

Magnet assisted fabrication of microtubule arrays

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Magnetic fields were used to fashion permanently aligned microtubule arrays allowing formation of highly ordered platforms for assembly, separation, and single molecule motor studies.

The integration of biological molecular motors with nanoscale materials provides new avenues for investigating biomotor function and sets the stage for their incorporation into functional micro- and nanoscale devices. Kinesin is a cytoskeletal motor protein that uses energy derived from the hydrolysis of ATP to transport intracellular cargo unidirectionally along poly-tubulin cylinders called microtubules.¹ In mammalian cells, radial microtubule arrays are formed in which the fast growing (*i.e.*, “plus”) ends of the microtubules are pointed to the periphery of the cell, while the slow-growing (*i.e.*, “minus”) ends are fixed in a cellular organelle called the centrosome. During cell division, the centrosomes are duplicated, forming overlapping microtubule arrays that play key roles in mitosis, including transporting the duplicated chromosomes to the center of the cell, pulling them apart, and then transporting them to the two centrosomes.² Even the most basic single cell eukaryotes organize polarized arrays of microtubules and use them to perform single molecule separations, yet the assembly and organization of aligned microtubule arrays by artificial means *in vitro* has proven to be extremely difficult and typically possible over only very short distances. Some success has been reported in developing arrays using lithographic techniques,³ using viscous drag forces,^{4,5} or by coupling nanoparticles to the microtubules,⁶ however alignment distance remains limited by microtubule length.

We previously showed that magnetic nanoparticle-functionalized microtubules could be aligned and deposited onto kinesin-coated glass surfaces by application of a magnetic field.⁶ The microtubule arrays were directed to the surface using simple and inexpensive permanent magnets, where they bound to immobilized motors. However, while the microtubules were arranged over wide lateral distances, this assembly strategy utilized microtubules that were both too short for useful arrays and rapidly lost their alignment upon introduction of ATP.⁷ To address these two issues, we present an alternative experimental approach to achieve alignment of large numbers of long (>20 μm) microtubules into linear arrays. Previous work from our lab used ATP-starved kinesin motors as a platform for microtubule alignment,^{6,7} however such platforms are not well suited

to permanent array formation because re-introduction of ATP initiates motor transport that erases the preformed arrays within minutes, preventing their use in subsequent motility assays.⁷ To avoid this, we now instead use modified kinesin motors that bind to microtubules but are not motile. A robust linkage to the glass surface is therefore created, and the aligned microtubules remain in place even in the presence of large fluid flow forces. We demonstrate that these microtubules may be subsequently elongated by introduction of added tubulin subunits to achieve very long tracks for force generation and biomotor-assisted assembly.

Biotin functionalized cobalt ferrite (CoFe₂O₄) nanoparticles were prepared and encapsulated as previously described.^{6,8} Biotinylated microtubules were formed by mixing 2.3% biotin-tubulin (BTU) with rhodamine-labeled tubulin (1 : 3 rhodamine : unlabeled tubulin, RTU),⁹ polymerizing them, and stabilizing them with paclitaxel following standard literature methods.¹⁰ Short microtubule segments (*i.e.*, “seeds”) were prepared by centrifuging the 2.3% BTU microtubules to remove any unpolymerized biotin-labeled tubulin, resuspending them in paclitaxel-free buffer, and shearing them by thrice passing through a 30-gauge needle. The resulting seeds were then stabilized by diluting to 32 nM in tubulin in a solution containing 10 μM paclitaxel in buffer.¹⁰ The biotinylated microtubules were then magnetically labeled with biotinylated-CoFe₂O₄ using a neutravidin crosslinker, schematically shown in Fig. 1A, following a previously published method.⁶ The exact

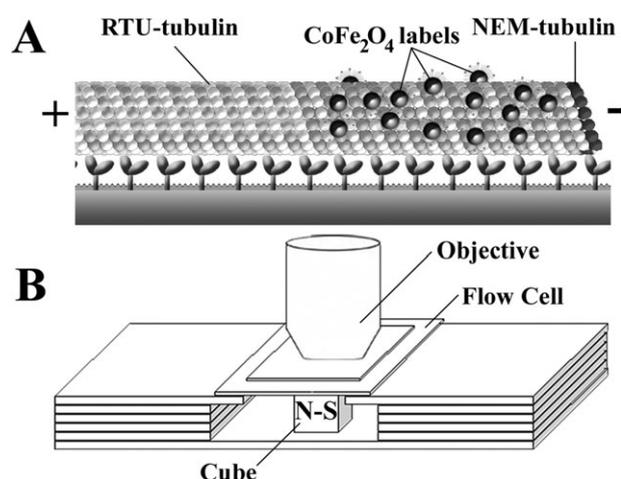


Fig. 1 (A) Schematic of a segmented microtubule on a kinesin surface. The spherical cobalt ferrite particles are conjugated to the biotinylated (BTU) segment of the microtubule *via* a neutravidin linkage. (B) Schematic of the magnet, flow cell and objective configuration.

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number of magnetic particles that are attached is not known, however it is assumed to be proportional to the amount of biotin functionalities on the microtubules' surfaces. In all alignment experiments, a flow cell (shown in Fig. 1B), prepared by affixing two glass coverslips with double-sided tape, was perfused sequentially with a casein solution (0.5 mg mL^{-1}) for 5 minutes and then a solution of inactive *Drosophila melanogaster* kinesin, a preparation of hexa-His tagged kinesin that is truncated at amino acid 559, is not motile, and displays irreversible binding to microtubules. After 5 min incubation with kinesin, the magnetic microtubule solution was added, and the flow cell was then exposed to a magnetic field using a 5 mm edge length cube NdFeB permanent magnet (Engineered Concepts, Inc., Birmingham, AL) for 15 minutes, and imaged with epifluorescence microscopy.

Microtubules that are introduced to the flow cell in the presence of the permanent magnet bind to the kinesin motors and are aligned parallel to the magnetic field lines.⁶ Standard microtubule preparations result in a distribution of microtubule lengths; in a typical preparation the average length is $9 \pm 7 \mu\text{m}$ with a maximum length of $40 \mu\text{m}$, however very long microtubules ($>20\text{--}30 \mu\text{m}$) are prone to buckling, bending, and misalignment during surface patterning. To create microtubule arrays for use in separations and assembly, these assemblies must span large distances and be resistant to flow-induced realignment. To achieve this, we first magnetically align microtubule seeds into arrays such as that shown in the fluorescence microscopy image in Fig. 2A. The alignment of the bound microtubules was analyzed by measuring the length and angle of 104 microtubules in the $4 \times 10^3 \mu\text{m}^2$ area. The inset of Fig. 2A shows the wide distribution of the lengths and angles for these microtubules, which have an average length of $6 \pm 4 \mu\text{m}$ and a standard deviation in orientation of 45° (i.e., the long axis of the microtubule *versus* the angle of the applied field). The data indicate a relationship between length and orientation: short microtubules are less aligned ($<10 \mu\text{m}$, $\pm 47^\circ$) than longer microtubules ($10\text{--}20 \mu\text{m}$, $\pm 26^\circ$), however very long microtubules ($>20\text{--}30 \mu\text{m}$) are prone to buckling and bending during array formation. These results are consistent with re-alignment observed for microtubules in suspension: the larger viscous drag forces on the long microtubules induce more complete re-alignment.⁶ For the longest microtubules, the observed buckling may be caused by bending following an initial binding event (i.e., in the middle of the microtubule). Because shorter

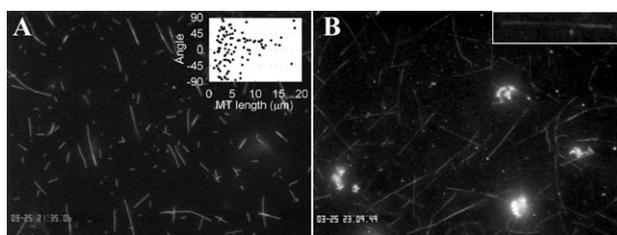


Fig. 2 Fluorescence micrographs showing microtubule array alignment (A) before and (B) after NEM-controlled growth with “dim” tubulin. Inset A: Deviation angle from mean of microtubule seeds as a function of length. Inset B: Magnified microtubule showing a bright seed segment and a dim segment grown by elongation. Scale bar is $20 \mu\text{m}$.

microtubules experience smaller drag forces, they are incompletely aligned.

To extend the length of the microtubule arrays, these seeds were used as nucleation sites for the polymerization of tubulin. Microtubules were grown uniquely from the plus end by using $1.5 \mu\text{M}$ *N*-ethylmaleimide labeled tubulin (NEM-tubulin), added to the polymerization solution together with $6 \mu\text{M}$ dim-RTU (1 : 7 rhodamine : unlabeled tubulin).¹⁰ The dim tubulin enables visualization of the extended segment relative to the RTU seed segments. During microtubule growth, the tubulin solution was replenished every 15 minutes (typically for 3 cycles) to maintain a high tubulin concentration. Fig. 2B contains a representative fluorescence microscopy image of the microtubules after the polymerization. Close examination of the microtubules (Fig. 2B, inset) shows that they contain a short, bright minus-end segment and long, dim plus-end segment.

Analysis of the resulting microtubule patterns indicates that the dim sections maintain minimal alignment ($\pm 48^\circ$) from the original magnetic pattern, and that the plus (e.g., dim) ends are not oriented. We next sought to improve the alignment and encourage polarization by adding an additional magnetic segment to the microtubule plus-ends, which could be used to pull the antiparallel (non-polarized) arrays to a uniform isopolar formation. To achieve this, $0.6 \mu\text{M}$ BTU, $5.4 \mu\text{M}$ RTU, and $1.5 \mu\text{M}$ NEM-tubulin were added to extend the aligned microtubules. The filaments were then stabilized with paclitaxel and exposed to neutravidin and biotinylated- CoFe_2O_4 nanoparticles to magnetically label the 10% BTU segment. However, when the permanent magnet was placed under the flow cell (at a location several millimeters from its original location), no alignment of the new magnetically-labeled termini was observed. The most likely cause is incomplete functionalization of the microtubule while it is affixed to the surface, which results in insufficient quantities of attached magnetic cargo to affect magnetic field-based alignment.

To circumvent this problem, segmented microtubules were prepared *ex situ* and deposited in aligned arrays onto the kinesin-modified glass surface. In this case, the microtubules contain heterofunctional segments that were prepared by growth of dim tubulin on 2.3% BTU microtubule seeds using NEM-tubulin regulated growth at the same concentrations used above. The biotinylated segment was labeled with biotinylated CoFe_3O_4 nanoparticles by coupling with neutravidin. Alignment was performed with a cube magnet, as before, resulting in non-polarized arrays, with brighter segments (2.3% BTU) oriented towards and away from the magnet. The fluorescent micrograph in Fig. 3 shows that the microtubules average $13 \pm 5 \mu\text{m}$ in length and align well with the magnetic field ($\pm 26^\circ$). When these immobilized filaments were subjected to flowing streams of solution by filling the $20 \mu\text{L}$ flow cell in several cycles, the patterns were retained even in the presence of excess ATP in solution. The observed alignment deviations were $\pm 26^\circ$, $\pm 41^\circ$, and $\pm 39^\circ$ following 1, 3, and 5 flow cell rinses, respectively. Thus, the microtubule patterns have remarkably greater stability against flow induced re-alignment as compared to the microtubules grown from aligned seeds above.

The greater stability may be the result of improved motor binding along the length of the microtubule, since during elongation (above), short tubulin polymers may have nucleated

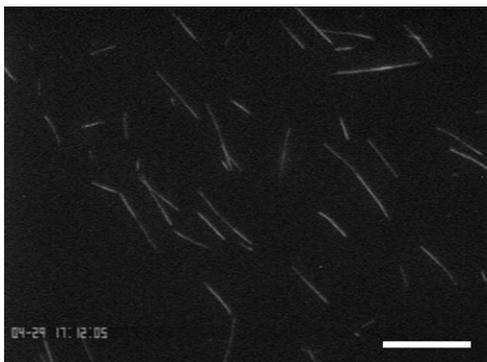


Fig. 3 Magnetic alignment of a microtubule array formed from pre-grown segmented microtubules. Bright segments are magnetically labelled plus-end of the microtubule, while dim segments contain fewer rhodamine tags and no biotin. Scale bar is 20 μm .

and bound or blocked access to motor binding sites. The facile assembly of long, aligned, and heterofunctional microtubule arrays provides a unique platform for the investigation of controlled kinesin motor transport. Our continuing efforts seek to obtain uniform polarity of these immobilized microtubules and to further elongate and functionalize the microtubules to

produce arrays with size and structure analogous to the mitotic spindle.

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