moving microtubules on a kinesin-coated surface are physically guided by microfabricated walls.[5−8] In the other approach, microtubules are functionally oriented and immobilized on a glass surface. Then kinesin-coated objects are unidirectionally transported on microtubules.[9−13] Those reports describe orientation methods and some experimental results. However, the quantitative analysis of the orientation technique from the engineering point of view was not performed about how well microtubules were oriented or objects were transported unidirectionally. Therefore it is essential to measure the orientation yield quantitatively in order to optimize the process condition for the unidirectional conveyance of micro/nano objects.

We have chosen the latter approach because it offers much better control over transportation direction and device preparation: (1) multiple tracks may be defined in different directions on a chip. (2) Bidirectional motion on the same track will be possible by selectively using particles coated with kinesin or dynein. (3) A chip with predefined tracks of microtubules may be kept undamaged over a few months. Microtubules keep their functionalities longer than kinesin in liquid nitrogen. Both microtubule and kinesin must be prepared immediately before the experiment in the approach which physically guides microtubules by walls. On the other hand, oriented and immobilized microtubules in devices can be preserved for future experiments when kinesin carry objects on those microtubules. (4) It is a straightforward approach to bind kinesin molecules and objects in the same way as realized in the bead assay.

Materials and Methods. Kinesin heavy chain (110 kDa) derived from *Drosophila melanogaster* with GST-tag (26 kDa, total = 136 kDa) was expressed in *E. coli*. First purification was performed with glutathione-Sepharose (17-5132-01, Amersham Biosciences). The affinity between kinesin and microtubules was utilized in the second purification step to remove inactive kinesin. The final concentration of kinesin was ~40 μg/mL in 25 mM Tris-acetate, 200 mM K-acetate, 10 mM $MgSO_4$, 10 mM ATP, 1 mM EGTA, and stored in the liquid nitrogen. Tubulin, which would be polymerized to microtubules, was prepared from four porcine brains by two cycles of temperature-dependent polymerizations and phosphocellulose chromatography.[14] The tubulin was stored in the liquid nitrogen at the concentration of 4 mg/mL in 0.5 mM guanosine 5′-triphosphate sodium salt hydrate (GTP) in PC buffer (100 mM PIPES−NaOH (pH = 6.8), 1 mM EGTA, 1 mM $MgSO_4$). They were polymerized at 37° C in PC buffer containing 1 mM $MgSO_4$ and 1 mM GTP. The general assay buffer used in our system was BRB80 containing 80 mM PIPES−NaOH (pH 6.8), 1 mM $MgCl_2$, 1 mM EGTA.

Two different concentrations of proteins were prepared for kinesin and microtubules, respectively, to achieve the functional orientation using the in vitro gliding assay. The concentrations of kinesin prepared in 1 mg/mL casein in BRB80 were 4 μg/mL and 40 μg/mL. The microtubule concentrations of 40 μg/mL and 400 μg/mL were diluted from 4 mg/mL in PC buffer with 20 μM paclitaxel. Gliding microtubules can be observed with any combination of protein concentrations, and the combination of 40 μg/mL for kinesin and 400 μg/mL for microtubule brings the highest microtubule density among four combinations after the orientation process. The bead assay was performed to evaluate the polarity of oriented microtubules. Kinesin-coated beads were prepared by incubating 40 μg/mL kinesin with casein-coated beads (320 nm in diameter, 5.4% solids, PC00N/3079, Bangs Lab.) for 15 min. By dividing the number of kinesin molecules by the number of beads, the number of kinesin molecules per bead was estimated at approximately 3×10^2.

Starting from the simple method, the orientation process in the micrometer scale was realized by using three different kinds of channels: (i) a flow cell, (ii) a coverslip channel, and (iii) a PDMS channel. The flow cell was utilized for the bead assay on microtubules immobilized on the glass surface without the orientation process. The process was conducted in the coverslip channel while being monitored by the dark-field microscope. The objective of using the PDMS channel was to realize the process in micrometer scale based on the process established by the coverslip channel. A flow cell is a well-known technique to enclose proteins between two coverslips to observe motor proteins.[15−16] It was prepared using spacers (1 × 25 mm paper slips) coated with grease (Apiezon M grease, Apiezon) between two coverslips (18 × 18 mm (top), 24 × 36 mm (bottom), No. 1: 0.12−0.17 mm in thickness, Matsunami). The flow cell had the dimensions of 18 mm in length, 6−7 mm in width, and 40 μm in height. The coverslip channel was composed of two coverslips (24 × 60 mm (top and bottom), No. 1: 0.12−0.17 mm in thickness, Matsunami) with the dimensions of 40 mm in length, 2 mm in width, and 107 μm in height.[10] The outlet was connected to a syringe pump to generate a steady flow, which makes buffer replacements easier in a channel, even when the channel is on a microscope stage as shown in Figure 1a. The PDMS channel was composed of a coverslip and PDMS replica on which micro channels were transferred from a mother mold. The channel dimension was 30 mm in length, 30−500 μm in width, and 80 μm in height. Thick negative photoresist (SU-8 2050, Microchem) was

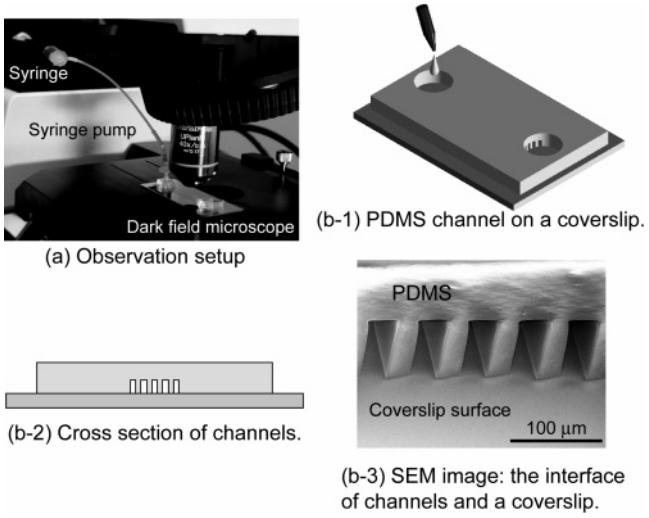

Figure 1. (a) Observation setup for monitoring the orientation process using a flow cell and a coverslip channel. (b-1) A PDMS channel fabricated to orient microtubules in the micrometer scale channel. Figure (b-2) and (b-3) are its cross section.

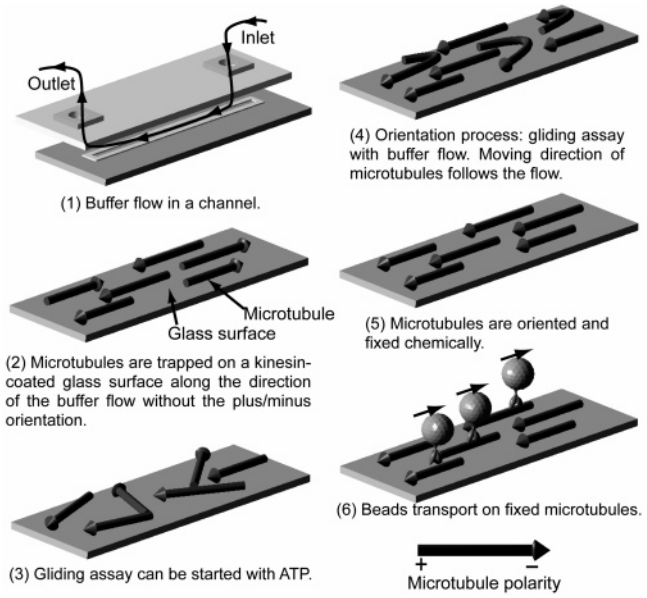

Figure 2. Orientation methodology of microtubules by a buffer flow. An arrow indicates a moving microtubule of which arrowhead is the minus end of the microtubule. Panels (2)−(6) illustrate the orientation process focusing the microtubule movement on a glass surface inside a channel.

patterned on a silicon wafer as a mother mold for channels. PDMS prepolymer (Sylgard 184, Dow Corning) was then poured on the mold and cured. Multiple channels were transferred on the PDMS surface as shown in Figure 1b-2 and b-3. The replica was placed on a coverslip and pressed to remove bubbles without any adhesive as shown in Figure 1b-1−b-3. The adhesion between the coverslip and PDMS is high enough to manipulate buffers without leakage. The observation setup was the same as Figure 1a with inlet and outlet connections for buffer replacements.

The orientation process was performed in the coverslip channel and the PDMS channel as illustrated in Figure 2. The buffer flow in a channel was from right to left as shown in Figure 2-(1); please note this direction is always the same in the following drawings. Figures 2-(2) to (6) are close-up illustrations of the channel surface focusing on the movement of microtubules during the process. Kinesin in BRB80 was incubated for 10 min to coat the glass surface and the excessive kinesin was washed out by BRB80. Microtubules were trapped parallel to the buffer flow on the kinesin-coated surface. Although microtubules are physically aligned by the fluid force, those microtubules have the random polarity in respect to the functional plus/minus orientation. They are represented by arrowheads directed to both right and left in Figure 2-(2). The continuous buffer flow with ATP was utilized to orient the immobilized microtubules.[9−11] This is because the pulse injection of ATP (1 mM) induces random movement of microtubules as called in vitro gliding assay (Figure 2-(3)). The continuous flow, however, removes microtubules gliding upstream, while almost all the microtubules gliding downstream remained on the kinesin-coated glass surface (Figure 2-(4)). Therefore, 0.5 mM ATP in BRB80 was flown into the channel for the orientation for 2 min. The flow rate was varied from 0 (in vitro gliding assay) to 80 μm/s to optimize the orientation condition. BRB80 including 0.1% glutaraldehyde was kept flowing for 5 min to crosslink kinesin and microtubule; this results in the immobilization of gliding microtubules with physical and functional orientation. 0.1 M glycine in BRB80 was also injected for 10 min to block aldehyde groups (Figure 2-(5)). Kinesin-coated beads were injected in the channel and trapped on microtubules after replacing glycine by BRB80. Beads started to move on the 1 mM ATP injection. Because the plus end and the minus end of microtubules are placed upstream and downstream, respectively, beads move upstream unidirectionally (Figure 2-(6)).

Microtubules and beads were observed by the following setup. The orientation process was monitored by a dark-field microscope (BX50, UPlanFl 40×, Olympus) with a CCD camera (CCD300-RC, DAGE-MTI), an image intensifier unit (C9016−01, Hamamatsu), a video enhancement system (XL-20, Olympus), and a digital videocassette recorder (DSR-11, SONY). The setup shown in Figure 1a allows the real-time observation of the orientation process even on the microscope stage. Microtubules were visualized in the case of (i) the flow cell and (ii) the coverslip channel, because these channels can contain samples between smooth glass surfaces which are applicable to the dark-field microscopy. Though intricate channels can be fabricated in (iii) the PDMS channel case, microtubules cannot be visualized by the dark-field microscope due to the light scattering by the thick and rough PDMS layer. We established the orientation process by monitoring the (i) and (ii) cases, and investigated only the bead movement in the case of (iii). The moving beads were observed by a DIC microscope (IX71, UPlanFl 100× oil, Olympus) and captured by a CCD camera (C5405−50F, Hamamatsu), the video enhancement system, and the digital videocassette recorder. This setup was utilized for all three cases (i)−(iii) for a clear picture of beads, though the dark-field microscope could visualize both microtubules and beads for (i) and (ii) cases. The fluorescently labeled microtubules

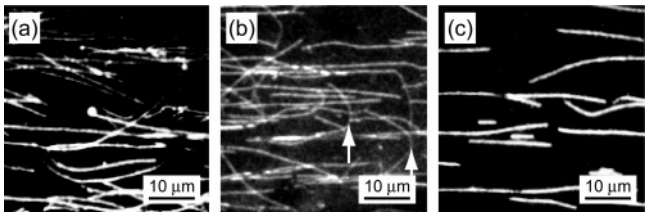

Figure 3. Microtubules monitored by the dark-field microscope. Images show microtubules (a) before the orientation process, (b) during the process, and (c) after the immobilization.

can be an option, but photobleaching and depolymerization prevent us from the long-time visualization in a PDMS channel.[3]

Results and Discussion. The orientation process was successfully established in the coverslip channel based on the theory shown in Figure 2 and monitored as shown in Figure 3. When the concentrations of kinesin and microtubule are high, numerous microtubules are captured on the kinesin-coated surface and each microtubule cannot be identified. To visualize the movement of each microtubule, the experiment was performed with the concentrations of 40 μg/mL both for kinesin and microtubules; this condition was frequently used for the in vitro gliding assay. Figure 3a represents the physically aligned microtubules by the fluid force, when they were injected to the kinesin-coated glass surface as shown in Figure 2-(2). During the orientation process microtubules gliding upstream were removed as indicated by white arrows in Figure 3b, which corresponds to the orientation process illustrated in Figure 2-(4). Sequential injections of glutaraldehyde and glycine immobilized microtubules on the kinesin-coated glass surface. They were oriented parallel to each other due to the flow as shown in Figure 3-(c) which corresponds to Figure 2-(5). The standard deviation of the angle between them is only 3.2 degree.

We found out that almost all the beads moved upstream unidirectionally by conducting the bead assay on the immobilized microtubules. For the evaluation of the microtubule polarity, the kinesin-coated beads were introduced on the microtubules and ATP was injected to activate kinesin as illustrated in Figure 2-(6). The bead movement was recorded on a digital videocassette of which fast-forward mode enhanced the unidirectional movement of beads. We could not find any bead moving perpendicular to the direction of buffer flow in the experiments. This indicates that microtubules moving perpendicular to the flow were removed in the alignment process.

The quantitative evaluation of the orientation yield was performed in relation to the buffer flow rate. The orientation ratio is defined as the ratio of the number of beads moving upstream to the number of all the moving beads; this represents how well microtubules are oriented. The ratio increases as microtubules are functionally oriented including the plus/minus polarity. The total number of moving beads counted in each experiment was over 100 to minimize the deviation. Since channel profiles are different among experiments, the flow rate at 100 nm above the glass surface was adopted as the parameter to compare results by assuming the parabolic flow profile.[10−11]

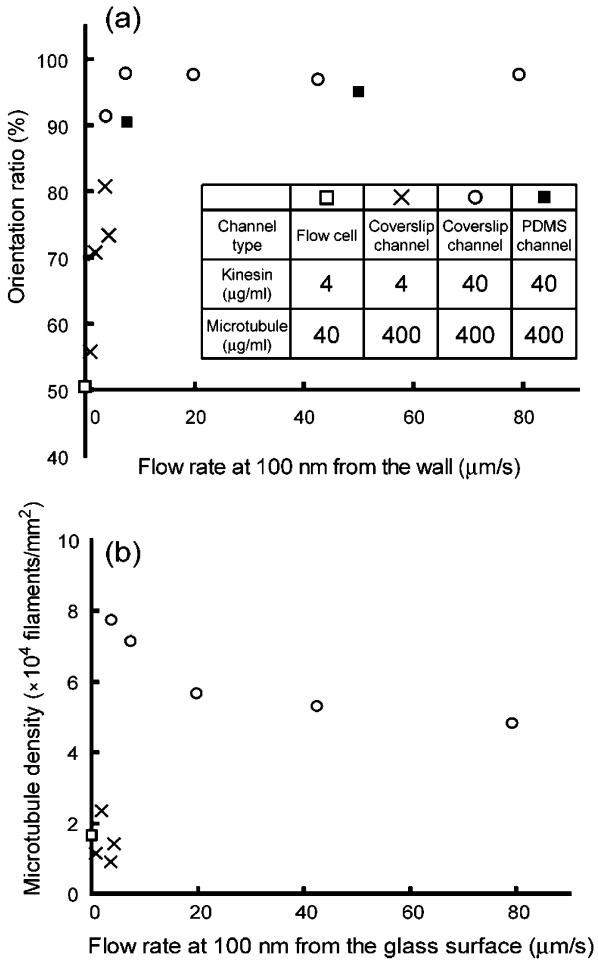

Figure 4. (a) Orientation ratio and (b) microtubule density are shown in relation to the flow rate at 100 nm from the glass surface. The legend shows the channel type and protein concentrations.

It is proved that the higher flow rate increases the orientation ratio but decreases the density of microtubules immobilized after the orientation process. The orientation ratio and the microtubule density are plotted in relation to the buffer flow rate in Figure 4a and b, respectively. Protein concentrations used in experiments are listed in the legend of Figure 4a. As a control experiment, microtubules (40 μg/mL) were injected to a flow cell coated with 4 μg/mL kinesin and immobilized without the orientation process. The orientation ratio was 50.3%, plotted by the open-square. The result is consistent with the fact that microtubules were physically aligned with the random plus/minus orientation. The orientation ratio of over 80% was achieved among four data points, expressed as crosses, in which the high concentration of microtubules (400 μg/mL) were oriented in a coverslip channel coated with 4 μg/mL kinesin. The microtubule densities of 0.9×10^4−2.3×10^4 filaments/mm^2 were, however, too low to transport many beads because the bead density immobilized on oriented microtubules was 6×10^4 beads/mm^2, which corresponds to only six beads in a 10 μm square. This phenomenon was caused by the low density of kinesin coated inside the channel. Therefore, the higher kinesin concentration of 40 μg/mL was used to coat inside the coverslip channel in other experiments. Results with this

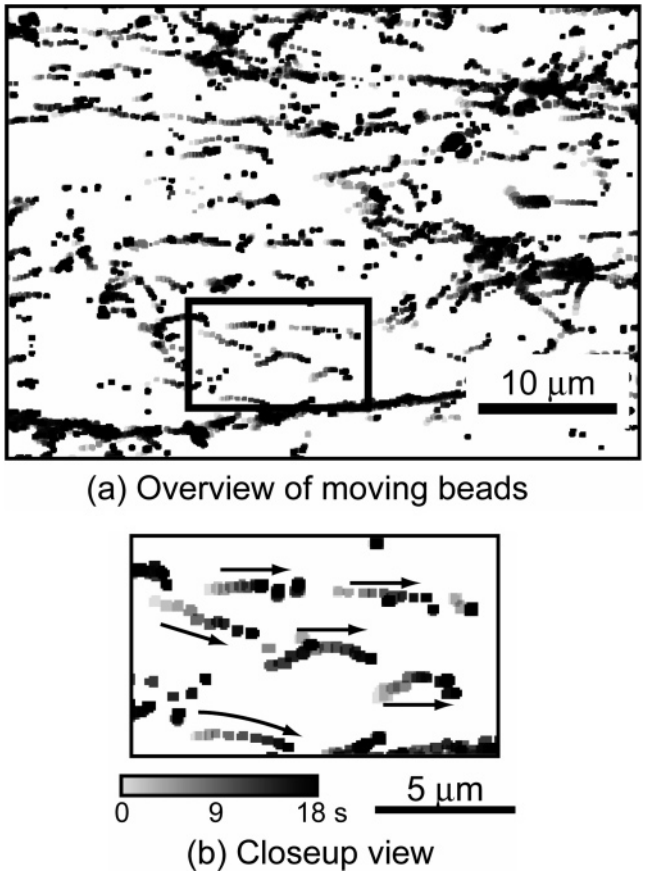

Figure 5. The superposition of ten consecutive images with two-second intervals showing the moving direction of beads. The gradation from bright gray to black corresponds to the time scale from 0 to 18 s. Figure (b), an expansion of (a), clearly shows the unidirectional movement of beads as indicated by arrows.

condition are indicated as open circles in Figure 4. The concentration yielded enough binding between microtubules and kinesin against the high flow rate to bring the high microtubule density to over 5×10^4 filaments/mm^2. The bead density was increased to $(2-3) \times 10^5$ beads/mm^2 (20−30 beads in a 10 μm square).

The orientation ratio reached about 97% over the flow rate of 8 μm/s. The larger shear stress by higher flow rate removes even the oriented microtubules from the glass surface. The flow rate of 8 μm/s is the best for both the orientation and the microtubule density due to their tradeoff. To visualize the bead movement, 10 still images were extracted with two-second intervals from the movie. They were converted to gray scale image and processed to have gradation from gray to black with the time course. The superposition of 10 images shows the unidirectional transport to right-hand side upstream as shown in Figure 5.

Based on those results obtained by the flow cell and the coverslip channel, we applied the technique to PDMS channels. One is the single channel with the dimension of 30 mm in length, 500 μm in width, and 20 μm in height. The other is parallel multiple channels composed of 16 channels with 30 μm spacing (length: 30 mm, width: 30 μm, height: 80 μm). Only the orientation ratio was measured and plotted as solid-squares in Figure 4a, since microtubules could not be visualized. The orientation ratios for the single channel and the multiple channels were 90% and 95%, respectively. The result proves that the orientation process can be applied to the sealed channel fabricated by the MEMS process.

In the present study, the fabrication method of channels with oriented microtubules was quantitatively evaluated. In addition, the unidirectional transportation of cargos was demonstrated. Some of previous reports showed the orientation technique only without the evaluation of transportation of cargos[10,13] or the observation of a few moving cargos for several micrometers only.[11] Our experiment includes both the orientation and immobilization technique of microtubules and the quantitative analysis of microtubule polarities. The evaluation of polarities by the bead movement is a very effective way to prove not only the unidirectional polarity but also the functional integrity of immobilized microtubules to be utilized for the nanotransport system. Microtubules in a channel with high density of 5×10^4 filaments/mm^2 realized the efficient transportation of beads with $(2-3) \times 10^5$ beads/mm^2, which was almost the same density of moving beads as seen in the conventional in vitro bead assay.

We have established the orientation technique of microtubules and evaluated the polarity of those microtubules quantitatively by the bead assay. The unidirectional transport was achieved with the orientation ratio of over 95%, even in a PDMS microfluidic channel. It will be the essential component in μTAS energized by the kinesin microtubule biomolecular motor system. Further complex 2-D transport systems can be realized on a chip by integrating multiple channels coated with the oriented microtubules.

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Supporting Information Available: The unidirectional transport of kinesin-coated beads on oriented and immobilized microtubules is recorded in the .mpg file with 5× speed. This material is available free of charge via the Internet at http://pubs.acs.org.

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