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A Polarized Microtubule Array for Kinesin-Powered Nanoscale Assembly and Force Generation

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ABSTRACT

Kinesins are biological motors that transport cargo unidirectionally along microtubule tracks. These motors are attractive candidates for carrying out biomolecular separations, directed assembly of nanoparticles, or for powering nano- or microscale devices. However, a prerequisite for harnessing kinesins is properly aligning the microtubule tracks that they walk along. We describe a method for constructing an array of aligned microtubules on a two-dimensional surface. The process involves immobilizing short microtubule seeds, polymerizing long microtubules uniquely from one end, and then attaching the elongated filaments to the surface. To quantitate the extent of microtubule alignment, we analyzed microtubule orientations from four different arrays and found a standard deviation of 12.8°, which is comparable to the alignment of oriented microtubule arrays observed in migrating fibroblasts. By producing a field of aligned microtubules, this array provides a launching point for employing kinesins for directed assembly or nanoscale force generation.

In eukaryotic cells, the task of transporting intracellular cargo and positioning organelles is carried out by motor proteins that move along cytoskeletal tracks. Much of this intracellular transport is accomplished by kinesins, a family of molecular motors that use the energy derived from ATP hydrolysis to transport vesicles, chromosomes, and protein complexes throughout the intracellular environment.[1] Conventional kinesin (Figure 1) has two heads with dimensions 7 nm × 4 nm × 4 nm (ref 2) that "walk" in a coordinated manner along microtubules, while the motor's tail acts as an attachment domain to bind intracellular cargo.[3] The microtubule tracks that kinesin walks along are cylindrical polymers, 25 nm diameter and up to tens of microns long, made from the protein tubulin.[4] Because controlling motion and assembling structures at submicron dimensions is a major research thrust in nanoscale science and technology, the ability of these motors to convert chemical energy into mechanical work at the level of single molecules has led to considerable interest in harnessing these protein machines for nanoscale bioengineering.[5−10] Possible applications include biomolecular separation systems, directed assembly of nanoparticles, or actuators for pumps or valves in MEMS microfluidic applications.

To harness kinesins for biotechnological applications, it is necessary to gain control over the placement of motors and microtubules on surfaces. Conventional kinesin binds well to glass and other surfaces through its tail domain, and the motor contains a swivel such that the heads can orient to bind a microtubule.[11] Microtubules, on the other hand, have a structural polarity: one end (termed the plus end) polymerizes at a faster rate than the other, and kinesin motors move unidirectionally along microtubules.[12,13] Hence to generate useful movement, microtubules must be properly oriented on surfaces. To date there have been two classes of approaches to orienting kinesin and microtubules for directed transport: immobilizing motors to transport cargo-laden microtubules,[6,8−10] and immobilizing microtubules to transport cargo-bound motors.[5,9] In the first case, kinesins are attached to selected regions of a surface or along surface features, and microtubules are transported along these surfaces. Creating ridges and valleys on a surface, either by aligning deposited polymers or by embossing or lithographic patterning, has been shown to successfully restrict microtubule motion to one dimension.[6,7] However, in these cases microtubules still move bidirectionally, resulting in no net

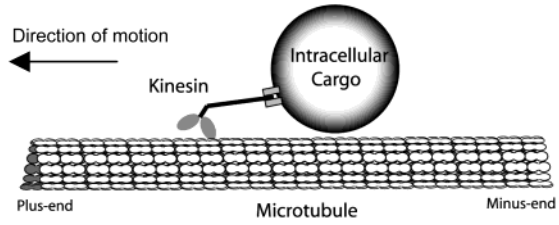

Figure 1. Kinesin function in vivo. Conventional kinesin has two head domains that "walk" along microtubules and transport intracellular cargo to the cell periphery. Other motors transport cargo in the opposite direction.

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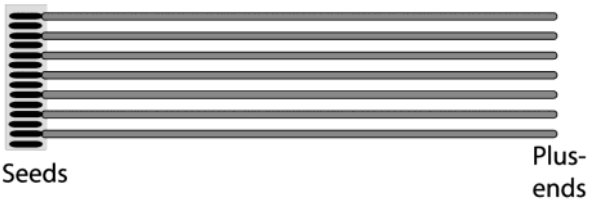

Figure 2. Diagram of the microtubule array. Microtubule seeds are adsorbed to an adhesive surface and microtubules are polymerized from the plus ends of these seeds to form an array with all of the microtubule plus ends facing the same direction.

movement. The most successful approach to directing microtubule movement has been to lithographically pattern arrowhead shapes in 1 μm thick photoresist on glass.[8] When kinesin was adsorbed to the exposed glass and microtubules were introduced to the surface, the edges created by the photoresist acted to steer the gliding microtubules along the tracks, while microtubules moving against the arrowhead pattern collided with the base of the structure, bent, and were redirected.

The second approach for harnessing kinesin motility, which is analogous to the geometry found in cells, is to immobilize microtubules to guide the movement of cargo-bound kinesins. The advantages of this approach are: (a) microtubules can be precisely oriented to direct motors to desired locations, (b) kinesin motors can be modified by protein engineering to possess a range of cargo attachment domains, increasing the utility of the motors, and (c) it enables the use of motors with opposite directionality on the same track, enabling bidirectional sorting of cargo. To date, two approaches have been reported for immobilizing oriented microtubules on surfaces. Stracke et al.[9] used fluid flow in a custom flow chamber to orient microtubules gliding over a motor-coated surface. When the leading edges of microtubules came free from the surface, they were bent downstream by the flow. Although this approach led to an oriented field of moving microtubules, the microtubules were not immobilized so when the flow was abolished the directions randomized. A second and more elegant approach by Limeris et al.[5] used surface-immobilized antibodies complementary to microtubule minus ends to capture microtubules and flow to orient the microtubule plus ends downstream. This approach produced a field of aligned and end-immobilized microtubules, with the next challenge being to attach the rest of the microtubules to the surface to preserve the proper orientation.

Here, we report a novel method for producing an array of aligned microtubules fixed to a surface, which can be used for nanoscale assembly applications or for harnessing the force generating capabilities of kinesins attached to microfabricated structures or particles. The approach involves immobilizing short microtubule seeds in desired locations and polymerizing long oriented microtubules from these seeds (Figure 2). The advantages of this system are that the microtubules can be positioned in precisely defined locations, the array can be grown in confined geometries, and the approach can achieve a high density of aligned microtubules, a prerequisite for generating appreciable forces.

The first step in creating the microtubule array was to immobilize microtubule seeds at defined locations on a glass surface. For all of the microtubules described here, tubulin was isolated from bovine brains, fluorescently labeled with rhodamine, and polymerized according to standard procedures.[14−16] Polymerized microtubules were stabilized by diluting 1:100 into a solution of 10 μM paclitaxel, and sheared using a 30-gauge needle to lengths 1−5 μm. Unincorporated free tubulin was removed by pelleting the microtubules for 10 min at 30 psi in a Beckman Airfuge (Beckman-Coulter, Inc., Fullerton, CA) and resuspending in BRB80 buffer plus 10 μM paclitaxel. A number of surface treatments were tested for both adsorbing microtubules and resisting microtubule adsorption. While bare glass and most surface treatments led to moderate microtubule binding, we found that either 3-aminopropyltriethoxysilane (APTES, Pierce Biotechnology, Inc.) or immobilized kinesin motors resulted in the highest density of surface-immobilized microtubules. For passivating surfaces, we found the protein casein to be the most effective treatment, resulting in no measurable microtubule adsorption over the course of the experiment.

To pattern microtubules, a microscope coverslip (Corning 1, 18 mm square) was masked with cellophane tape and a selected region was exposed to kinesin motors.[17] Following motor adsorption, the tape was removed and the remaining surface was blocked with a 0.5 mg/mL solution of casein, the coverslip assembled into a flow cell configuration, and microtubules flowed into the ~20 μL chamber in the presence of 1 mM adenosine 5′-(β,γ-imido) triphosphate (AMP−PNP, Sigma, Inc.), an ATP analogue that promotes strong attachment of kinesin motors to microtubules. Individual kinesins detach at a rate of 0.001 s^{-1} in AMP−PNP;[18] microtubules bound to a surface by many motors are effectively irreversibly bound. Following a 10 min incubation, unbound microtubules were washed out and the immobilized microtubules visualized by epifluorescence microscopy (Nikon E600, 100×, 1.3 N.A. Plan Fluor objective).

The second technique for immobilizing microtubule seeds was to mask a narrow strip of an APTES coated coverslip with tape, block the rest of the surface with 0.5 mg/mL casein, and then attach seeds to the remaining adsorbent region. The advantage of this method is that a greater density of immobilized seeds can be obtained and the microtubules are bound more tightly to the glass. An example of an interface between adsorbed microtubules and a passivated surface is shown in Figure 3.

The microtubule array was grown by polymerizing microtubules from surface-adsorbed seeds under conditions where no new microtubules formed and growth from existing microtubules occurred at the plus ends only. In control experiments to determine the optimal free tubulin concentration for elongation, the critical concentration for nucleation of new microtubules was found to be approximately 10 μM and the critical concentration for growth from existing microtubules was approximately 1 μM. An intermediate concentration of 6 μM tubulin was chosen for all elongation

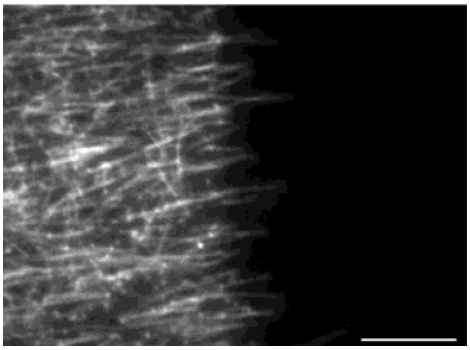

Figure 3. Interface between an adherent region of glass that binds microtubules and a region passiviated by casein treatment. Rapidly flowing the microtubules into the flow cell aids microtubule alignment. Scale bar is 10 μm.

experiments. To ensure that all of the microtubules extended exclusively from their plus ends, minus-end growth was inhibited by polymerizing in the presence of *N*-ethylmaleimide modified tubulin (NEM tubulin). This covalently modified tubulin, prepared as previously described,[15] blocks polymerization from microtubule minus ends while having no effect on plus-end polymerization. In control experiments microtubule seeds incubated in equimolar native tubulin and NEM tubulin polymerized uniquely from their plus ends (Figure 4).

To grow the microtubule array, a mixture of 6 μM rhodamine-labeled tubulin and 6 μM NEM tubulin was introduced into the flow chamber under polymerizing conditions, the chamber heated to 37° C, and the tubulin solution replenished every 15 min to maintain sufficient tubulin concentration for growth. The net microtubule growth rate under these conditions was found to be 1−2 μm/min. Following 30 min of growth, the microtubules were stabilized by flowing in a solution of 10 μM paclitaxel in BRB80, resulting in microtubules that are stable for days at room temperature. To aid visualization, bright fluorescent tubulin was used to polymerize the seeds and dim tubulin was used to elongate the seeds, similar to Figure 4.

This procedure resulted in microtubules emanating from the immobilized strip, all with their plus ends facing out, but they were not fully aligned and their long (∼50 μm or more) extended regions were flexible and not well anchored to the surface. To align and immobilize the array, a solution of buffer was flowed through the chamber at a rate of 5−15 mm/s (5−15 μL/s), which caused the microtubules to bend downstream and align parallel to the fluid flow. Finally, a solution of full-length kinesin motors was flowed in to the chamber in the presence of AMP−PNP. Because these motors bind by their tail to the casein-coated glass coverslip (they presumably can access the glass between the casein particles while microtubules are sterically blocked from the surface) and they bind to microtubules by their heads, they effectively cross-link the microtubules to the surface.

For long-term stability of the array and for use in the presence of ATP, the microtubules were cross-linked to the surface-adsorbed motors by flowing a 1% gluteraldehyde solution through the chamber. In control experiments, gluteraldehyde-fixed microtubules supported kinesin movement, demonstrating that this treatment preserves the fidelity of the microtubule tracks. An alternative approach to attach the microtubules to the surface would be to use mutant kinesins that are defective for ATP hydrolysis and bind microtubules irreversibly.[19,20]

An image of a polymerized microtubule array is shown in Figure 5, demonstrating the alignment produced by the combination of fluid flow and surface attachment. To quantitate the extent to which the microtubules are aligned parallel to one another, we measured microtubule orientations in a total of eight array images taken from four different arrays, and analyzed the angular distribution for each image. Because the filaments were not perfectly straight, we divided each image into a series of 9-μm strips oriented parallel to the line of immobilized seeds and analyzed the orientation of every microtubule segment in each strip (a total of 535 microtubule segments measured). For each image the distribution of microtubule angles around the mean was used to quantitate the relative alignment. The standard deviation of angles for the eight images ranged from 8.4° to 20.6°, with an overall standard deviation of 12.8° for the 535 segments counted. A distribution of the angles of all

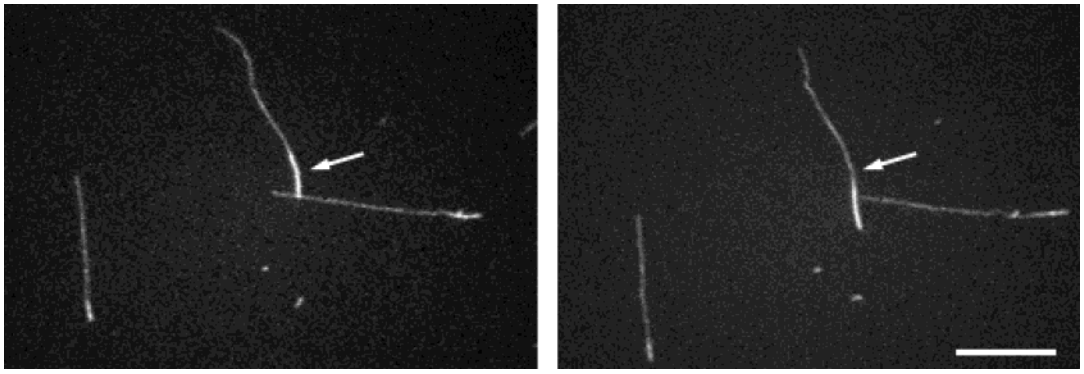

Figure 4. Extension from microtubule plus ends. Bright and dim tubulin populations were made by mixing different ratios of native and fluorescently labeled tubulin. Microtubule seeds (arrowhead at left) were polymerized from bright tubulin and were extended by incubating at 37 °C in 6 μM dim tubulin plus 6 μM NEM tubulin. To confirm that the extension was uniquely from the plus ends, movement of these microtubules was observed over a surface of immobilized conventional kinesin motors in 1 mM ATP. Because these immobilized motors move to the microtubule plus ends, the free microtubules move with their minus ends leading. Scale bar is 5 μm and images are 5 s apart. Reference arrow is at constant position.

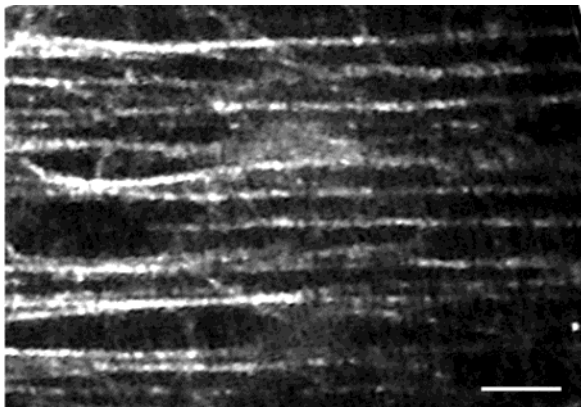

Figure 5. Image of the polymerized microtubule array showing oriented microtutubles bound to a glass surface. Microtubule seeds are out of frame to the left, microtubule plus ends are at the right. Scale bar is 5 μm.

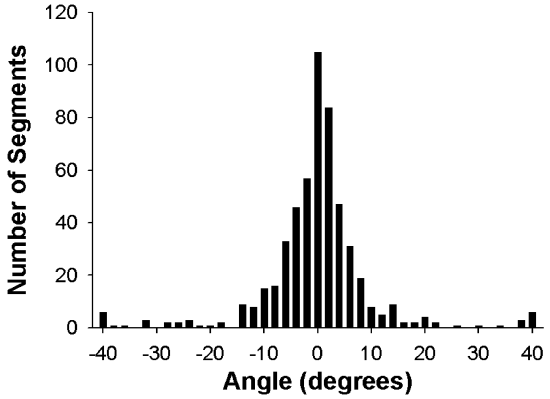

Figure 6. Distribution of angles for table of 535 microtubule segments taken from 8 microtubule array images.

microtubule segments measured is shown in Figure 6. Overall, 84.7% of the segments measured were within $\pm 10°$ of the mean.

To determine how microtubule alignment varied along each array, we compared the standard deviation of each 9-μm wide segment as a function of distance from the immobilized seeds. Characterizing the arrays in this manner is important both to determine the consistency of the alignment (how large is the useful region of the array) as well as to understand the mechanisms driving alignment. In seven of eight cases the standard deviation of microtubule angles fell with increasing distance from the seeds, indicating the microtubules were somewhat better aligned further away from the seeds, though overall there was only a weak correlation ($R^2 = 0.21$) of alignment with distance when the data were pooled. The most important difference was that the region closest to the seeds had an average standard deviation of 17.0° while all others had an average standard deviation of 9.0°. One explanation for this consistency in alignment is that the fluid flow efficiently bends the filaments, so any misalignment of the original seeds is rectified in the proximal portion of the array and the distal regions are more uniformly aligned. Consistent with this, radii of curvature as small as 2 μm were observed in microtubules emanating from the immobilized strip. Hence, the microtubules are well aligned

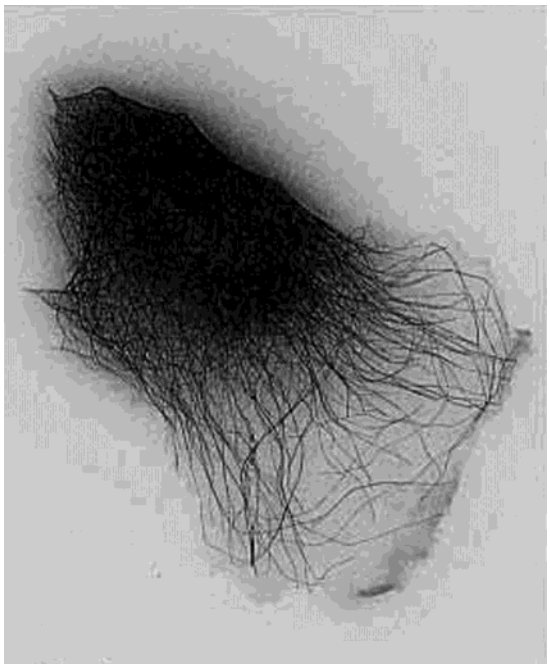

Figure 7. Microtubule array in a migrating Swiss 3T3 fibroblast showing alignment of microtubules in the direction of movement (to the bottom right). Tubulin was fluorescently labeled and the contrast of the image inverted to show the filaments more clearly. Image taken from ref 21 with permission.

for most of their length (>40 μm) and, combined with the ability to make the arrays arbitrarily wide, these arrays can span considerable areas.

An interesting comparison can be made between the in vitro alignment achieved here and the in vivo alignment seen in living cells. In cultured fibroblasts injected with fluorescently labeled tubulin, microtubules can be seen oriented toward the leading (migrating) edge of the cell, emanating from the microtubule organizing center positioned near the nucleus (Figure 7).[21] These microtubules are aligned by a combination of polymerization of existing microtubules toward the leading edge and actomyosin driven remodeling of the microtubule cytoskeleton, and this array is thought to provide structural integrity for the migrating cell and act as tracks to enable the transport of material toward the leading edge.[21,22] Using the microtubule orientation analysis developed above, we analyzed the distribution of microtubule angles from two studies in the literature.[21,22] In migrating Swiss 3T3 fibroblast cells,[21] the standard deviation of microtubule orientation was found to be 28.3° from two images at successive time points. In hepatocyte growth factor-treated PtK2 cells,[22] the standard deviation was found to be 11.8°. Hence, the alignment achieved in vitro (12.8° overall or 9.0° excluding the region adjacent to the seeds) is as good as or better than the alignment found in cells.

One consideration for useful application of the array is the degree of microtubule crossing. With the >50 μm microtubules employed here, if the filaments are spaced 1 μm apart then they would need to be within 1° of parallel to prevent crossing; if spacings were 100 nm or less, as may be useful for assembling particles on the array, the alignment

would need to be within 0.1°. To obtain this precision of alignment will require a different alignment approach such as creating microgrooves on the surface or patterning functional groups at submicron scales.

A future improvement to the array will be to reduce the width of the adsorbent seed strip and allow growth from only one side of the strip. In the current configuration, microtubules emanate from both sides of the strip of seeds, although the fluid flow tends to fold the upstream microtubules over, reversing their direction. One approach to creating a one-sided array would be to position the immobilized strip of seeds next to a nanofabricated wall that blocks microtubule polymerization. These improvements will be the goal of future work.

This system solves one of the most significant hurdles of harnessing the intracellular motility system for micro- and nanoscale applications − aligning the filament tracks. The approach taken here is to use the natural polymerization characteristics of microtubules to ensure uniform orientation. In this way, the approach is similar to the way a cell defines the orientation of its microtubules: the minus ends are capped and stabilized at the centrosome and the plus ends emanate from there. The formation of the mitotic spindle in dividing cells follows a similar strategy: dynamic microtubules emanating from each pole stochastically lengthen and shorten until they encounter a chromosome or another microtubule, which stabilizes the filament in the polymer form.[23,24]

One potential application of molecular motors is force-generating actuators in micro- or nanoscale bioengineered systems. While individual microtubules can be interfaced with surface adsorbed motors for this purpose, single microtubules will likely have an insufficient number of motors interacting with them to achieve appreciable forces. In contrast, the microtubule array described here has an ideal geometry for interfacing with a fabricated cantilever or rotor to which many motors are attached. Because kinesins can swivel to find a microtubule,[11] the microtubules determine the direction of movement when two such surfaces come together. If an array can be generated that has microtubules spaced 100 nm apart and kinesins (which can produce 7 pN per motor[25]) can be adsorbed at densities of 2000 motors/μm^2,[26] this array should be able to support forces in the range of nN per μm^2, comparable to the forces exerted by cells moving on flexible substrates.[27,28]

The second potential application of kinesins and microtubules is in molecular transport devices. While most approaches discussed to date have envisioned microtubules as the transporters,[6,8−10] using motors as the transporters opens more opportunities for cargo attachment because kinesins are much more amenable to protein engineering than are tubulins. For instance, kinesin heads could be fused to receptors, DNA binding domains, or other protein motifs that bind to a cargo of interest. One hurdle in such a molecular transport system will be to overcome the natural mixing caused by diffusion. A protein complex with a diffusion coefficient of 10 μm^2/s that is transported by a motor at 0.8 μm/s will need to travel a distance of 25 μm to outrace diffusion.[29] Overcoming this mixing will require either increasing transport distances or slowing diffusion by increasing the viscosity of the solution or restricting the geometry around the transport track.

To engineer useful in vitro systems using biological components requires one to control protein attachment, maintain protein stability, and detect very weak signals, among other technical challenges. Along with strategies of patterning motors to align moving microtubules, this array of oriented microtubules will serve as an important addition to growing collection of tools for harnessing kinesins and microtubules for micro- and nanoscale assembly and transport.

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(17) Full-length Drosophila conventional kinesin, purified as previously described (ref 26) was used at a concentration of 1−5 μg/mL. Before kinesin adsorption, surfaces were exposed to 0.5 mg/mL casein for 3 min to prevent motor denaturation. Any unbound motors were washed off with BRB80 buffer supplemented with 0.2 mg/mL casein and 0.1 mM MgATP.
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