Supporting Online Material for

Cilia-Like Beating of Active Microtubule Bundles

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Materials and Methods

Tubulin purification and handling

Tubulin was purified from bovine brain through two cycles of polymerization-depolymerization in high-molarity PIPES buffer according to the protocol described in Castoldi and Popova (*2*). The purified protein was frozen at 7.4 mg/ml in M2B buffer (80 mM PIPES pH 6.8, 1 mM EGTA, 2 mM $MgCl_2$). Tubulin was subsequently recycled and flash-frozen at a concentration of 13.2 mg/ml in liquid nitrogen using thin-walled PCR tubes. One batch of tubulin was labeled with Alexa Fluor 647 (Invitrogen, A-20006) via a succinimidyl ester linker, using the protocol provided by the Mitchison lab (*3*). Recycled tubulin was copolymerized with 3% fluorescently labeled tubulin, which itself had 29% of monomers labeled. The polymerization mixture consisted of 4.5 μL unlabeled tubulin, 0.9 μL of 7.4 mg/ml Alexa-labeled tubulin, 1.4 μL glycerol (to 15% of final), 3 mM GTP, 1mM DTT, and M2B buffer a final volume of 8.7 μL (for a tubulin concentration of 6.8 mg/ml). This solution was incubated for 30 minutes at 37°C. Subsequently, 0.5 mM taxol (1:1 of 1 mM taxol in DMSO to M2B) was used to dilute the microtubules to 6 mg/ml. The taxol was added in 1/4-volume increments, spaced 7 minutes apart and lightly vortexed each time to avoid precipitation of microtubules. This resulted in an average microtubule length of 4.8 μm, with a standard deviation of 4.1 μm. Microtubules were stable at room temperature for our experimental purposes for up to two days.

Kinesin-streptavidin complexes

Biotin-labeled Kinesin 401 (K401) was a gift from Jeff Gelles. K401 which consists of 401 amino acids of the N-terminal motor domain of *D. melanogaster* kinesin, was purified as previously published (*4, 5*). The protein was frozen at 0.7 mg/ml in 50 mM imidazole (pH 6.7), 4 mM $MgCl_2$, 2 mM DTT, 50 μM ATP and 36% sucrose buffer. Kinesin-streptavidin complexes were assembled by mixing 7 μL of freshly thawed K401 solution with 1 μM ATP, 3 mM DTT, 5.5 μL of 0.28 mg/ml streptavidin (Invitrogen, Cat. # S-888), and M2B to a final volume of 14 uL. The mixture was allowed to incubate on ice for at least 10 minutes before use.

Preparation of active microtubule bundles

Several initial mixtures were prepared separately and then combined into the final solution. Each individual component of the following mixtures was dissolved and frozen separately in M2B, and thawed freshly for use within a day. The first mixture is a taxol-$MgCl_2$ solution, composed of 1.3 uL of 1 mM taxol in DMSO and 18.7 uL of 6 mM $MgCl_2$, 80mM PIPES and 1 mM EGTA buffer. Next, we included anti-oxidant components (listed below) and trolox (Sigma, 238813) to avoid photo-bleaching during fluorescence imaging. Anti-oxidant solution 1 (AO1) contained 15 mg/ml glucose and 25 M DTT. Anti-oxidant solution 2 (AO2) contained 10 mg/ml glucose oxidase (Sigma G2133) and 1.75 mg/ml catalase (Sigma C40). Lastly, ATP-regenerating components were included in the final mixture, which included the enzyme mixture PK/LDH (Sigma, Cat. # P-0294) and phosphoenol pyruvate (PEP). As ATP is hydrolyzed by kinesin activity, the PK/LDH uses PEP as a fuel source to convert ADP back into ATP at a higher rate than the ATP hydrolysis, maintaining a constant ATP concentration until the PEP is exhausted (*6*).

The components described above were mixed without microtubules and in large volumes to reduce pipetting inaccuracies, while not wasting valuable tubulin. This "active pre-mixture" contains the following: 1.3 μL AO1, 1.3 μL AO2, 1.7 μL ATP (50 mM), 1.7 μL PK/LDH, 2 μL BSA (10 mg/ml), 2.9 μL taxol-$MgCl_2$ solution, 6 μL trolox (20 mM), 14.3 μL PEP (40 mM), X μL kinesin-streptavidin mix, Y μL PEG (6 % w/w in M2B) in (18.8 - X - Y) μL M2B buffer. Most samples were prepared with X=1.5 μL of kinesin-streptavidin and Y=4 or 5 μL of PEG. To prepare the final active mixture, 5 μL of the active pre-mixture was mixed with 1 μL of the 6 mg/ml microtubule solution , resulting in a final microtubule concentration of 1 mg/ml.

Flow cell and assembly of attached, active bundles

Microscope glass slides and coverslips were coated with a poly-acrylamide brush to prevent non-specific adsorption of protein (*7*). The microscope glass slides and coverslips were cleaned through consecutive 5 minute sonication and rinse cycles in millipore-filtered water. Slides were first rinsed and sonicated with hot water containing 0.5% detergent, then with ethanol, then with 0.1 M KOH. Cleaned glass surfaces were then coated with a poly-acrylamide polymer brush by first soaking slides in a mixture of 98.5% ethanol, 1% acetic acid, and 0.5% of the silane-bonding agent 3-(Trimethoxysilyl)poropylmethacrylate (Acros Organics, 216551000) for 15 minutes. Slides were rinsed a final time and immersed in a 2% w/V poly-acrylamide solution in water. 35 μL/100 ml of TEMED and 70 mg/100 ml of ammonium persulfate were both added to the acrylamide solution immediately before use to promote polymerization of acrylamide polymer off the glass surfaces. Slides and coverslips were stored in poly-acrylamide solution and used for up to 2 weeks. Each slide was rinsed and air dried immediately before use.

3-mm-wide flow chambers were constructed using 10-μm-thick double-sided tape (Nitto Denko Corporation) to connect pre-treated slides and coverslips. Active mixtures were flowed into the chambers and the flow spontaneously caused bubbles to form within the chamber. These bubbles were desired, as they served as attachment sites for active bundles. Samples were allowed to develop for 45 minutes. During this time, many microtubules became trapped between the air bubbles and the glass. At this stage, dilute distributions of active bundles could be observed beating at bubble edges. Next, the active pre-mixture (see previous section) was flowed through the chamber to remove background microtubules from the bulk volume. During this process, thick swaths of microtubules were forced against bubbles. As excess microtubules in these swaths diffused into the now dilute bulk, they left behind comparatively dense bundle fields at the bubble-glass interface, which routinely synchronized to produce metachronal waves (movie S6).

Light microscopy

All observations of active microtubule bundles were obtained using standard epi-fluorescence microscopy (Nikon Eclipse Ti microscope). Alexa Fluor 647-labeled microtubules were illuminated with a 120W metal halide light source (X-cite 120) and a Semrock Cy5-4040B-NTE filter set. Image sequences were acquired with an Andor Clara camera. To monitor large areas, the motorized stage of the microscope and the Ti's Perfect Focus hardware were used to acquire adjacent fields of view, which were stitched together using Matlab.

Inter-filament sliding in microtubule pairs under depletion

Acrylamide-coated slides were made as described above with modifications: after silane treatment slides were sonicated in methanol for ten minutes before rinsing with water. Acrylamide concentration was 1% and polymerization was allowed to proceed for 1-2 hours, after which slides were stored in water. Microtubule samples were prepared in M2B buffer with 20 μM taxol and imaged with darkfield microscopy. The exposure time was 50 ms. Trajectories were analyzed with custom filament tracking software (*8*).

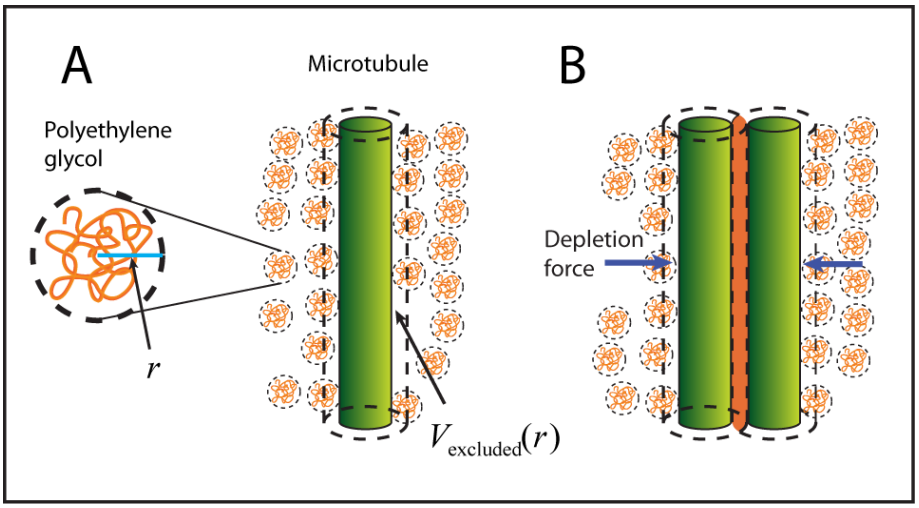

Fig. S1. Schematic illustration of the depletion effect in which addition of a non-adsorbing polymer induces effective attractive interactions between microtubule filaments, leading to their bundling transitions (*1*). **(A)** Non-adsorbing polymers exert uniform osmotic pressure onto an isolated filament. The presence of a filament leads to volume that is excluded to the center of mass of all depleting polymers. This volume ($V_{excluded}$) is larger than the bare volume of filaments and is indicated with dashed line. **(B)** As two filaments approach each other, there is an overlap of excluded volumes indicated in red. When the overlap distance is less than the diameter of the depletion polymer, the polymers are excluded from the overlap region. Consequently, the polymer concentration is higher outside of the filaments than between them, leading to an imbalance of osmotic pressures and effective attractive interactions between the filaments. The range and strength of the depletion interaction can be tuned by controlling polymer size and concentration, respectively.

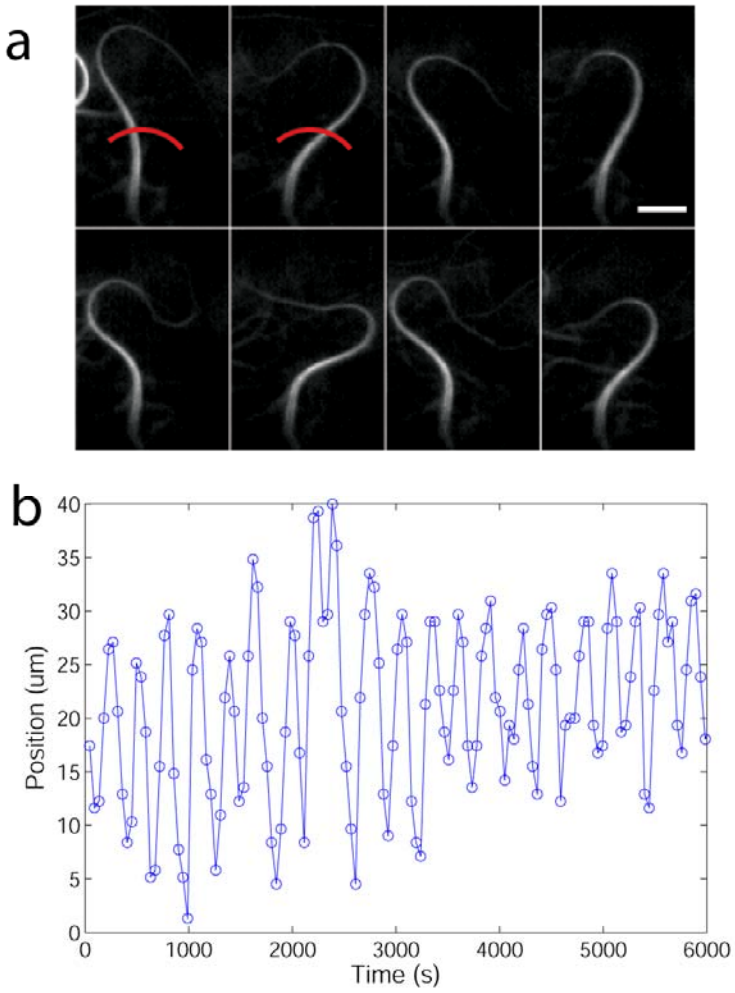

Fig. S2. Beating patterns of an isolated active bundle in which oscillations persisted over a long time period (more then two hours) and many beating cycles. This analysis corresponds to the bundle in movie S3. **(A)** Images of a bundle close to the leftmost and rightmost reversal point in the beating cycle shown over four consecutive periods. These images demonstrate that the active bundle returns to a similar position after each beating period. **(B)** To estimate the periodicity of the beating we measured the position of the bundle's midpoint along an arc drawn perpendicular to the bundle (indicated in red). Plotting this position as a function of time, reveals that periodic motion persisted for more then 20 beating cycles, at which point the data collection was stopped. The data contains one dominant frequency, $4.4 \cdot 10^{-3}$ Hz, with other frequencies present, indicating that the beating pattern is not perfectly sinusoidal. Similar analysis was used to extract the depedence of beating periodicity on bundle length shown in Fig. S3.

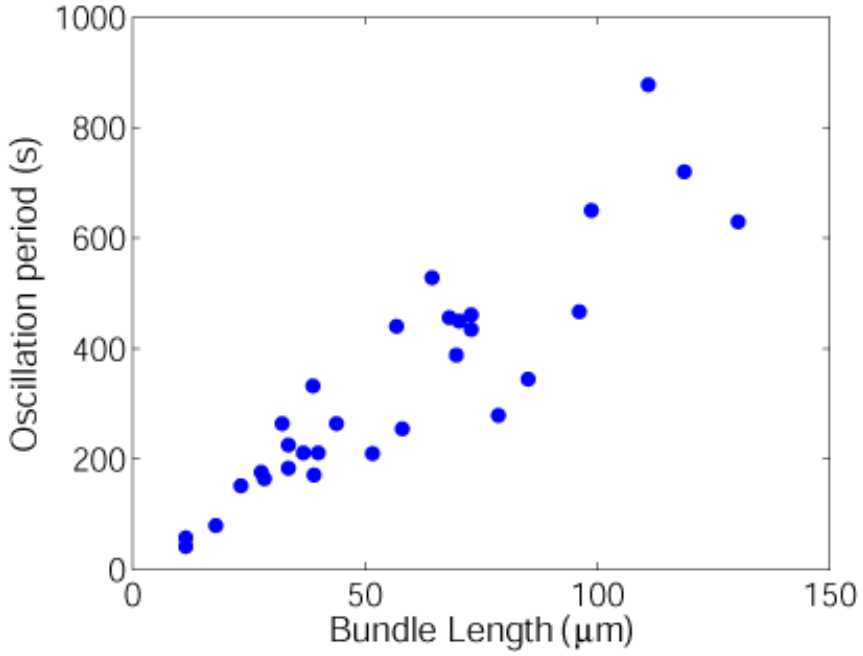

Fig. S3. The dependence of the oscillation period on the length of active bundles. This analysis included filaments anchored to chamber boundaries as well as air bubbles. It also included fairly isolated filaments, as well as filaments that were weakly synchronized with neighbors, but did not include highly synchronized filaments beating as part of a metachronal wave. The data indicates that the beating period increases monotonically with bundle length. A similar analysis was performed for a subset of filaments in order to determine the dependence of the oscillation period on bundle thickness, but specific trends were not obvious, indicating that beating period is independent of or only weakly dependent on bundle thickness.

Supporting Materials References:

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Movie S1
Movie of a long active microtubule bundle observed with fluorescence microscopy (bundle length 130 μm). The freely beating bundle consists of thousands of microtubules and is anchored at the edge of a 10-μm-high tape boundary. The 10 μm vertical confinement of the chamber is much smaller than the length of the filament, causing the beating pattern to be quasi-two-dimensional and stay in focus. Scale bar is 15μm.

Movie S2
Movie of a few active bundles of intermediate size (40-50 μm length) which are attached to the edge of an air bubble. The movie illustrates three-dimensional beating patterns with bundles passing in and out of the focal plane. These comparatively smaller bundles beat more quickly than the bundle shown in movie S1. Scale bar is 10μm.

Movie S3
A medium-sized active microtubule that exhibits sustained oscillations for over 2 1/2 hours. A clear periodicity is present with a dominant frequency. Scale bar is 10 μm.

Movie S4
Dynamics of two microtubules observed with darkfield microscopy which are attached to each other via a depletion interaction. The filaments are confined to a quasi 2D chamber. To suppress surface interactions the glass coverslide and slip are coated with poly-acrylamide brush. The microtubules slide past each other due to thermal forces. Their positions can be extracted and the calculated relative MSD shows that the microtubules exhibit sub-diffusive behavior, indicating a viscoelastic coupling between the two filaments. Scale bar is 5μm.

Movie S5
Multiple active bundles beating in close proximity. At sufficiently high density, active bundles synchronize their beating patterns, due to hydrodynamic and/or steric interactions. This synchronization can persist over many beating cycles. Scale bar is 5μm.

Movie S6
An air bubble confined in a 10-μm-thick chamber, traps microtubules at the air-glass interface. These microtubules nucleate assemblies of active bundles perpendicular to the edge of the bubble. At lower coating density, bundles beat asynchronously. The average length of bundles in this movie is around 10 μm. Scale bar is 20μm.

Movie S7
This video features the same air bubble as shown in Fig. 3A. At a high density of active bundles we observed the emergence of propagating density waves along the contour of the bubble. Scale bar is 20μm.

Movie S8
An air-bubble (with ~200 μm diameter) is coated with active microtubule bundles. The length of these bundles is, on average, 15 μm. Scale bar is 20μm.

Movie S9
Metachronal waves are capable of transporting debris in a preferred direction along the surface of the bubble, mimicking the biological process of fluid transport along the surface of a cell. Scale bar is 20μm.

Movie S10
Active microtubule bundles lodged beneath an air bubble in a 10 μm chamber. The composite image sequence shows the bubble at two different z-positions: (left) at the coverslip surface and (right) 5 μm off the surface, in the middle of the chamber. Bundles can be seen in focus at both positions, indicating a three-dimensional beating pattern. This observation provides additional evidence that bundle beating is not due to surface bound motors. Scale bar is 5μm.