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Depletion Force Induced Collective Motion of Microtubules Driven by Kinesin

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Abstract

Collective motion is a fascinating example of coordinated behavior of self-propelled objects, which is often associated with the formation of large scale patterns. Nowadays, *in vitro* gliding assay is being considered a model system to experimentally investigate various aspects of group behavior and pattern formation by self-propelled objects. In the *in vitro* gliding assay, cytoskeletal filaments F-actin or microtubules are driven by surface immobilized associated biomolecular motors myosin or dynein respectively. Although F-actin/myosin or microtubule/dynein system was found promising in understanding the collective motion and pattern formation by self-propelled objects, the most widely used biomolecular motor system microtubule/kinesin could not be successfully employed so far in this regard. Failure in exhibiting collective motion by kinesin driven microtubules is attributed to the intrinsic property of kinesin, which was speculated to affect the behavior of individual gliding microtubules and mutual interaction among them. In this work, for the first time, we have demonstrated the collective motion of kinesin driven microtubules by regulating mutual interaction among the gliding microtubules, by employing depletion force among them. Proper regulation of the mutual interaction among the gliding microtubules through employment of the depletion force was found to allow the exhibition of collective motion and stream pattern formation by microtubules. This work offers a universal means for demonstrating the collective motion using *in vitro* gliding assay of biomolecular motor systems and will

help obtain a meticulous understanding of the fascinating coordinated behavior and pattern formation by self-propelled objects.

Introduction

Collective motion is a common display of coordinated behaviour which emerges from moving objects such as animal, birds, fishes, insects, bacteria, cells and self-propelled particles¹⁻⁷. One of the fundamental properties of collective motion is the evolution of fascinating large scale patterns, such as stream and vortices pattern¹⁻⁷. Computer simulation based approaches have been employed so far to understand the mechanism of the pattern formation associated with the collective motion of self-propelled objects⁸⁻¹⁵. These *in silico* works revealed that large scale patterns arise mainly from repeated local interactions among individual moving objects. Recently, biomolecular motor systems such as F-actin/myosin and microtubule/dynein have been used as model systems for experimentally demonstrating collective motion of self-propelled objects by employing them in the *in vitro* gliding assay where cytoskeletal filaments are driven by biomolecular motors immobilized on a surface in the presence of adenosine triphosphate (ATP)¹⁶⁻¹⁹. For instance, Schaller *et al.* reported highly concentrated F-actin driven by myosin exhibited collective motion which resulted in fascinating wave-like or polar patterns over time¹⁶⁻¹⁸. Sumino *et al.* reported the emergence of stream and lattice of vortices pattern from collective motion of microtubules driven by ‘inner-arm dynein c’¹⁹. These experimental evidences have emphasized the importance of local interaction between gliding cytoskeletal filaments in the collective motion and pattern formation. Thus, the *in vitro* gliding assay offers a

simple means to investigate experimentally roles of parameters that govern collective motion and pattern formation. However, the investigation using biomolecular motor system was not always successful due to failure of biomolecular motor systems in exhibiting collective motion. For instance, it was reported that microtubule/kinesin, the most widely used biomolecular motor system²⁰, failed to exhibit collective motion and consequently formed no pattern which has been attributed to the intrinsic property of kinesin that is speculated to affect the behavior of individual microtubules and local interaction among them¹⁹.

To overcome this drawback, we for the first time demonstrate that regulation of local interaction among gliding microtubules allows them exhibit collective motion even on a kinesin coated surface. We have regulated the interaction of gliding microtubules by employing depletion force among them which is an attractive interaction known to work between colloidal particles or macromolecules suspended in polymer solution such as methylcellulose (MC) or polyethylene glycol (PEG)²¹⁻²⁸. MC induced depletion force was found to increase the probability of attractive collision between gliding microtubules, which consequently allowed emergence of collective motion and finally resulted in stream pattern of microtubules. Depletion force mediated collective motion and subsequent pattern formation by microtubules on a kinesin coated substrate indicates that emergence of collective motion is independent of the type of biomolecular motor used in the *in vitro* gliding assay, if the interaction among the cytoskeletal filaments is properly

regulated. This work offers a universal means for demonstrating the collective motion of biomolecular motor driven cytoskeletal protein filaments using the *in vitro* gliding assay, which in turn is expected to widen the applications of biomolecular motor systems and might foster the present understanding on coordinated behavior and pattern formation by self-propelled objects.

Material and Methods

Purification and Labelling of Proteins

Tubulin was purified from porcine brain using a high-concentration PIPES buffer (1 M PIPES, 20 mM EGTA, and 10 mM MgCl₂; pH adjusted to 6.8 using KOH). The high-concentration PIPES buffer and 80 mM PIPES buffer (BRB80) were prepared using PIPES from Sigma, and the pH was adjusted using KOH²⁹. GFP-tagged kinesin consisting of the first 560 amino acids of human kinesin-1 was prepared by partially modifying the expression and purification methods described in reference 30. Rhodamine-labelled microtubules were obtained by polymerising a mixture of rhodamine-labelled tubulin (TT) and non-labelled tubulin (WT) (TT: WT = 4:1; final tubulin concentration, 55.6 µM) at 37°C in the presence of 1 mM GTP according to the standard technique³¹. The solution containing the microtubules was then diluted with motility buffer (80 mM PIPES, 1 mM EGTA, 2 mM MgCl₂, 0.5 mg mL⁻¹ casein, 1 mM DTT, 10 µM paclitaxel and ~1% DMSO; pH 6.8). MAP4 fragment which was used as isoform fragment polypeptides (PA₄T) containing the microtubule-binding domain, which was a gift from Prof. Tokuraku^{32,33}.

***In Vitro* Gliding Assay**

In vitro gliding assay of microtubules was performed as described³⁴. Flow cells with dimensions of 2 × 5 × 0.15 mm³ (W × L × H) were assembled from two (5 × 7) mm²

and (40 × 50) mm² cover glasses (MATSUNAMI), and double-sided tape was used as the spacer. Anti-GFP antibody (Invitrogen) at 0.2 mg mL⁻¹ (2 μL) was applied to the flow cell. After incubation for 3 min, the flow cell was washed with 5 μL of motility buffer. Then, 3 μL of GFP-tagged kinesin solution (~80 mM PIPES, 1 mM EGTA, 2 mM MgCl₂, 0.5 mg mL⁻¹ casein, 1 mM DTT, 10 μM paclitaxel/DMSO, ~1% DMSO; pH 6.8) was introduced and incubated for 3 min to bind the kinesin to the antibody. The flow cell was washed with 5 μL of motility buffer. Next, 3 μL of microtubule solution of prescribed concentration was introduced and incubated for 3 min, followed by washing with 5 μL of motility buffer with 2 times. Then, 10 μL of 10 mM ATP buffer (~80 mM PIPES, 1 mM EGTA, 2 mM MgCl₂, 0.5 mg mL⁻¹ casein, 1 mM DTT, 10 μM paclitaxel/DMSO, ~1% DMSO; pH 6.8) was added to the flow cell. For the experiments where we investigated the effect of MC, 0.1 and 0.3 wt% MC in the 10 mM ATP buffer were used. For these experiments, the flow cell was placed inside the inert chamber and humid nitrogen gas was passed through the chamber to remove oxygen from the chamber^{35, 36}. Then ATP buffer was applied to the flow cell and the time of ATP addition was set as 0 min, after which microscopic observation was initiated. Next, where the MAP4 fragments were used, the ATP buffer was applied to the flow cell after application of 0.25 μM MAP4 fragment and subsequent wash with motility buffer. All the aforementioned experiments were performed at room temperature.

Quantitative Analysis of Collective Motion of Microtubules by Nematic Order Parameter, S

The circular histogram of angles of microtubules was obtained by using image J plugin, orientation J³⁷. Orientation degree of microtubules in streams was characterized by the nematic order parameter, S (Supplementary Fig.S1) which was calculated using following equation^{38, 39}.

$$S = \frac{1}{N_{MT}} \sqrt{\left(\sum_{i=0}^{180} R_i \cos 2\theta_i\right)^2 + \left(\sum_{i=0}^{180} R_i \sin 2\theta_i\right)^2} \quad (eq. 1)$$

Where N_{MT} refers to total number of microtubules in the frame, R_i is frequency of the angle of individual microtubules θ_i ($i = 0$ to 180). The range of θ_i was fixed from 0° to 180° based on the symmetry of horizontally or vertically aligned microtubules with respect to the x axis.

Analysis of the Density Fluctuation of the Microtubules in the Streams

Density fluctuation, D_f was calculated by the following equation:

$$\text{Density fluctuation, } D_f = \frac{1}{n} \cdot \frac{1}{P_{ave}} \sum_{i=1}^n (P_i - P_{ave})^2 \quad (eq. 2)$$

for all tiles A_i ($i = 1$ to n , the size of each tiles; $1.3 \times 1.3 \mu\text{m}^2$) covering a frame. P_i is an estimated microtubule number in tile A_i (Supplementary Fig. S4). P_{ave} is average microtubule density when assuming the total number of microtubules are equally distributed over the frame and can be defined as follows (eq. 3),

$$P_{ave} = \frac{1}{n} \sum_{i=1}^n (N_i) \quad (eq. 3)$$

where N_i is number of microtubules observed in 1 tile.

The number of microtubule in a tile is estimated by quadratic function ' f ' of average signal intensity ' x ' available from pixels in a tile:

$$f(x) = ax^2 + bx + c, \quad a = -0.045, b = 6.2, c = -4.5$$

Values for a , b and c were obtained by fitting a curve (Supplementary Fig. S3) to the experimental data observed by a high-resolution fluorescence microscopy image (Supplementary Fig. S2). The signal intensity was saturated when the number of microtubules was 68 in each tile which was experimentally confirmed.

Microscopic Image Capture

The samples were illuminated with a 100-W mercury lamp and visualised with an epifluorescence microscope (Eclipse Ti; Nikon) using an oil-coupled Plan Apo 60× 1.40 objective (Nikon). Filter blocks with UV-cut specifications (TRITC: EX540/25, DM565, BA606/55; GFP-HQ: EX455-485, DM495, BA500-545; Nikon) were used in the optical path of the microscope to allow for visualisation of samples while eliminating the UV portion of the radiation and minimising the harmful effects of UV radiation on the samples. Images and movies were captured using a cooled CMOS camera (Neo sCMOS; Andor) connected to a PC. To capture a field of view for more than several minutes, ND filters (ND4, 25% transmittance) were inserted into the illuminating light path of the

fluorescence microscope to avoid photobleaching.

Computational Environment for Image Analysis

Movies of microtubules captured by fluorescence microscopy were analysed using Adobe Photoshop CS6 and image J, and we have developed our algorithm in MATLAB R2015a using image processing toolbox.

Results and Discussion

The effect of depletion force induced by MC on the assembly of microtubules was firstly investigated by monitoring the organization of microtubules suspended in MC solutions of different concentrations. Microtubules were prepared by polymerising fluorescence dye labelled tubulin heterodimers and stabilised using paclitaxel. The average length of microtubules was $\sim 7.0 \pm 5.4 \mu\text{m}$ (mean $\pm$ s.d., $n = 300$). Low concentration microtubules, prepared from 200 nM tubulin, was suspended in 0.1 and 0.3 wt% MC (methylcellulose 4000, Junsei Chemical Co., Ltd, MW=140 kDa) solutions and observed under fluorescence microscopy. At 0.1 wt% of MC solution, microtubules were found to form aster like aggregations (Fig. 1). On further increasing the MC concentration to 0.3 wt%, thick and long microtubule bundles were formed owing to increased attractive interaction among microtubules (Fig. 1). In contrast, aggregation and bundle formation of microtubules were not observed in the absence of MC (0 wt%) as

shown in Fig. 1. This result indicates that depletion force induced by MC can effectively enhance the attractive interaction among microtubules and allow their assembly formation, which coincides well with the previous report²³.

To investigate the effect of depletion force on the behaviour of gliding microtubules, we performed an *in vitro* gliding assay of microtubules in the presence of MC. The gliding assay system consisting of microtubules and kinesin in a glass flow cell is schematically shown in Fig. 2A. GFP-tagged kinesin (recombinant conventional kinesin consisting of 560 amino acid of human kinesin-1) was immobilized on the glass substrate via anti-GFP antibody. Then microtubules were deposited at the kinesin coated glass surface. Motility of microtubules was initiated by adding ATP buffer at 25 °C. On addition of ATP buffer, microtubules started to move randomly at a mean velocity of $110 \pm 20 \text{ nm s}^{-1}$. Investigation on the behaviour of gliding microtubules filaments revealed that during movement, microtubules randomly approached each other and collided resulting in either snuggling (Fig. 2B) or crossing over (Fig. 2C). During snuggling, gliding microtubules interacted showing a parallel (Fig. 2B left) or antiparallel alignment (Fig. 2B right) where the terms indicate that snuggling microtubules are aligned following same or opposite polarity, respectively. Snuggling is considered the most important behaviour of gliding microtubules for producing collective motion that often leads to formation of stream or vortex patterns of microtubules¹⁹. Fig. 3A shows the probability of snuggling and crossing over of microtubules in the gliding assay in the

absence of MC. To avoid excessive crowding, the concentration of microtubules was fixed keeping tubulin concentration constant at 0.2 μM which resulted in a density of 0.9×10^4 filaments mm^{-2} ; the kinesin concentration was varied from 20 to 1000 nM to investigate whether kinesin density has any role in the behaviour of gliding microtubules. Surface density of kinesin, estimated by Quartz Crystal Microbalance (QCM)⁴⁰, was within 700 to 1900 molecules μm^{-2} . As shown in the Fig. 3A, approximately 20% of events resulted in snuggling which was almost insensitive to the kinesin density on the substrate. Under this condition, no collective motion was observed even when high concentrations of microtubules were used and this could be accounted for by the high probability of crossing over as reported in literature¹⁹.

Next, we investigated the behaviour of gliding microtubules in the presence of depletion force induced by MC. Here, we initiated the motility of microtubules by applying ATP buffer containing MC of two different concentrations (0.1 and 0.3 wt%). In the presence of 0.1 wt% MC, the probability of snuggling event was increased to 30% and increasing MC concentration further to 0.3 wt%, the probability of snuggling also increased to 50 %. In these two cases, the probability of snuggling remained insensitive to the change in kinesin density on the substrate (Fig.3B, C).

Here, to discuss the effect of depletion force on the snuggling probability of gliding microtubules, we estimated the depletion force between two microtubules induced by 0.3 wt% MC from the calculation of excluded volume associated with each

microtubules by treating it as a cylinder with radius $R_{MT} = 12.5$ nm surrounded by depletion layer^{22, 41}. The radius of gyration (R_g) of 140 kDa MC is ~ 30 nm. To apply the Asakura-Oosawa model⁴², flexible polymers like MC are typically treated as freely interpenetrating hard spheres of radius R_{AO} , which are excluded from the colloid surface by a thin layer of thickness R_{AO} ⁴³. R_{AO} should be determined by $2R_g/\sqrt{\pi} = 33.85$ nm. Then the size of the depletion force was calculated as ~ 0.11 pN which was almost comparable to that of PEG or MC reported in previous literatures^{23, 44}. Here, if we consider the force of each kinesin as ~ 5 pN⁴⁵, the total force of kinesins interacting with each microtubule on the surface should be several tens of pN. The total force of kinesin is much larger than the depletion force. Despite the increase in snuggling by the depletion force, it was not enough to form stable bundle of microtubules moving on the kinesin coated surface, although stable bundles were formed in a solution (previously discussed).

To explore the condition for obtaining collective motion of microtubules at fixed kinesin and MC concentrations of 1000 nM and 0.3 wt%, respectively, we investigated how the microtubule density affects their moving behaviour by varying the concentration of microtubules prepared by tubulin solution of 0.2 to 5.0 μ M. Density of microtubules, ρ was manually measured and found within range of $0.9 \sim 65 \times 10^4$ filaments mm^{-2} . Initially, just after addition of ATP (time set as 0 min), the microtubules at any of the concentrations moved randomly without showing any specific directional preference (Fig. 4A). Over time (~ 30 min), randomly moving microtubules showed collective motion

which resulted in the formation of large streams. Collective motion of microtubules was observed above a certain concentration of microtubules, i.e. at a tubulin concentration of $2.0 \mu\text{M}$ (Fig. 4A, Movie 1). The size of streams was increased with increasing the concentration of microtubules.

To evaluate emergence of collective motion, we quantified the orientation of microtubules by analysing fluorescence microscopy images of collectively moving microtubules at 30 min using ‘Image J plugin Orientation J’. First, we obtained the distribution of orientation angles of gliding microtubules from which we calculated the nematic order parameter, S which is the degree of orientation of microtubules. The $S = 0$ and ~ 1 represent random and uniaxial orientation distribution of microtubules, respectively. Fig. 4B shows the change in S with change in microtubule density. As shown in this figure, there exists a critical density of microtubules, ρ_c at $\sim 28 \times 10^4 \text{ mm}^{-2}$ above which the S remained constant and was close to 1. This transition in S depending on microtubule concentration well represents a phase transition of liquid crystal from an isotropic to a nematic phase⁴⁶.

Fig. 5A shows the fluorescence microscopy images of collective motion of microtubules over time. To quantitatively characterise the change in orientation and density of microtubules as observed in this figure, we adopted two parameters: nematic order parameter, S and density fluctuation, D_f . Here, D_f is a parameter that can characterize the density fluctuation of microtubules in different gathering states, i.e.

crowded and scattered by determining quantitatively the variance of microtubule density for a fixed certain area ($281.6 \times 237.6 \mu\text{m}^2$) (Supplementary Figure 4). In the crowded state, microtubules locally form highly dense streams, whereas the scattered state is manifested by a reduction of numbers of microtubules in the streams over time. Fig.5B and C show change in the S and the D_f of microtubules over time. The S of microtubules initially increased with time but it decreased after 1h. This time-dependent change in the S reflects the change in orientation of microtubules with time and after 1h, alignment of microtubules started to be random (detailed image was provided in Supplementary Figure 5). For the D_f , it increased due to the formation of local streams over time. However, after 1h, the D_f started to decrease perhaps due to the decreased microtubule density in the streams. Thus, although collective motion of microtubules was successfully demonstrated on the kinesin coated surface, it is necessary to maintain the stability of collective motion to investigate time evolution of the pattern. Perhaps, inducing attractive interaction among the microtubules in the streams might help improve the stability of the streams. Therefore, to increase the stability of the streams, we introduced microtubules-associated protein, MAP4 fragment in the gliding assay system, which is well known to induce an attractive interaction among microtubule filaments^{32, 33}. By using MAP4 fragment of varying concentrations (0.25 and 2.5 μM), we investigated the stability of microtubules streams. In the presence of 0.25 μM MAP4 fragment with 0.3 wt% MC, microtubules formed the streams that were found to be stable for more than 2h (Fig. 5A,

Supplementary Fig. S6). On the other hand, at 2.5 μM MAP4 fragment, microtubules formed small bundles instead of the streams and the bundles easily detached from the surface with time (Supplementary Fig. S6). Detachment of the bundles with time could be accounted for by decreased interaction between microtubules and kinesin due to the excessive coverage of microtubule bundle surface by MAP4 fragment³³.

To discuss the role of MAP4 fragment in maintaining the stability of microtubule streams, we compared nematic order parameter, S and density fluctuation, D_f of microtubules with and without MAP4 fragment in the presence of 0.3 wt% MC. In the presence of 0.25 μM MAP4 fragment, the S increased until 30 min and remained constant indicating a prolonged orientation of microtubules, which was in contrast to that observed in the absence of MAP4 fragment (Fig. 5B). Besides, in the presence of MAP4 fragment, the D_f increased and similar to the S remained constant showing the preservation of high density of microtubules in streams (Fig. 5C). To understand the effect of MAP4 fragment on the evolution of collective motion, we performed *in vitro* gliding assay in the presence of 0.25 μM MAP4 fragment without 0.3 wt% MC, at fixed kinesin and microtubule densities of ~ 1900 molecules μm^{-2} and $\sim 65 \times 10^4$ filaments mm^{-2} , respectively. We observed that microtubules also exhibited collective motion and generated streams. It is considered that MAP4 fragments may induce transient attractive interaction between microtubules like the depletion force induced by MC. To separately characterize the effect of MC and MAP4 fragment on evolution of collective motion of microtubules, we

performed *in vitro* gliding assay in the presence and absence of 0.25 μM MAP4 fragment, which were carried out both with and without 0.3 wt% MC. Here, also we fixed the kinesin and microtubule densities at ~ 1900 molecules μm^{-2} and $\sim 0.9 \times 10^4$ filaments mm^{-2} , respectively. The snuggling probability was 17.9 % in the absence of both MAP4 fragment and MC, which increased to 35.0 % when 0.25 μM MAP4 fragment without MC was present (Supplementary Fig. S7). On the other hand, snuggling probability dramatically increased to 62.3 % in the presence of 0.3 wt% MC but without MAP4 fragment. However, presence of both the MAP4 fragment and MC, further brought a little increase in the snuggling probability (67.8 %). These results clearly reveal that it is the MC which mainly influences the collision event of the microtubules. Although MAP4 fragments can generate the streams and improve the stability of the streams, it has less pronounced effect on mutual interaction between microtubule filaments compared to that of MC. Therefore, it can be concluded that depletion force due to MC and weak attractive interaction induced by MAP4 fragment can together ensure high stability of microtubule streams and both interactions can be useful for demonstrating collective motion of microtubules on kinesin coated surface. It is to note that, in this work, we found the velocity of microtubules were almost similar in and out of streams (data not shown). To understand the effect of velocity of microtubules on the collective motion, a detailed investigation is required in future.

Conclusion

In conclusion, by employing a macromolecule (methylcellulose) induced depletion force, we demonstrated the first-ever collective motion and stream pattern formation by microtubules on a kinesin coated surface. This method offers a simple and universal technique to investigate the coordinated behaviour of self-propelled objects using biomolecular motor systems. Consequently, this will be helpful in understanding not only the collective behaviour of self-propelled objects such as birds, animals or fishes, but also may provide new insight into emergent structures obtained through a non-equilibrium process⁴⁷⁻⁴⁹. Recently microtubule/kinesin system has attracted attention in the field of molecular robotics as the smallest self-propelled objects^{50, 51}. Molecular robots, relying on a large number of collectively moving self-propelled objects such as gliding microtubules, enables parallel processing in transporting a large number of small cargos and assembling building blocks into an ordered structure. Therefore, ideas obtained from the present study on collective motion of gliding microtubules are expected to expand the boundaries in the field of molecular robotics.

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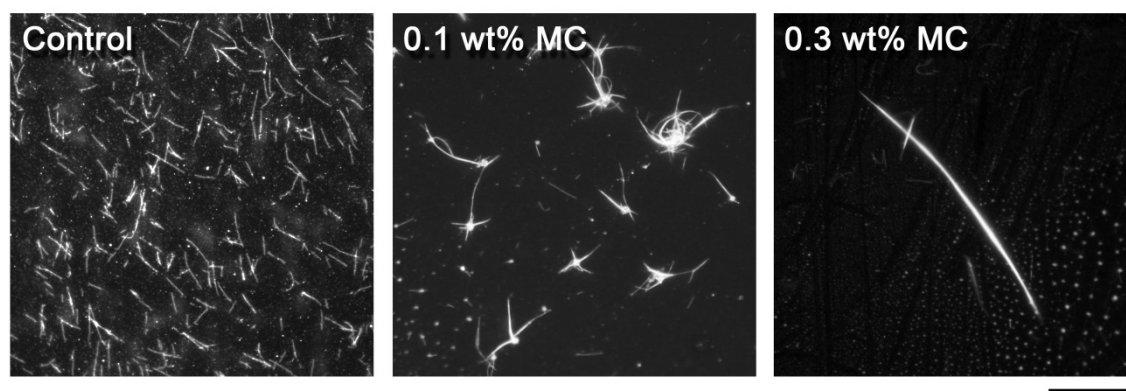


Fig. 1 Depletion force induced bundle formation of microtubules. Fluorescence microscopic images of microtubules which were suspended in solution (left) without MC and (centre and right) with MC of 0.1 and 0.3 wt%, respectively. Scale bar: 10 μm .

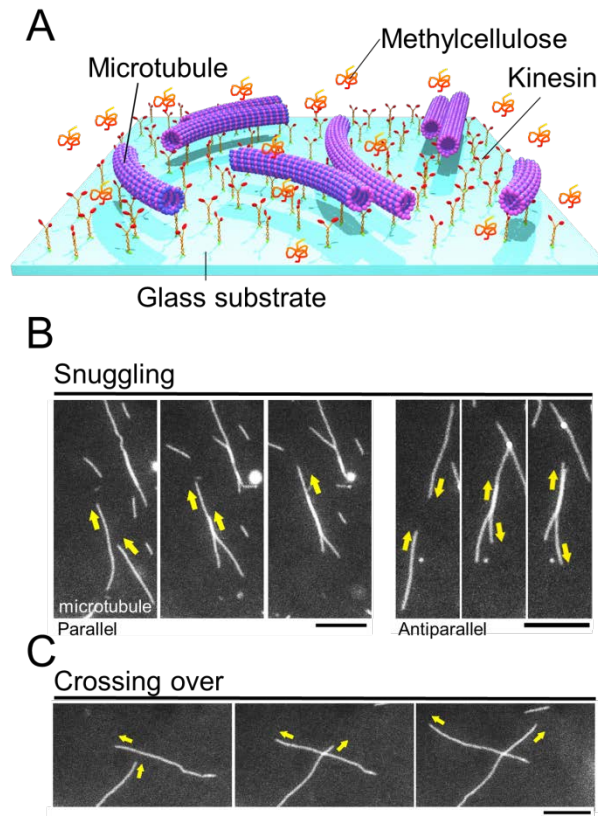


Fig. 2 *In vitro* gliding assay of microtubules on a kinesin coated surface in the presence of MC. (A) Schematic diagram of *in vitro* gliding assay. (B) Different types of collisions among microtubules with different orientations. Snuggling of two microtubules moving toward same (left) and opposite directions (right). (C) Crossing over of moving microtubules. Scale bars: 10 μm .

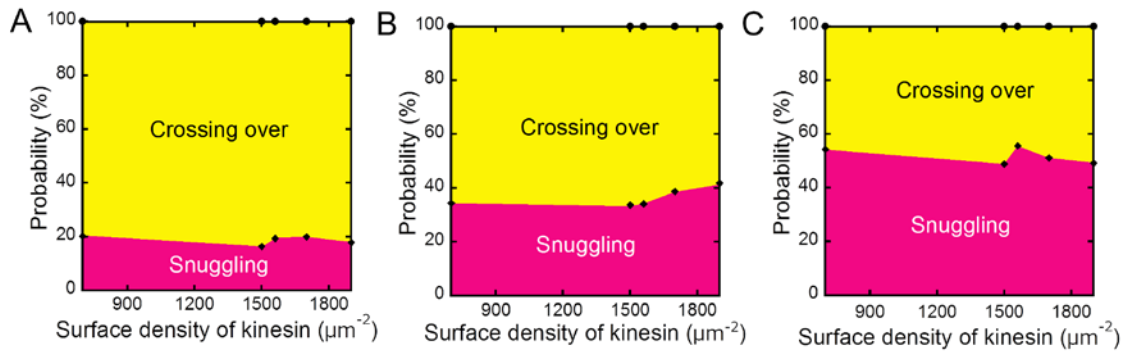


Fig. 3 Effect of depletion force on the behaviour of gliding microtubules at different MC concentrations and surface kinesin density. Probability of snuggling and crossing over of microtubules (A) in the absence of MC and (B), (C) in the presence of 0.1 wt%, 0.3 wt% MC, respectively.

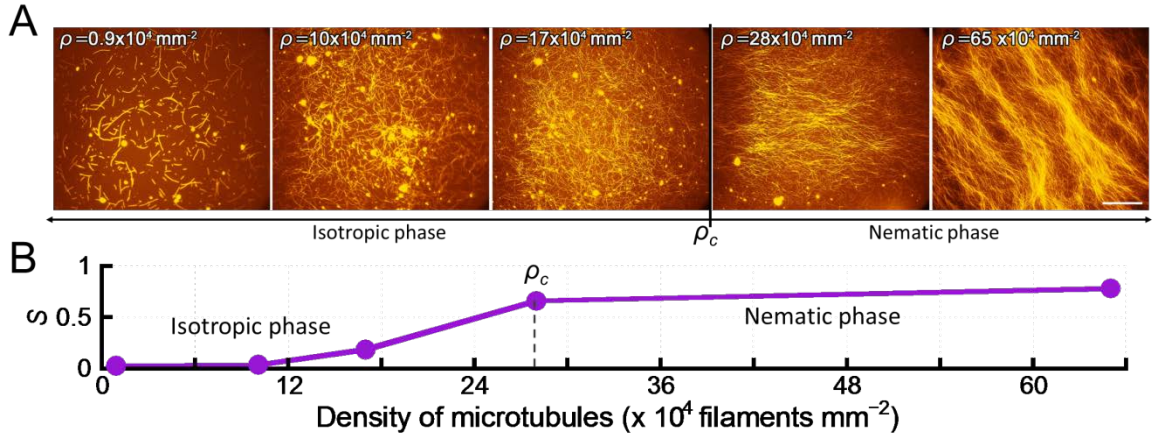


Fig. 4 Effect of density of microtubules on evolution of collective motion. (A) Fluorescence microscopy images of microtubules at different densities of microtubules, ρ which are varied from 0.9 to $65 \times 10^4 \text{ filaments mm}^{-2}$. Scale bar: $50 \mu\text{m}$. (B) Classification of moving behaviour of microtubules by using the nematic order parameter, S . All the images shown here were captured after 30 min of ATP addition.

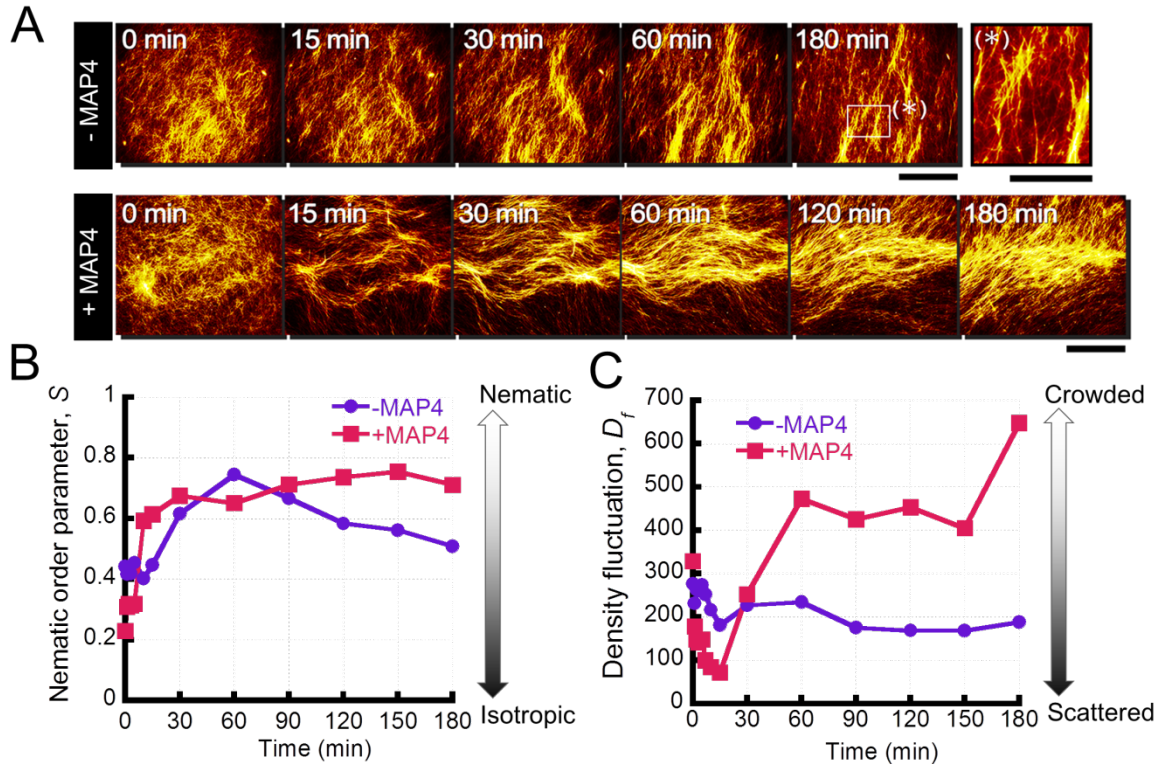


Fig. 5 Stability of the streams of gliding microtubules. (A) Time course of the streams of microtubules in the absence and in the presence of MAP4 fragment. ‘-MAP4’ and ‘+MAP4’ indicate the absence and presence of MAP4 fragment, respectively. Scale bars: 100 μm , enlarged image: 25 μm . Analysis of stability of the streams by using (B) the nematic order parameter, S and (C) the density fluctuation, D_f .