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Patterning Surface-bound Microtubules through Reversible DNA Hybridization

Gayatri Muthukrishnan, Caitlin A. Roberts, Yi-Chun Chen, Jeffrey D. Zahn, and William O. Hancock*

Department of Bioengineering, Pennsylvania State University, University Park, Pennsylvania 16802

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ABSTRACT

Biomolecular motors have great potential as transporters and actuators in microscale devices. Existing efforts toward harnessing kinesin motors have involved microtubule movements over immobilized motors. The reverse geometry has distinct advantages, but progress has been hindered by the difficulty of immobilizing patterned and aligned microtubules on surfaces. Here we show that microtubules can be reversibly patterned with microscale resolution through DNA hybridization, and that these DNA-functionalized microtubules support the movement of kinesin-coated beads.

Because of the unique transport and force generating capabilities of biological molecular machines, there is considerable interest in incorporating them into nano- and microscale hybrid devices. Kinesins are transport motors that use the chemical energy of ATP hydrolysis to carry intracellular cargo in eukaryotic cells. Their tracks, microtubules, are protein polymers, 25 nm diameter and tens of microns long. By isolating these proteins from cells and reconstituting their activity in engineered microscale systems, these biological machines provide a powerful system for transporting material at microscale dimensions, for microactuation of MEMS devices, and for manipulating and assembling nanoparticles into useful materials.

Until now, most applications of the kinesin-microtubule system have utilized the upside-down geometry, in which motors are immobilized on surfaces and microtubules are transported over them. By fabricating microscale channels and functionalizing them with kinesin motors, a number of groups have shown that the direction of microtubule motion can be controlled, a prerequisite for useful transport.[1−5] Because the microtubules can be functionalized with biotin or antibodies, this geometry works well for microscale transport. However, to fully realize the potential of these motors, methods must be developed to immobilize microtubules with control over their position and orientation. This orientation is key: in cells microtubules are oriented with their slow growing minus-ends at the center of the cell and their fast growing plus-ends at the periphery, and motors move unidirectionally along these tracks. The first advantage of replicating this cellular geometry is that it provides greater control over cargo transport−recombinant kinesins can be engineered with an almost limitless range of cargo attachment domains, and the arrangement of microtubules determines their transport direction. A second motivation is that to generate the nanoNewton forces required to power MEMS and NEMS devices, it is necessary to team the activity of many motors working along an array of parallel microtubules. The goal of this study is to develop methods to reversibly pattern microtubules on engineered surfaces, with the eventual goal of controlling the orientation of surface-immobilized microtubules and 3D microtubule assemblies.

It is well established that microtubules can be noncovalently attached to surfaces while retaining their functional properties as substrates for kinesin movement. Microtubules, which have a net negative charge,[6] bind well to amino-silane functionalized surfaces;[7] biotinylated microtubules bind well to streptavidin-functionalized surfaces;[8] and, in the presence of the non-hydrolyzable nucleotide analog AMP−PNP, microtubules bind tightly to kinesin-functionalized surfaces.[9] Using these methods, it has been shown that kinesin can carry beads,[9] gold nanowires,[4] or micron-scale silicon microchips[10] along surface-immobilized microtubules, but in these studies there was no control over the transport direction. There has been some success to date in aligning microtubules on surfaces. Both fluid flow[11] and the application of electric fields[12] have been shown to orient gliding microtubules. In another study, Limberis et al. used antibodies complementary to alpha-tubulin to bind the minus-ends of microtubules to surfaces and then used fluid flow to align the filaments on the surface.[13] To make an aligned microtubule array, Brown and Hancock immobilized short microtubule seeds on a patterned surface, polymerized long microtubules selectively

* Corresponding author. E-mail: wohbio@engr.psu.edu. Phone: 814-863-0492. Fax: 814-863-0490.

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Table 1. List of Oligonucleotides[a]

oligonucleotide name	oligonucleotide sequence
Oligo A	5′−(biotin) taacatt CGCATTCAGGAT−3′
Oligo A′	5′−(biotin) taacatt ATCCTGAATGCG−3′
Oligo B	5′−CCGGAATTGGCC taacatt (biotin)−3′
FITCOligoA′	5′−(fluorescein) taacatt ATCCTGAATGCG−3′

[a] T_M of A−A′ overlap = 36 °C.

from these plus-ends, and then aligned the filaments using fluid flow and cross-linked them to the surface.[14] However, while these techniques achieved a degree of microtubule alignment, they provide little control over positioning, they do not result in a large number of immobilized microtubules, and they are not optimal approaches for incorporating microtubules into microscale devices.

Our approach is to use the specificity and reversibility of DNA hybridization to immobilize oriented microtubules on surfaces. In other systems, DNA oligonucleotides have been successfully used to create nanostructural assemblies. For example, to exploit the optical properties of colloidal gold nanoparticles for sensor applications, particles functionalized with different oligonucleotides were reversibly aggregated by a bridging strand of DNA, causing a shift in the optical absorbance spectrum.[15] DNA-directed assembly of Au nanowires on surfaces has also been achieved by coating nanowires with oligonucleotides through Au−thiol linkages.[16]

Here, we use DNA hybridization to pattern immobilized microtubules on surfaces. The eventual goal is to functionalize the two ends of microtubules with different DNA oligonucleotides, pattern complementary oligonucleotides at defined sites on a surface, and use the specificity of DNA hybridization to properly orient the microtubules. In this study we develop a method to attach single-stranded oligonucleotides to microtubules, we use two approaches to pattern complementary oligonucleotides on glass surfaces, we establish proper buffer conditions to enable reversible hybridization while maintaining microtubule stability, and we demonstrate that these DNA-functionalized microtubules remain functional tracks for kinesin motors.

To make microtubules, tubulin protein was isolated from calf brains using established procedures,[17] polymerized at 37 °C, and stabilized with 10 μM paclitaxel in BRB80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) as previously described.[18] Microtubules were fluorescently labeled with rhodamine, and a subset of tubulin was covalently labeled with biotin as previously described.[18] Microtubules were polymerized with different proportions of unlabeled, rhodamine-labeled, and biotinylated tubulin.

Single-stranded oligonucleotides (Proligo, Inc.) were attached to microtubules via biotin−avidin chemistry using two different methods. Both take advantage of the fact that avidin has four biotin binding sites and can be used as a bridge. In the first approach, microtubules (684 nM final tubulin concentration with a ratio of 4:4:1 unlabeled/ rhodamine/biotinylated tubulin) were polymerized and stabilized with 10 μm paclitaxel, 76 nM neutravidin was added,

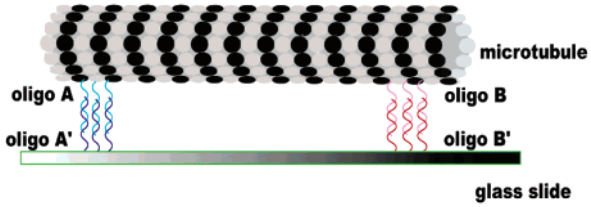

Figure 1. Diagrammatic representation of microtubule immobilization through DNA hybridization, showing the eventual goal of functionalizing the microtubule with different oligonucleotides on each end.

the solution vortexed for 20 s, and then incubated with 760 nM of OligoA (Table 1) for 20 min to make OligoA-functionalized microtubules (Mt-OligoA). To determine whether the microtubule-bound oligonucleotides retained their function, the microtubules were incubated with 760 nM of a fluorescently labeled complementary strand, FITCOligoA′ (Table 1), and visualized by fluorescence microscopy using a Nikon E600 microscope (1.3 NA, 100× objective) coupled to a CCD camera (Genwac GW902H, gamma factor = 0.45) with G-2E/C filter for TRITC (rhodamine) and B2E/C for FITC (fluorescein). Since the FITC fluorescence signal was very low, the concentration of biotinylated tubulin was increased (820 nM final tubulin concentration with 0.8:0.8:1 ratio of unlabeled/ rhodamine/biotinylated tubulin), and the neutravidin (300 nM) and both oligonucleotide concentrations (3 μM) increased proportionally. To remove background fluorescence, this solution was centrifuged for 5 min at 30 psi in a Beckman Airfuge, resuspended in BRB80 buffer plus 10 μM paclitaxel, and visualized. Oligo-functionalized microtubules were clearly seen both under TRITC and FITC filters, while the nonfunctionalized microtubules were seen only under TRITC (Figure 2).

In the above method, removing free neutravidin by centrifugation and resuspension before adding the oligonucleotides caused the microtubules to aggregate and depolymerize, especially at higher biotinylated tubulin concentrations. To remove the unbound neutravidin, another method using magnetic beads was devised. In this method, 1.9 μM OligoA was incubated with a 5-fold excess of neutravidin for 10 min to bind ≤ 1 oligonucleotide per neutravidin. Simultaneously, 3.84 μM streptavidin-coated magnetic beads (0.83 μm diameter, Bangs Laboratories, Inc.) were incubated for 10 min with an identical concentration of complementary oligonucleotide (OligoA′, Table 1). These two solutions were then mixed and the oligonucleotides allowed to hybridize for 20 min. A magnetic separator (Bangs Laboratories, Inc.) was used to separate the magnetic beads and OligoA-neutravidin complexes from free neutravidin. The pellet was then resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and heated at 42 °C for 2 min to denature the DNA, and the magnetic beads were pulled out of solution, leaving a supernatant of OligoA-labeled neutravidin. Finally, 50 μL of this solution was incubated with 100 μL of biotinylated microtubules (684 nM final tubulin concentration with ratio of 4:4:1 unlabeled/rhodamine/biotinylated tubulin) to obtain oligonucleotide-functionalized microtubules (Mt-OligoA).

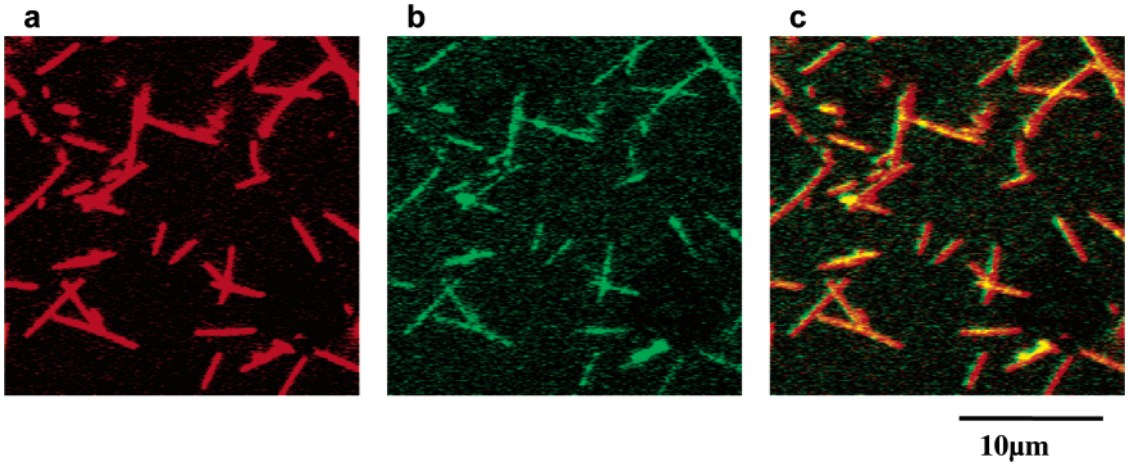

Figure 2. Oligonucleotide-functionalized microtubules. (a) View under TRITC filter showing rhodamine labeled microtubules, (b) same view under FITC filter showing FITCOligoA′ hybridized to OligoA functionalized microtubules. (c) Digitally overlapped image of (a) and (b). Nonfunctionalized microtubules are not detectable under the FITC filter.

To attach these oligo-functionalized microtubules to surfaces and determine the sequence specificity of microtubule binding, glass coverslip (Corning, no. $1^1/_2$, 18 mm squares) surfaces were functionalized with different oligonucleotides. To quantify microtubule binding, the number of microtubules bound to the surface after 20 min was compared to a surface of kinesin in adenosine 5′-(β, γ-imido)triphosphate (AMP−PNP).[19,20] Flow cells were constructed from coverslips, glass slides (FisherFinest Premium), and two-sided tape. Neutravidin (10 μM) was flowed in, followed by 10 μM biotinylated oligonucleotide and the Mt-OligoA solution (456 nM tubulin). The sample was incubated for 20 min. Finally, unbound microtubules were washed out with motility buffer (0.2 mg/mL casein, 10 μM paclitaxel, 20 mM D-glucose, 0.02 mg/mL glucose oxidase, 0.008 mg/mL catalase, 0.5% β-mercaptoethanol in BRB80), and the surface visualized. The results obtained by the two methods described to make Mt-OligoA gave results within 20% of each other, so the data were pooled.

Microtubules bound with very high specificity. There was 95% specific binding on the complementary OligoA′ surface, and 3.53% and 2.77% nonspecific binding on OligoA and OligoB surfaces, respectively (Figure 3b). The relative binding to a neutravidin surface devoid of oligonucleotides was 1.02%. In these experiments, the buffer (BRB80: 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH 6.9) was optimized for microtubule stability and is of much higher ionic strength than standard DNA buffers. We hypothesized that the elevated ionic strength may decrease the hybridization specificity somewhat, so in an attempt to reduce the nonspecific binding even further, we repeated the experiment in a lower salt buffer BRB12 (12 mM PIPES, 1 mM EGTA, 2 mM $MgCl_2$, pH 6.9). This more dilute buffer reduced the absolute amount of nonspecific binding, but it also reduced the degree of specific binding to an equal degree (Figure 3c). Finally, since casein was used as a blocking agent while patterning in further experiments, the relative amount of Mt-OligoA binding to a surface treated with 0.5 mg/mL casein was tested and found to be $< 0.5\%$.

To test the reversibility of hybridization, we heated the flow cells to denature the double-stranded DNA hybrid. Increasing the temperature by placing the flow cell at 52 °C for 10 min on a heat block and washing in warm buffer (60 μL BRB80 + 10 μM paclitaxel at 52 °C) effectively detached the microtubules from the surface. Before heating, the number of microtubules on the surface was 179 ± 11 ($N =$ 10 screens from 2 flow cells); after heating, only 3 ± 1.7 microtubules remained. To confirm that microtubules were not simply depolymerizing from this procedure, a solution of microtubules in a flow cell was heated in a similar manner for 10 min at 52 °C. No measurable depolymerization was detected. Importantly, when oligo-functionalized microtubules were introduced back into the flow cell, they bound to the surface, demonstrating that detachment was due to melting of the DNA duplex and not due to the DNA coming off of the surface.

After establishing that DNA hybridization can be used to attach microtubules to homogeneous surfaces, we investigated whether this approach can be used to pattern microtubules at defined locations on surfaces. As a first approach to patterning, we used microcontact printing, a soft lithography technique that uses a PDMS (silicone) stamp to deposit molecules on surfaces.[21] The importance of binding biological ligands to surfaces for bioassays and bioelectronic devices has expanded the use of this technique to biological molecules such as enzymes, antibodies, other proteins, and DNA.[22,23] After lithographically etching a pattern into a silicon wafer, the stamp was made by pouring PDMS over the wafer, curing it overnight and peeling it off. The stamp was first 'inked' with a solution of 10 μM neutravidin that adsorbed onto the PDMS. The stamp was then dried and pressed onto a coverslip, transferring the neutravidin (Figure 4a). A flow cell was prepared using this coverslip, 0.5 mg/mL casein was introduced for 5 min to block the nonpatterned surface, and then 1 μM OligoA was flowed in and allowed to bind for 20 min. Finally, 10 μM biotin was flowed in to block any free biotin-binding sites. To confirm that the oligonucleotide was properly patterned, 1 μM fluores-

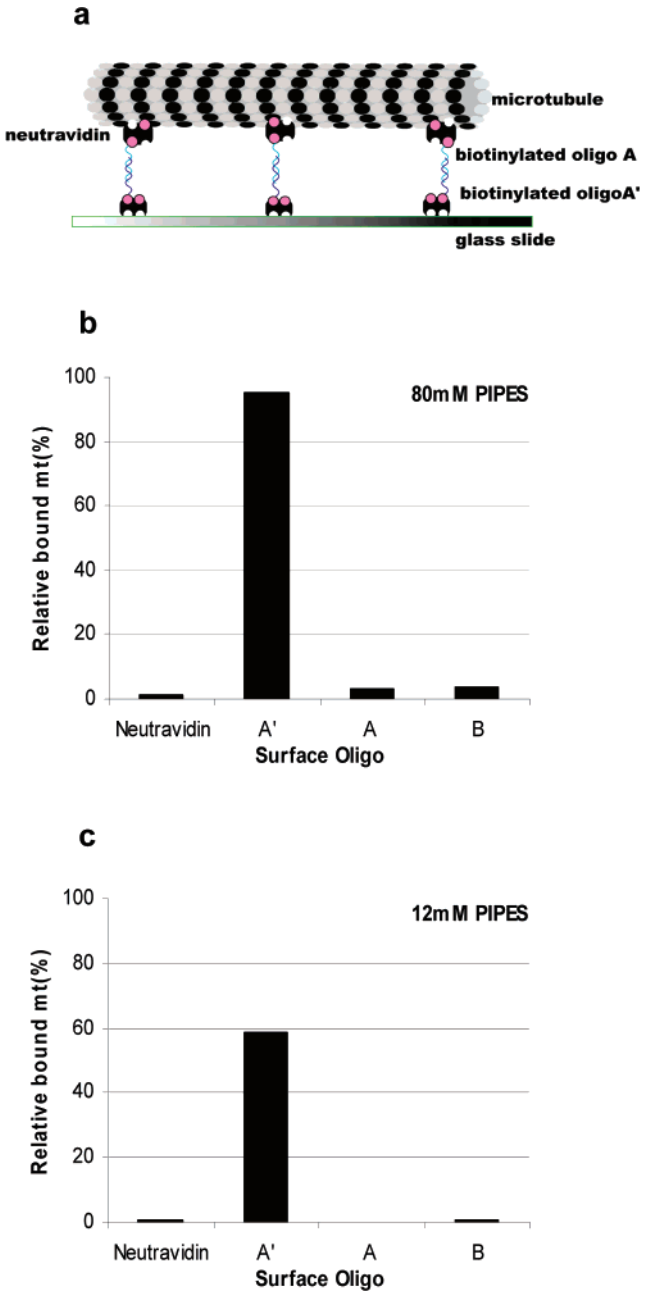

Figure 3. Relative microtubule binding to oligo-functionalized surfaces. (a) Sketch of oligonucleotide functionalization and hybridization strategy. (b) Results of Mt-OligoA binding to surfaces in 80 mM PIPES buffer, normalized to binding to a kinesin surface in AMP−PNP. (c) Results in 12 mM PIPES buffer. Oligonucleotide sequences are listed in Table 1.

cently labeled complementary oligonucleotide (FITCOligoA′) was introduced into the flow cell, incubated for 20 min, and imaged. High specificity was observed (Figure 4b).

Having patterned the oligonucleotides, we proceeded to pattern microtubules by incubating oligo-functionalized microtubules together with complementary oligonucleotide patterned surfaces. After 20 min of incubation, unbound microtubules were removed by washing thoroughly with motility buffer (described above). We observed clear patterns of microtubules with limited nonspecific binding (Figure 5a). Figure 5b shows limited bridging of microtubules across two

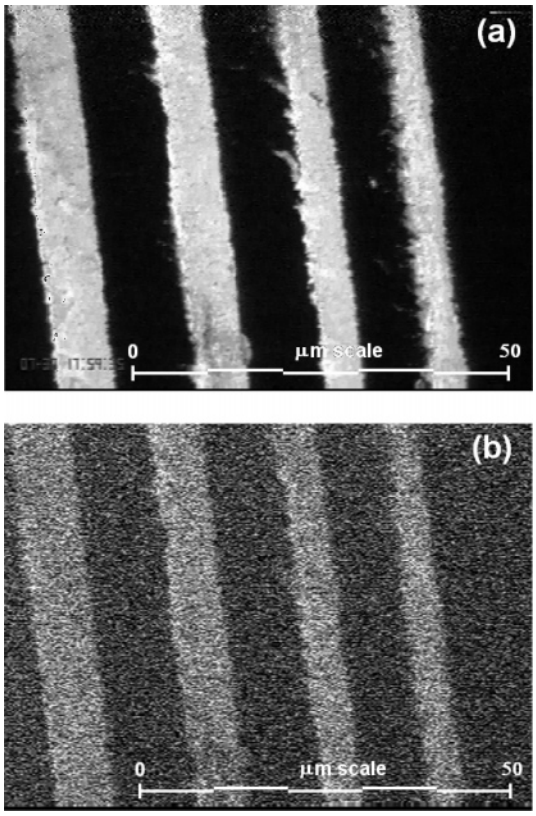

Figure 4. Microcontact printing of neutravidin on a glass coverslip. (a) View under TRITC filter, showing rhodamine-labeled neutravidin. (b) View under FITC filter, showing FITCOligoA′ hybridized to OligoA. Reduced pattern contrast is a result of the higher camera gain needed for the faint fluorescence signal. When the slide was observed through the FITC filter before introducing FITCOligoA′, no fluorescence from the rhodamine was observed.

patterned areas (around two per screen). In future work, smaller islands of immobilized DNA will be used to create high-density polarized bridges for force generation or biomolecular separations.

As an alternative to microcontact printing, we also used parylene dry lift-off, a technique developed by the Craighead group to pattern biomolecules on surfaces.[24] This process, which involves lithographically patterning a weakly bound polymer on a surface, adsorbing biomolecules to the patterned surface, and then peeling off the polymer, overcomes both the elastomer sagging and protein transfer problems of PDMS stamps. The technique has been used to immobilize *E. coli* and rat basophilic leukemia cells through patterned antibodies and poly-L-lysine.[24]

Our approach followed published procedures.[24] Parylene was vapor deposited on glass coverslips and coated first with the adhesion promoter hexamethyldisilizane, and then 1.8 μm of Shipley 1818 positive photoresist. The sample was soft baked at 95 °C for 90 s followed by exposure with UV light through a photomask for 6 s, and developed with Shipley CD27. The exposed parylene and remaining photoresist were then removed by an oxygen plasma etch (300 milliTorr, 300 W for 15 min). After patterning the parylene, 10 μL of 10 μM neutravidin was allowed to adsorb to the glass surface for 30 min, and the parylene was peeled off using a razor blade and tweezers. A flow cell was then

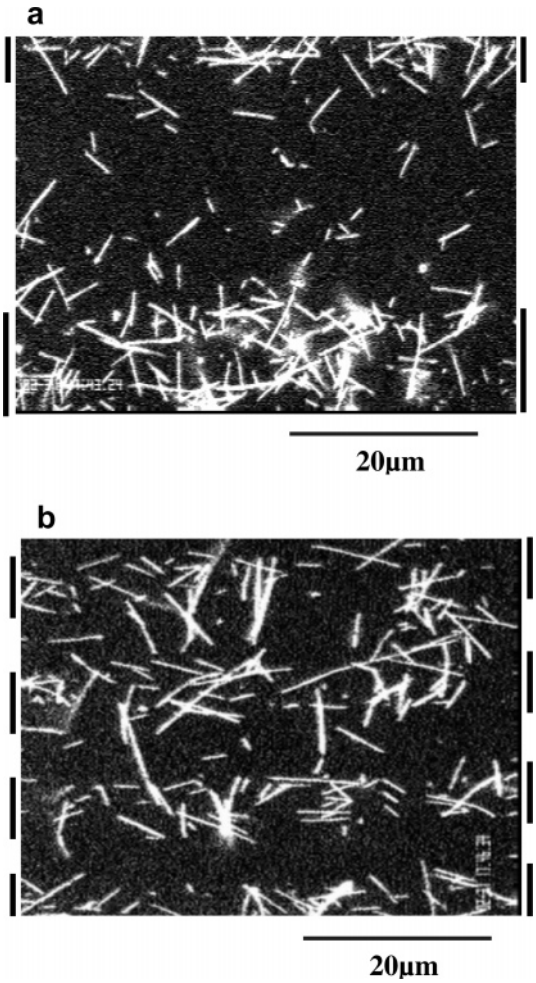

Figure 5. Microtubules immobilized on patterned surface. Clear microtubule patterns can be observed with limited nonspecific binding. In (b) bridging between to patterns 10 μm apart can be seen. Bands on figure edges are estimated positions of the oligonucleotide patterns.

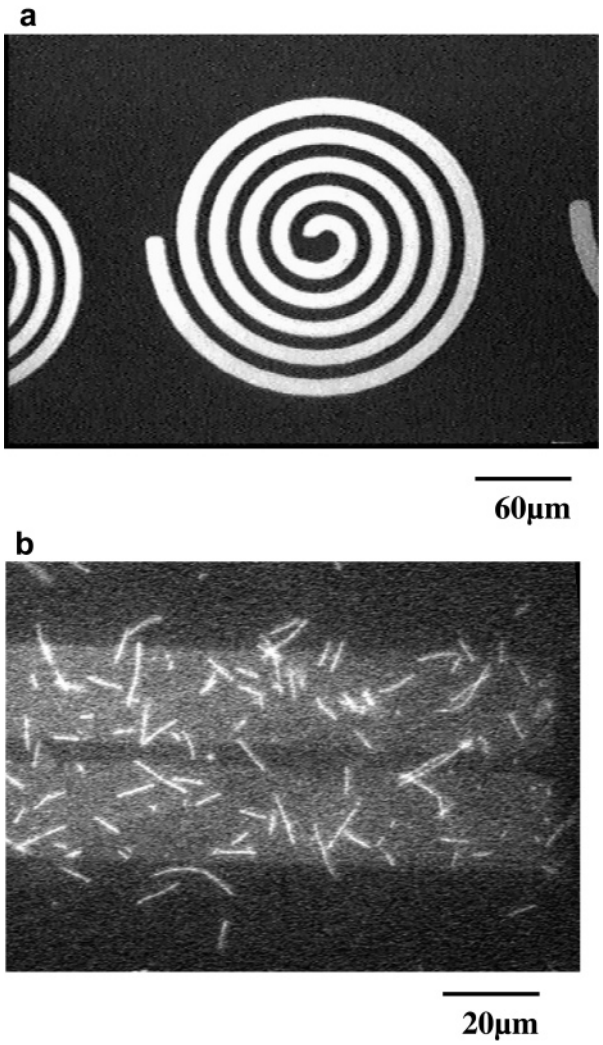

Figure 6. Parylene dry lift-off patterning. (a) Image of fluorescently labeled oligonucleotides hybridized to complementary oligonucleotides on the neutravidin surface. (b) Oligo-functionalized microtubules (Mt-OligoA) bound to complementary oligonucleotide (OligoA′) patterned surfaces.

constructed using this coverslip, and the oligonucleotide of interest was flowed in. Figure 6a shows a sharp pattern of a fluorescently labeled oligonucleotide (FITCOligoA′) hybridized to patterned complementary oligonucleotides (OligoA) on the surface. Figure 6b shows oligonucleotide-functionalized microtubules (Mt-OligoA) binding to surfaces patterned with complementary oligonucleotides (OligoA′). Like the microcontact printing approach, high specificity of microtubule binding was observed. Microtubules could be removed from the pattern by heating the sample at 52 °C for 10 min.

Although oligonucleotide functionalization works well to immobilize microtubules on surfaces, it is important that this microtubule modification does not disrupt the ability of kinesins to walk along the filaments. To confirm that oligonucleotide-functionalized microtubules remain good substrates for motors, we first performed a microtubule gliding assay. His-tagged *Drosophila melanogaster* kinesin heavy chain motors were adsorbed to a glass surface and oligonucleotide-functionalized microtubules were flowed in along with ATP and antifade following standard procedures.[25] DNA-functionalized microtubules moved at 592 ± 67 nm/s (mean ± SD, $N = 10$), compared to 583 ± 55 nm/s ($N = 10$) for standard rhodamine-labeled microtubules.

We next tested whether surface-bound microtubules can support the movement of kinesin-coated beads. Kinesin motors were adsorbed to 200-nm silica beads as previously described[26] at a stoichiometry of approximately 50 motors/bead, and microtubules were attached to the surface of a coverslip by DNA hybridization as described above. The beads were then introduced into the flow cell in the presence of 1 mM MgATP, and the sample was visualized by differential interference contrast microscopy. Beads moved along DNA-functionalized microtubules at a speed of 658 ± 79 nm/s (mean ± SD, $N = 3$) (Figure 7), while beads moved along unlabeled microtubules adsorbed to a standard amino-silane coated surface at 760 ± 432 nm/s ($N = 3$). Hence, DNA functionalization and surface attachment did not affect the ability of the microtubules to support kinesin-based motility. For long-distance transport, both the length and density of surface-bound microtubules will need to be increased significantly.

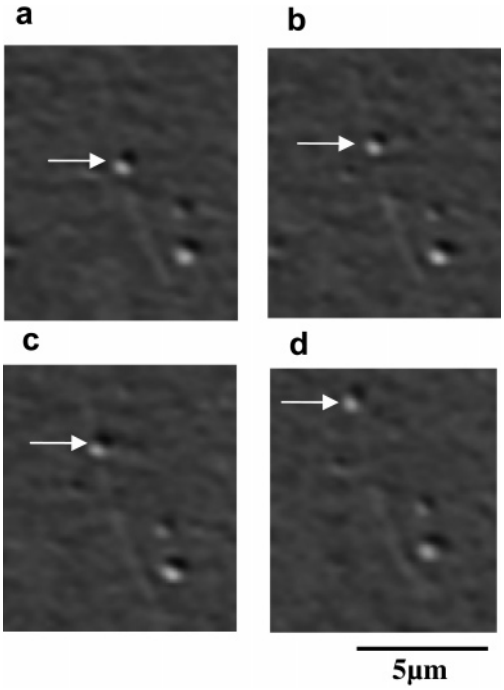

Figure 7. Kinesin-functionalized beads moving along microtubules immobilized via DNA hybridization. The pictures are 1 s apart. In (d), the bead has reached the end of the microtubule and is diffusing away. Images were obtained with a Nikon TE2000 inverted microscope, 100× 1.3 NA oil objective, and Sony XC−ST-50 CCD camera with background subtraction by an Argus-20 (Hamamatsu, Inc.).

The approach described here shows specific immobilization of microtubules, no loss in microtubule functionality, and reversibility of the attachment by denaturing the double-stranded DNA. The ability to pattern oligonucleotides on the surface using microcontact printing or parylene lift-off as shown, or by other potentially better methods such as dip-pen lithography or microarray spotting enables microtubule patterning with high resolution.

With the present technique, the surface and the microtubules use the same biotin−avidin binding chemistry, creating the potential for cross-linking. This can be improved in the future by, for example, using thiolated oligos on Au surfaces, or covalently cross-linking oligonucleotides to amino-terminated glass surfaces. Also, covalently cross-linking oligonucleotides to microtubules may increase microtubule stability and the potential for long-term storage.

The next step toward aligning microtubules on surfaces is defining their polarity and, in turn, the path taken by motors associated with them. To achieve this, we envision making segmented microtubules with different oligonucleotides on each end and immobilizing them on surfaces patterned with different complementary oligonucleotides. In this way, the patterns of the surface-bound oligonucleotides define the position and orientation of the microtubules, enabling the assembly of a range of 2D and 3D microtubule structures. Using this geometry, recombinant kinesins can be engineered with a range of different cargo binding domains to transport biomolecules, nanoparticles, cells, or other objects along these tracks. Finally, for actuating microdevices using the kinesin-microtubule system, this microtubule immobilization approach allows parallel arrays of microtubules to be created, enabling the activity of many kinesins to be summed.

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